POTENTIAL MECHANISMS FOR DRUG-INDUCED PROLONGATION OF QT INTERVAL AND GENESIS OF TORSADES DE POINTES EVALUATED IN THE FAILING RABBIT HEART

DISSERTATION

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Torsades de pointes (TdP) is a polymorphic ventricular tachycardia characterized by a distinctive pattern of undulating QRS complexes that twist around the isoelectric line. TdP is usually self-terminating or can subsequently degenerate into ventricular fibrillation, syncope, and sudden death. TdP has been associated with QT interval prolongation of the electrocardiogram; therefore, the QT interval has come to be recognized as a surrogate marker for the risk of TdP.

International guidelines have been developed to harmonize both the preclinical and clinical studies for the evaluation of drug-induced TdP. However, currently preclinical in vitro and in vivo methods as well as biomarkers for proarrhythmias have been imperfect in predicting drug-induced TdP in humans. It is clear that relevant biomarkers together with appropriate models are needed to assess the arrhythmic risk of new chemical entities.

The goal of the present dissertation is to create rabbit with myocardial failing heart as an in vivo animal model to predict TdP in humans and to determine mechanism(s) underlying TdP in this model. Electrocardiograms were recorded from bipolar transthoracic leads in 7 conscious healthy rabbits previously trained to rest quietly in slings. The RR and QT relationship,
QT = 2.4RR^{0.72} (r^2 = 0.79, p < 0.001) was obtained by slowed the heart rate with 2.0 mg/kg zatebradine, and the algorithm for removing effect of heart rate on QT is QTc = QT/(RR)^{0.72}. QTc lengthened significantly in all conscious rabbits given intravenous cisapride, dofetilide or haloperidol (p < 0.05), and QTc did not change with DMSO (vehicle control), propranolol or enalaprilat.

The rabbit with myocardial failure was created by coronary ligation and validated with drugs known to be torsadogenic or non-torsadogenic in humans. A greater percentage of rabbits with failing hearts developed TdP following intravenous infusion of escalating doses of dofetilide (85%), clofilium (100%), or cisapride (50%) than did normal rabbits exposed to the same drug protocol (20%, 33% and 0%, respectively). None of the rabbits in either group developed TdP when exposed to escalating doses of amiodarone, verapamil, or quinidine. These results suggested that a rabbit with myocardial failure possesses specificity and sensitivity to assess drugs that tend to induce TdP when given to humans. This may be partly due to conscious rabbits with myocardial failing hearts had a slower heart rate, prolongation of QT and QTc intervals, and increase short-term variability when compared with the normal conscious group. Furthermore, the results from isolated myocytes of myocardial failure suggested that (1) the re-entrant circuits emanating from action potential durations of different duration, (2) triggered activity in the form of EADs, and (3) increased dispersion of repolarization are responsible for genesis of the TdP in the failing rabbit heart.

It is thought that abnormal calcium cycling is the proximate cause of EADs, and it is known that calcium cycling is abnormal in heart failure. The torsadogenic-modifying
effects of verapamil, ryanodine, KB-R7943, W-7, KN-93, and H-8 on ventricular premature depolarizations (VPDs) and TdP were then evaluated in the conscious failing rabbit heart. In the vehicle control hearts, VPDs and TdP were induced in all rabbits and the times to onset of VPD and TdP were 3.6 ± 1.3 min and 10.3 ± 1.4 min, respectively. Verapamil, ryanodine and H-8 significantly delayed onset of VPDs ($p<0.05$) and suppressed the occurrence of TdP ($p < 0.01$). On the other hand, KB-R7943, W-7, and KN-93 accelerated the onset of TdP. These results demonstrated that calcium entry via $I_{\text{CaL}}$ channel, calcium overload by SR Ca$^{2+}$ release, and blocking of PKA may play a major role in the appearance of TdP suggested by the preventive effect of verapamil, ryanodine and H-8. Also, the data indicates that blockade of sodium/calcium exchange, calmodulin, and calcium/calmodulin dependent protein kinase II may favor the development of TdP in the setting of heart failure, and suggests potential new avenues for development of antiarrhythmics in the presence of heart failure.
Dedicated to my parents
Ue Tiew and Boonpa Haeng
Who gave me my life

Dedicated to my wife, Thanaporn Mongkolmaneepol
and my daughter, Pitchapa Kijtawornrat
Who gave me support and inspiration

Dedicated to my advisor
Professor Robert L. Hamlin
Who gave me a chance to pursue a PhD in the field of cardiology
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CHAPTER 1

INTRODUCTION

Since the late 1980s, assessment of the QT interval began to receive increased attention from regulatory agencies due to recurrent reports of torsades de pointes (TdP) and other arrhythmias occurring in patients treated with an overdose of terfenadine, a non-sedating H\textsubscript{1}-antihistamine (Shah, 2005). QT interval (also termed electrical systole), the period between the beginning of the QRS complex and the end of the T wave of the electrocardiogram, reflects the ventricular action potential duration (APD) and represents the time required for ventricular depolarization and repolarization. This duration is determined by the balance of inward and outward currents occurring during depolarization and repolarization phases of ventricular action potential (AP). Thus, QT interval prolongation is a result of an increase of inward current and/or a reduction of outward currents, mainly mediated by blocking of the rapid component of the delayed rectifier potassium (I\textsubscript{Kr}) channels but also attributable to many other currents.
(e.g., the sodium current, $I_{Na}$; the slow component of the delayed rectifier potassium current, $I_{Ks}$; the calcium current, $I_{Ca}$; the transient outward potassium current, $I_{TO}$).

Principally, prolongation of QT interval is a desired therapeutic strategy of antiarrhythmic class III drugs acting by blocking $I_{Kr}$ channels, resulting in delayed ventricular repolarization, and consequently increasing myocardial refractory period. This prevents so-called reentrant arrhythmias. However, excessive QT prolongation is associated with a potentially fatal, rapid, polymorphic, ventricular tachycardia known as TdP (Shah, 2002).

Torsades de pointes, a unique potentially life-threatening ventricular tachyarrhythmia, is a condition that is often heralded by severe (>60 ms from baseline value) QT interval prolongation and is distinguished by a constellation of symptoms including intermittent syncope, dyspnea, and polymorphic ventricular tachycardia (Blancett et al., 2005). TdP was first named by Dessertenne in 1966. The phrase "Torsades de Pointes" is French and literally means "twisting of the points", referring to the characteristic appearance of the electrocardiogram during the rhythm abnormality in which the QRS complexes rotate around the isoelectric line over a sequence of 5 to 20 complexes or more (Pater, 2005). TdP can subsequently degenerate into ventricular fibrillation (VF), syncope, and sudden death (Shah, 2004). In 1985, Salle and coworkers (1985) reported that the incidence of VF degenerated from TdP is approximately 20% in patients who had an episode of TdP and these arrhythmias are consequently followed by cardiac arrest and sudden death. The mortality is approximately 10-17% (Fung et al., 2000; Salle et al., 1985). The incidence of TdP in patients who have been treated with action potential prolonging compounds is quite rare. It was reported by Bauman and
colleagues (1984) that TdP occurred in only 0.5% to 8.8% of patients who were treated with quinidine. Another study by MacNeil et al. (1993) demonstrated that TdP occurs in about 2.6% to 4.1% in patients treated with sotalol. Interestingly, a retrospective analysis by Linquist and Edwards (1997) showed that the number of adverse reactions with terfenadine was less than 0.25 per million daily doses (Fermini and Fossa, 2003). Another torsadogenic compound is cisapride, in which TdP was reported to occur in only about 1 out of 120,000 patients prescribed this medication (Sanguinetti and Tristani-Firouzi, 2006).

Even though TdP is a rare arrhythmia, regulatory agencies are concerned about this adverse drug reaction, occurring with drug-induced QT interval prolongation, both for cardiovascular and non-cardiovascular drugs. Surprisingly, there was no regulatory guidance to the pharmaceutical industry for studying drugs that could effect cardiac repolarization until the late 1990’s. In 1997, the “Points to Consider” document from the Committee for Proprietary Medicinal Products (CPMP) was issued by the European Agency for the Evaluation of Medicinal Products (EMEA) in order to outline a series of experiments on clinical and non-clinical models for assessing the potential for QT prolongation by non-cardiovascular agents (CPMP, 1997). Since that time, regulatory agencies have removed from the market drugs that were associated with QT interval prolongation, and have relabeled or relegated to restricted use many drugs (Sanguinetti and Tristani-Firouzi, 2006). For instance, from June 1990 to March 2001, eight non-cardiovascular drugs (e.g. terodiline, terfenadine, sertindole, astemizole, grepafloxacin, cisapride, droperidol, and levacetylmethadol) were removed from the market in the USA and elsewhere because of their propensity to delay cardiac repolarization, prolongation.
of QT interval on the ECG, and increase of TdP (Shah, 2004). New guidelines have been
developed and proposed subsequently. The Food and Drug Administration’s Center for
Drug Evaluation and Research (FDA’s CDER) and Health Canada’s Therapeutic
Products Directorate issued “The clinical evaluation of QT/QTc interval prolongation and
pro-arrhythmic potential for non-antiarrhythmic drugs” in 2003 (FDA, 2003). Currently
the International Conference on Harmonization (ICH), a joint initiative involving both
regulators and research-based industry as equal partners in the scientific and technical
discussions of testing procedures required to ensure and assess the safety, quality and
efficacy of medicines, has proposed S7A entitled “Safety pharmacology studies for
human pharmaceuticals”, S7B entitled “Guideline on safety pharmacology studies for
assessing the potential for delayed ventricular repolarization (QT interval prolongation)
by human pharmaceuticals” (ICH Harmonized Tripartite Guideline, 2001; ICH S7B,
2003), and E14 guidelines for clinical trials. The ICH S7A guidelines describe the
objectives and principles of safety pharmacology as well as define a core battery of
pharmacological evaluations (cardiovascular, respiratory, and central nervous system)
that should be conducted prior to initial clinical trials (first in man dosing). The ICH S7B
provides guidelines on suitable levels and methods of preclinical testing in order to
further determine the potential for a drug to produce QT prolongation (Fermini and Fossa,
2003).

TdP has been associated with QT interval prolongation of the electrocardiogram;
therefore, the QT interval has come to be recognized as a surrogate marker for the risk of
TdP. However, TdP does not develop invariably in all individuals with equivalent
prolongation of the QT interval (Shah, 2005), and may not occur until many days of
therapy. Not all the drugs that prolong the QTc interval in clinical use have been reported to induce TdP (Belardinelli et al., 2003; Redfern et al., 2003). Also, not all drugs that have been linked with TdP cause significant lengthening of the QT interval or prolong it only slightly (e.g., terfenadine). Even though QT interval is widely used as a surrogate marker of the risk of TdP in humans, it is also recognized to be an imperfect marker (Shah, 2005). There are also several limitations of current test methods for torsadogenic potential of new chemical entities (NCE) e.g. false negatives (e.g., arsenic trioxide, diphenhydramine, geldanamycin, nifedipine, and pentamidine) and false positives (e.g., verapamil and clozapine) of hERG assay. The precise relationship between the drug-induced prolongation of ventricular repolarization and the risk of TdP arrhythmia is not fully elucidated; that is there is a questionable relationship between the degree of QT lengthening and torsadogenicity. Relevant biomarkers together with appropriate models are clearly needed to assess the arrhythmic risk of NCE.

The crucial goal of the present dissertation is to establish an appropriate in vivo animal model to predict TdP in humans and to evaluate mechanism(s) underlying TdP in this model. The aim of this literature review is to give an overview of APD, QT interval and its role in safety pharmacology. Mechanisms of drug-induced QT interval prolongation and TdP along with predisposing factors for genesis of TdP are also included. Subsequently, the current indices for proarrhythmias together with proarrhythmic models are reviewed at the end of this section. Since QT interval changes with heart rate in the absence of any other intervention and since data on QTc formula in conscious rabbit is limited, chapter 2 describes the existing QTc formulae and generates a novel QTc formula that appears to fit better for conscious rabbits and for rabbits
subsequently challenged with known positive and negative torsadogenic compounds in humans. Chapter 3 introduced an animal model that is believed to be appropriate to predict torsadogenic potential of NCE in humans. With tremendous help from high technology laser scanning confocal microscopy to measure calcium concentration and patch clamp technique to measure calcium current, a subsequent chapter will explore the effects of dofetilide on calcium transients and calcium currents in ventricular myocytes isolated from normal rabbit hearts compared with cells isolated from failing rabbit hearts. Chapter 5 evaluated the roles of calcium entry blockers, sarcoendoplasmic reticulum calcium release blockers, and calcium signaling pathways on dofetilide-induced TdP in conscious rabbit with rabbits with heart failure. Finally, this dissertation ends with a brief chapter that suggests a new direction for future study on the mechanism(s) of drug-induced QT interval prolongation and TdP.

**Cardiac action potential and electrocardiograms**

The ventricular action potential (AP) can be divided into 5 phases (Phases 0 to 4; Figure 1.1). When a wave of depolarization reaches ventricular myocytes, a rapid opening of voltage-gated sodium channels (I_{Na}), allows for the influx of Na^+ into the ventricular myocytes; this produces phase 0 of ventricular AP, and produces depolarization, which is represented by the QRS complex on the surface electrocardiogram (ECG). Immediately after maximal depolarization of Phase 0, I_{Na} is in the inactivated stage, and repolarization begins with activation of the transient outward potassium current (Yan and Antzelevitch, 1996). This process causes a brief rapid repolarization and yields a notch on the ventricular action potential known as
Phase 1. This phase is followed by a slower phase of repolarization called Phase 2 (the plateau). Phase 2 of the AP is generated mainly by the inward L-type calcium current ($I_{\text{cal}}$) and outward $K^+$ currents. The delayed rectifier potassium currents also begin to activate at this phase. The activation is slow and the currents have a reduced conductance at positive transmembrane potentials causing the prolonged AP (Sanguinetti and Tristani-Firouzi, 2006). Delayed repolarization not only ensures adequate time for influx of extracellular Ca$^{2+}$ into the myocytes for optimum excitation-contraction coupling but also makes cardiac muscle refractory to premature excitation. At this point in the cardiac cycle, the ECG has returned to the isoelectric line. Phase 3 represents the rapid phase of repolarization of the ventricular AP and returns the membrane potential to its resting level. This result from the opening of the delayed rectifier potassium currents, which is composed of two main components—rapid ($I_{\text{Kr}}$) and slow ($I_{\text{Ks}}$). The most important ionic current contributing to repolarization in the myocytes in Phase 3 is the $I_{\text{Kr}}$ through channels known as hERG channels (Sanguinetti et al., 1995; Trudeau et al., 1995). hERG stands for the human (h) ether (ER) a go-go (G) channel, since fruit flies lacking this channel undergo a dance-like motion mimicking the go-go dance. This phase is correlated with the T-wave on the surface ECG. Finally, activation of the inward rectifier potassium current ($I_{\text{K1}}$) and the sodium potassium exchange (NCX) complete the late phase of ventricular AP (phase 4), in which the membrane potential remains at its resting value until the initiation of the following cardiac cycle. This phase occurs during ventricular relaxation and is indicated on the T-Q of the ECG as a return to isoelectric line (Valentin et al., 2004). Therefore, the ventricular action potential has a closed relationship with the QT interval in ECGs on the body surface. Shortening and/or
lengthening of these durations (APD and QT interval) are useful indicators of effects of compounds principally on the repolarization phase of cardiac myocytes, since the depolarization phase represents only a fraction of the QT. APD$_{90}$ is the duration of cardiac action potential between onset of depolarization and when the AP achieves a value of 90% repolarization, and is measured from the midpoint of the upstroke until 90% repolarization (Valentin et al., 2004). Hence, understanding the electrophysiological characteristics of the changing of the APD and changes in AP morphology (e.g. triangulation, instability, etc.), and the ionic mechanism that underlie ventricular repolarization should provide a better insight into the arrhythmic risk of compounds that effects APD.

Afterdepolarizations (ADs)

Afterdepolarizations (ADs) are oscillations of the membrane potential that occur during phases 2 or 3 (EADs), or phase 4 (DADs) of the action potential. When the oscillations reach a critical threshold for activation of a depolarizing current, these oscillations can give rise to new action potentials known as “triggered events” and appear on the electrocardiogram as ectopic depolarizations (Volders et al., 2000).

There are two types of ADs (Figure 1.2): early afterdepolarizations (EADs) and delayed afterdepolarizations (DADs). EADs occur during phases 2 or 3 of the action potential, often in the setting of AP prolongation and with slow heart rates, because of an unbalance of the repolarization current (an increase in inward currents---slowed inactivation of I$_{CaL}$ or I$_{Na}$, a decrease in outward currents, or combinations).
Figure 1.1. Correlation between action potential duration and the QT interval on the body surface ECG. Notice that QRS complex is produced by upstroke of the action potential and the end of T wave represents the end of action potential duration. A schematic of the activity of the current during the action potential is shown in the center of the figure. The plateau of the action potential (between the two vertical lines) is a time of high membrane resistance and low current flow; therefore, small changes in depolarizing or repolarizing current at this time can have profound effects on the action potential shape and duration. (Courtesy of Dr. Robert L. Hamlin)
DADs are generated during phase 4 (after repolarization of an action potential) at more accelerated heart rates. The characteristics of EADs are slow rate of development, short duration, and small amplitude (Roden, 1993). The importance of EADs is that they can provide not only the trigger for premature ectopic depolarizations but also the substrate for initiation and perpetuation of torsade de pointes (Volders et al., 2000) by reentry due to slow propagation.

It has been known that DADs are caused by an arrhythmogenic transient inward current ($I_{TI}$) evoked by spontaneous calcium release from the SR under conditions that favor accumulation of calcium in the cytoplasm and result in intracellular calcium overload (Berlin et al., 1989). There are three different ionic currents, alone or in combination, implicated in the generation of $I_{TI}$: sodium-calcium exchange current ($I_{Na-Ca}$), non-selective cation current ($I_{NS}$), and calcium activated chloride current ($I_{Cl(Ca)}$) (Volders et al., 2000). However, the $I_{Na-Ca}$ is believed to be a major charge-carrying mechanism for $I_{TI}$ (Benndorf et al., 1993).

Major current generators for EADs are $I_{CaL}$ and $I_{Na}$ producing the upstroke (depolarizing) phase of EADs. They occur because cardiac cells enter into a relative refractory period, during which $I_{CaL}$ and $I_{Na}$ channels can be reactivated and undergo partial depolarization (Roden, 1993). The $I_{CaL}$ is thought to be responsible for EADs because of its voltage dependent activation falling in the range of membrane potential where repolarization is delayed (-35 to 0 mV; Volders et al., 2000). Another current thought responsible for EADs is $I_{Na-Ca}$. January and Riddle (1989) reported that inward $I_{Na-Ca}$ is likely to play a crucial role in the initial delay in repolarization, so-called “conditioning phase”, which appears as a transient delay in repolarization that may
or may not be followed by an EAD upstroke. Volders and co-workers (2000) also suggested that the primary role for \( I_{\text{Na-Ca}} \) is not only restricted to the conditioning phase of EADs related to calcium overload and spontaneous calcium release from the sarcoendoplasmic reticulum (i.e., isoproterenol-induced EADs), but applies also to EADs that occur in the setting of less extreme calcium loading. There is increasing evidence that inward \( I_{\text{Na-Ca}} \) has the primary role for initiating the so-called late EADs because of this phenomenon initiated at membrane potentials more negative than the range at which \( I_{\text{CaL}} \) can be activated (Szabo et al., 1995). Recently, Patterson et al. (1997) reported that \( I_{\text{Na-Ca}} \) has an important contribution in generating EADs when the action potential was prolonged by class III antiarrhythmic agents. In the chronic AV block dog, ryanodine, flunarizine and nisoldipine have been shown to terminate class III antiarrhythmic induced EADs, suggesting that calcium overload is the common mechanism responsible for EADs (Volders et al., 2000). In the new concept on EADs, increased cytoplasmic and/or subsarcolemmal calcium cycling determine the “go or no go” for EAD generation under various dynamic circumstances by setting the magnitude of inward \( I_{\text{Na-Ca}} \) (Volders et al., 2000).

An interesting study by Patterson et al. (1990) demonstrated that EADs and DADs can appear together in the same intact heart of the dog infused with cesium chloride (CsCl) which suggested that EADs and DADs could be based on similar cellular mechanisms via alteration of myocardial calcium homeostasis. This hypothesis was supported by the study of Vos and co-workers (1998) who found that ryanodine abolished both EADs and DADs induced by pacing in dogs with completed AV Block.
Figure 1.2. Schematic figure of two types of afterdepolarizations, A) early afterdepolarizations (EADs; both phase 2 and phase 3) and B) delayed afterdepolarizations (DADs). Both oscillations of the transmembrane potential depend on the preceding action potential and can trigger a new action potential when reach a critical threshold. (Courtesy of Burashnikov and Antzelevitch, 2006)
**Cardiac potassium channels**

Potassium channels in cardiac tissue can be divided into 1) voltage-activated channels (e.g. transient outward potassium channel, $I_{To}$; ultra rapid activated potassium channel, $I_{Kur}$; rapidly activated delayed rectifier potassium channel, $I_{Kr}$; slowly activated delayed rectifier potassium channel, $I_{Ks}$), 2) ligand-activated channels (acetylcholine activated potassium channel, $I_{KACb}$; ATP inhibited potassium channel, $I_{KATP}$; calcium activated potassium channel, $I_{KCa}$; potassium channel activated by fatty acid and amphiphiles, $I_{KAA}$), and 3) inward rectifier potassium channel, $I_{K1}$ conducts a background current that apparently does not gate ($I_{K1}$) (Carmeliet, 1999). All cardiac $K^+$ channels will carry outward current (repolarize the membrane during the action potential or stabilize the membrane at a hyperpolarized level) when activated because the equilibrium potential of $K^+$ is negative. Since the rapid component of the delayed rectifier potassium current ($I_{Kr}$) has a significant role in drug-induced QT prolongation, this review will emphasize only this channel. In the ventricle, the delayed rectifier potassium current ($I_{K}$) can be divided into two components: rapid component ($I_{Kr}$) and slow component ($I_{Ks}$). $I_{Kr}$ shows activation and inactivation, but $I_{Ks}$ shows only activation. Regional differences in the density of $I_{Kr}$ and $I_{Ks}$ exist transmurally (endo-epicarial), along the apico-basal axis, and between left and right ventricle, all contributing to the spatial heterogeneity of ventricular repolarization (Cheng and Kodama, 2004). In dog ventricle, $I_{Ks}$ density was shown to be significantly less in the midmyocardial ventricular (M) cell compared with sub-epicardial and sub-endocardial cells, whereas $I_{Kr}$ density was comparable within the three layers (Liu and Antzelevitch, 1995). In rabbit ventricle, $I_{Ks}$ density in sub-epicardial
myocytes was shown to be greater than that in sub-endocardial myocytes, whereas their $I_{Kr}$ densities were similar (Xu et al., 2001). In guinea pig, $I_{Kr}$ and $I_{Ks}$ densities in sub-endocardial myocytes are both smaller than those in mid-myocardial and sub-epicardial myocytes (Bryant et al., 1998). In rabbit ventricular myocytes, Cheng et al. (1999) demonstrated that the $I_{Kr}$ density was lower in the base than in the apex. In concordance with this observation, the QT interval was shown to be longer in the apex than in the base of isolated rabbit and intact canine hearts (Bauer et al., 2002; Iwata et al., 1996).

**hERG**

The human ether-a-go-go-related gene (hERG), also known as KCNH2, encodes the rapid component of the delayed rectifier potassium channel ($I_{Kr}$), which normally conducts an outward potassium current and is the key channel responsible for Phase 3 repolarization of the cardiac action potential (Lawrence et al., 2005). The KCNE2 gene encodes for the accessory protein of $I_{Kr}$ channel (Sides, 2002). The activation of this current plays a primary role in determining the duration of the action potential and consequently the QT interval. Mutations of hERG on chromosome 7 cause the second most common long QT syndrome (LQT2).

hERG is expressed in multiple tissues and cell types (i.e., neural, smooth muscle, and tumor cells) but it is most highly expressed in the heart (Sanguinetti and Tristani-Firouzi, 2006). hERG channel is formed by coassembly of four identical $\alpha$-subunits, each containing six $\alpha$-helical transmembrane domains, S1-S6 (Figure 1.3 and 1.4).
The channel pore is asymmetrical and its dimensions change when the channel gates from a closed to an open state (Figure 1.5). The extracellular end of the pore is a narrow cylinder called the \( \text{K}^+ \)-selectivity filter that is optimally constructed for conduction of \( \text{K}^+ \) ions. In each subunit, the side-chain hydroxyl group of Thr and the carbonyl oxygen atoms of the other four residues face towards the narrow ion-conduction pathway. Together these oxygen atoms form several octahedral binding sites that coordinate dehydrated \( \text{K}^+ \) ions arranged in a single file and separated by a single water molecule (Sanguinetti and Tristani-Firouzi, 2006; Zhou et al., 2001). Below the selectivity filter, the pore widens into a water-filled region, called the central cavity that is lined by the S6 \( \alpha \)-helices. In the closed state (Figure 1.5 bottom left), the four S6 domains criss-cross near the entire cytoplasmic interface to form a narrow aperture that is too small to permit entry of ions from the cytoplasm (Doyle et al., 1998; Sanguinetti and Tristani-Firouzi, 2006). In response to membrane depolarization (Figure 1.5 bottom right), the S6 \( \alpha \)-helices splay outwards and increase the diameter of the aperture to allow passage of ions (Sanguinetti and Tristani-Firouzi, 2006).

The primary voltage-sensing structure of hERG channels is the S4 \( \alpha \)-helical domain, which contains positively charged Lys or Arg residues in every third position. When the membrane is depolarized, the S4 moves outward. Voltage-dependent movement of the hERG S4 within the membrane electrical field can be detected as a small transient current or a change in the fluorescence of a fluorophore attached to an S4 domain (Sanguinetti and Tristani-Firouzi, 2006; Smith and Yellen, 2002). Negatively charged acidic residues in S1-S3 form transient salt bridges with specific basic residues in the S4 to stabilize the closed, intermediate and open states of hERG channels.
The outermost acidic Asp residues in S2 and S3 for a coordination site for external divalent cations that shield against salt-bridge formation and shift the voltage dependence of hERG opening to more positive potentials (Fernandez et al., 2005; Sanguinetti and Tristani-Firouzi, 2006). A unique feature of the I\text{Kr} channel is relatively slow activation-deactivation in comparison with rapid inactivation (Lehmann-Horn and Jurkat-Rott, 1999).

In clinical practice, drug-induced QT prolongation and TdP are caused by 1) direct blockage of hERG channels (Recanatini et al., 2005), 2) interference with hERG-channel trafficking to the cell surface (Kuryshev et al., 2005), or 3) drug-drug interactions (e.g., interference with metabolism) that ultimately lead to a reduction in hERG-channel current (Sanguinetti and Tristani-Firouzi, 2006).

hERG channels are blocked by chemicals with diverse structures that encompass several therapeutic drug classes, including antiarrhythmic, psychiatric, antimicrobial, prokinetic, and antihistamine so that the pharmaceutical companies need to screen compounds for hERG channel activity early during preclinical safety assessment (Sanguinetti and Mitcheson, 2005). The hERG channel is unusually susceptible to blockage by drugs in comparison with other voltage-gated K\textsuperscript{+} channels, suggesting that it has a unique binding site (Figure 1.6) (Sanguinetti and Tristani-Firouzi, 2006).

When the hERG channel is blocked there is a reduction in net repolarizing current causing the action potential to prolong and therefore the QT interval. It is this prolongation of the QT interval that has been linked to a potentially fatal but rare tachyarrhythmia known as TdP (Redfern et al., 2003; Yap and Camm, 2003).
Figure 1.3. Diagram of a single hERG subunit containing six α-helical transmembrane domains, S1-S6 (left panel). Notice the S4 domain contains multiple basic (+) amino acids while S1-S3 contains acidic Asp residues (-) which can form salt bridges with specific basic residues in S4 during gating. The N-terminal is a PAS domain and the C-Terminal is cyclic-nucleotide-binding domain (cNBD). Right panel is the crystal structure of a single α-subunit viewed from the side. Color coding of the helical domains is the same as in the top panel. Grey spheres represent K⁺ ions. (Courtesy of Sanguinetti and Tristani-firouzi, 2006)
Figure 1.4. Structural features of voltage-gated $K^+$ channels. View from the cytoplasmic side (left panel) and side view (right panel) of the membrane of the crystal structure of the complete, tetrameric Kv1.2 channel. Color coding of the helical domains is the same as in Figure 1.3. (Courtesy of Sanguinetti and Tristani-Firouzi, 2006)
Figure 1.5. Conformation of a single hERG channel. Single hERG channel (top panel), a voltage dependent channel, is either closed, open or inactivated, depending on transmembrane voltage. Channels are closed at negative voltages. Membrane depolarization slowly activates (opens) the channels, which then inactivate rapidly, especially at higher potentials. Repolarization of the membrane reverses the transitions between these channels states. C-type inactivation is thought to be caused by constriction of the selectivity filter (circled in red). Bottom left is the structure of a KcsA K\(^+\) channel crystallized in the closed state. Only two of the four subunits are shown. White spheres are K\(^+\) ions located within the selectivity filter. The Gly (red) and Tyr (yellow) residues of the selectivity filter are also indicated. Bottom right is the structure of the pore domain of a Kv1.2 K\(^+\) channel crystallized in the open state. Only two of the four subunits are shown. (Courtesy of Sanguinetti and Tristani-Firouzi, 2006).
Figure 1.6. Model of the hERG drug-binding site. Homology model of the hERG-channel pore module (S5, S6 and pore helix of two subunits) based on the crystal structure of KvAP. The key residues that interact with structurally diverse drugs are highlighted, including Thr 623 (orange) and Ser 624 (white) located at the base of the pore helix, and Tyr 652 (yellow) and Phe 656 (magenta) located on the S6 domain. (Courtesy of Sanguinetti and Tristani-Firouzi, 2006)
**QT/QTc prolongation**

As mentioned above, the QT interval is measured from the beginning of the QRS complex to the end of the T wave (Figure 1.7), therefore it reflects the duration of ventricular depolarization and ventricular repolarization which is roughly parallel to the ventricular refractory period. The QT interval consists of 2 components: the QRS complex represents ventricular depolarization and the JT interval, a measure of the duration of ventricular repolarization.

Since QT duration changes inversely with heart rate; the slower the heart rate the longer the QT interval. Hence, a QT correction formula is needed to substitute for each measured QT interval. The corrected QT (QTc) value corresponds to one that would have been measured had each ECG tracing been recorded at the same heart rate (Bednar et al., 2001). The three most common correction methods are Bazett’s equation (QTcB = QT/RR^{0.5}; Bazett, 1920), Fridericia’s equation (QTcF=QT/RR^{0.33}; Fridericia, 1920), and Van de water’s equation (QTcV=QT-0.087(RR-1000); van de Water, 1989).

The QT interval prolongation may arise from either a decrease in repolarizing cardiac membrane currents or an increase in depolarizing cardiac currents late in the cardiac cycle. Most commonly, QT interval prolongation is produced by delayed repolarization due to reductions in either the rapidly or the slowly activating delayed rectifier cardiac potassium currents. Less commonly, QT interval prolongation results from prolonged depolarization due to a small persistent inward leak in cardiac sodium.
current or from a sustained sodium current. QT interval prolongation can be characterized as acquired (drug-induced QT prolongation) or congenital known as long QT syndrome (LQTs), a rare genetic disorder associated with life-threatening arrhythmias.

Prolongation of ventricular repolarization and consequently lengthening of QT and/or QTc interval results in an increase in the absolute refractory period. This is the mechanism by which some antiarrhythmic drugs prevent or terminate ventricular tachyarrhythmias; however, prolongation of ventricular repolarization may be also implicated with arrhythmias especially torsades de pointes. Therefore, QTc prolongation is widely viewed as a surrogate marker of the arrhythmogenic potential of a drug. The precise relationship between the extent of QTc prolongation and the risk for TdP is unknown. Recently published data in humans showed that TdP rarely occurs unless the QTc exceeds 500 ms (Bednar et al., 2001).

A recent review of the value of preclinical cardiac electro physiological data to assess the risk of TdP induced by drugs in clinical use concluded that delayed ventricular repolarization per se is not proarrhythmic (Yap and Camm, 2003). Thus, other factors must coincide to enable QT interval prolongation to become a proarrhythmic event (Schneider et al., 2005). Hondeghem et al. (2001a) suggested that the prolongation of the APD and the QTc interval is antiarrhythmic, as long as it is not associated with instability identified in Poincaré plots, or by triangulation of the action potential or reverse-use dependency.
Figure 1.7. Schematic representation of normal electrocardiogram tracing with waves, segments, and intervals labeled. QT interval, the period between the beginning of the QRS complex and the end of the T wave of electrocardiogram, reflects the ventricular action potential duration (APD) and represents the time required for ventricular depolarization and repolarization.
Mechanisms underlying drug-induced QT prolongation and Torsades de pointes

Multiple ion currents contribute to the repolarization phase of the action potential; therefore, the pharmacological agents that can produce prolonged repolarization could be acting through these mechanisms: 1) blockade of I_{Kr} and/or I_{Ks} currents (i.e., dofetilide, ibutilide, sotalol, quinidine, cisapride, terfenadine), 2) blockade of I_{to} current (i.e., 4-aminopyridine), 3) prolonged opening of I_{Ca} (i.e., Bay K 8644), 4) delay inactivation of I_{Na} (i.e., aconitine, veratridine, batrachotoxin, DPI-201106 and ATX-II).

As mention previously, drug-induced QT interval prolongation and TdP most often occurs with drugs that block the efflux of K^+ through the I_{Kr} channel. The factors that might increase susceptibility of I_{Kr} channel to blockade are the large size of the I_{Kr} channel vestibule and the amino acid constituents of the pore-forming region (Sides, 2002). Drug access to the channel is from the cytoplasm and enters the channel vestibule when the activation gate is open. The drug is trapped in the voltage-gated channel with a following depolarization (Mitcheson et al., 2000) consequently causing decreased efflux of K^+ from the myocyte. This results in prolonged APD and QT interval. The amino acid residues important for a high-affinity block of hERG by many compounds have been described previously (Lees-Miller et al., 2000; Mitcheson et al., 2000; Kamiya et al., 2001; Sanchez-Chapula et al., 2002, 2003). Two S6 residues, Tyr652 and Phe656, are critical for channel block (Perry et al., 2004). Aromatic residues can bind drug molecules by \pi-stack interactions with phenyl groups and cation-\pi interactions with protonated
nitrogens (Dougherty, 1996; Perry et al., 2004). In addition to the S6 aromatic residues, three residues (Thr623, Ser624, and Val625) located at the C-terminal end of the pore helices just before the GFG selectivity filter were found to be involved in drug block (Mitcheson et al., 2000; Perry et al., 2004). For instance, clofilium and ibutilide have been shown to block hERG channels at Tyr652 and Phe656 residues (Perry et al., 2004) (Figure 1.8) whereas dofetilide has been shown to block hERG channel at Thr623, Ser624, and Val625 residues in the open state (Kamiya et al., 2006).

Another mechanism of drug-induced QT prolongation is by altered trafficking of protein that transfers channel from nuclear stimulation to cardiac membrane. This alteration reduces the number of functional channels expressed in the sarcolemmal membrane, contributing to a reduction in repolarizing current, but the biophysical properties of the channel are still functioning normally (Furutani et al., 1999). Roden and Kupershmidt (1999) have reviewed the multiple processes determining the way in which the nucleotide sequence of the DNA molecule is inserted as channel protein into the membrane of the cardiomyocyte (Figure 1.9). The first step is gene transcription from DNA to mRNA. The primary transcript is processed extensively in the nucleus before it is translated into a protein in the endoplasmic reticulum (ER). hERG undergoes alternative splicing, which results in the formation of two hERG isoforms; only one of them results in functional currents in the heart. The hERG channel protein is synthesized in the lumen of the rough endoplasmic reticulum and processed by cytosolic chaperons Hsp70 and Hsp90. The chaperones are crucial for productive folding and maturation of hERG (Ficker et al., 2005). The Hsp70 acts by holding most newly synthesized proteins in a folding competent state, whereas Hsp90 is required for folding of a small subset of
proteins (Ficker et al., 2003). The processed hERG channel protein is inserted into transport vesicles and taken to the cell surface where membrane fusion results in insertion of the protein into the cell membrane. The assembly of different α-subunits likely occurs prior to transport to the cell surface (Hoffmann and Warner, 2006). Arsenic trioxide, a compound that is used in treatment of leukemias, was the first compound that has been reported by Ficker and coworkers (2004) to interfere with the formation of hERG/chaperone complexes and to inhibit hERG maturation causing abnormal cardiac repolarization. Other compounds that inhibit hERG trafficking are geldanamycin (Ficker et al., 2003) and pentamidine (Kuryshev et al., 2005). Geldanamycin, an ansamycin antibiotic that blocks Hsp90, is used to target oncogenic kinases for degradation and is being tested in preclinical as well as clinical trials against various cancers (Neckers, 2002). Pentamidine isethionate, an antiprotozoal agent, is used in developing countries for the treatment of parasitic diseases such as trypanosomiasis and antimony-resistant visceral leishmaniasis but it is used in the treatment of Pneumocystis carinii pneumonia in the United States (Kuryshev et al., 2005).

In addition to direct blocking of hERG channels and interfering with hERG trafficking, drug-drug interaction can interfere with QT prolongation intensity of test articles, e.g. terfenadine, astemizole, pimozide, cisapride, and levacetylmethadol are metabolized by CYP3A4 enzyme. This enzyme is highly susceptible to liver disease and can be inhibited by azole antifungals and macrolide antibiotics (Shah, 2002).

The mechanism for the generation of TdP is not clearly understood. An EAD-induced extrasystole is believed to be responsible for the premature beat that initiates TdP, but the maintenance of the arrhythmia is generally thought to be due to circus
movement re-entry (Antzelevitch and Shimizu, 2002). Amplified electrical heterogeneity principally in the form of transmural dispersion of repolarization by 1) agents that reduce net repolarizing currents, 2) ion channel mutations or 3) ventricular remodeling (e.g., hypertrophic and dilated cardiomyopathy) were proposed by the Antzelevitch group to be responsible for creating vulnerable windows for the development of re-entry (Figure 1.10; Antzelevitch, 2005).
Figure 1.8. Docking of clofilium within the inner cavity of a homology model of the hERG channel. A, side view of one possible binding mode that is highly consistent with the experimental results. The S5-S6 domains of the channel are represented by shaded ribbons, with the four subunits distinguished by different colors. B, stereoview of the region outlined by the yellow box in A. The model in A was rotated about the vertical axis to get the best view of the perpendicular \( \pi \)-stacking interactions of the phenyl group of clofilium with Tyr652 and hydrophobic interactions of the aliphatic tail of clofilium with Phe656. Clofilium is represented by ball and sticks, with the chlorine atom in green, nitrogen atom in blue, and carbon atoms in orange. In this binding mode, the clofilium is interacting with residues from the cyan subunit only, which is shown in line-ribbon mode with key residues highlighted with colored sticks: Tyr652 (green), Phe656 (red), Thr623 (blue-green), Ser624 (pink), and Val625 (blue). (Courtesy of Perry et al., 2004)
Figure 1.9. Schematic view of mechanisms underlying ion channel expression. The specific steps underlying channel expression are indicated under the figure. DNA transcription and mRNA processing occur in the nucleus. mRNA translation (to ion channels, transcription factors, or other proteins) occurs in the endoplasmic reticulum (ER), and channels are then transported to the cell surface by vesicles secreted by the Golgi apparatus. Available evidence suggests that channels likely complex with multiple other proteins (such as anchoring elements, β-subunits, or kinases) at the cell surface. (Courtesy of CDI).
Figure 1.10. Mechanism by which transmural dispersion of repolarization and transseptal dispersion of repolarization precipitate torsades de pointes. APD = action potential duration; EAD = early afterdepolarization; M cells = midmyocardial cells. (Courtesy of Antzelevitch, 2005).
Predisposing factors for generation of drug-induced TdP

1. Bradycardia

Physiologically, when the heart rate slows, the repolarization time increases—an $I_{Kr}$ effect. It has been confirmed in numerous experiments (Brachmann et al., 1983; Levine et al., 1985; Vos et al., 2001) that slowing of the heart rate induces increase in APD which can be an essential factor for genesis of TdP. Recently, Volders et al (1998) have shown that sustained slow heart rate (e.g., chronic AV block) induced electrical remodeling which will increase electrical dispersion of repolarization (Antzelevitch et al., 1999).

2. Hypokalemia/hypomagnesemia

In experimental studies, diuretic administration resulting in hypokalemia will increase the incidence of TdP (Weissenburger et al., 1991). This could be explained by the fact that hypokalemia decreases the $I_{Kr}$ current, which consequently prolongs the APD (Yang et al., 1997). Hypomagnesemia has been reported in patients who have episodes of TdP (Marsepoil et al., 1985; Takanaka et al., 1997). Moreover, intravenous magnesium sulfate has been used to control episodes of TdP effectively (Takanaka et al., 1997). In barium-induced spontaneous action potentials in canine Purkinje fiber preparations, magnesium has been shown to shorten APD$_{20}$, decrease the amplitude, and diminish the maximum upstroke velocity of the action potential (Takanaka et al., 1997). In humans, administration of magnesium sulphate results in shortening of sinus cycle length and
decreased MAPD$_{50}$, MAPD$_{90}$, and ventricular effective refractory period (Parikka and Toivonen, 1999). Therefore, magnesium may suppress TdP by direct inhibition of the development of triggered potentials (Takanaka et al., 1997).

3. Gender

The literature, Makkar et al (1993), shows that females (approximately 70% of reported cases) are predisposed to drug-induced TdP. It could be because women had intrinsically longer repolarization times than men (Vos et al., 2001). Another possible explanation, suggested by Pham et al (2001), is that testosterone has a protective effect against drug-induced TdP.

4. Electrical remodeling

Chronic heart failure, chronic AV block, and post-myocardial infarction have been shown to have an influence on drug-induced TdP due to electrical remodeling (e.g., dog and rabbit with chronic AV block) (Verduyn et al., 2001a). These remodeling processes contribute to an enhanced susceptibility to drug-induced TdP arrhythmias by 1) increases in ventricular APD, 2) higher incidence of EADs and DADs, 3) enhanced amount of interventricular dispersion of QT (de Groot et al, 2000; Sipido et al., 2000; Volders et al., 1998; Vos et al., 1998). Sipido et al (2000) suggested that the electrical remodeling is partly due to down-regulation of both I$_{Kr}$ and I$_{Ks}$, and up-regulation of NCX.
5. Congenital long QT syndrome

The congenital LQTs is caused by mutations of genes that encode cardiac ion channels (e.g., KVLQT1, minK, and hERG encoding for potassium channels; SCN5A encodes for the cardiac sodium channel) (El-Sherif et al., 1999). These mutations cause abnormal delay between the electrical excitation and relaxation of the ventricles. Individuals with LQTs have prolongation of the QT interval on the ECG. It is associated with syncope (loss of consciousness) and occasionally with sudden death due to ventricular fibrillation. Arrhythmias in individuals with LQTs are often associated with exercise or excitement, in contradistinction to TdP occurring due to drugs, which occur at low heart rates. LQTs can be inherited in an autosomal dominant or an autosomal recessive fashion. The autosomal recessive forms of LQTs tend to have a more severe phenotype (i.e., leading to sudden death), with some variants being associated with syndactyly (LQT8) or congenital neural deafness (LQT1). An autosomal recessive form of LQTs with associated congenital deafness is called the Jervell and Lang-Nielsen syndrome (JLNS) (Ching and Tan, 2006). It is caused specifically by mutation of the KCNE1 (LQT5) (10%) and KCNQ1 (LQT1) (90%) genes. An autosomal dominant form of LQTS that is not associated with deafness is called Romano-Ward syndrome.

Generally, during adrenergic states, repolarization currents will also be enhanced thus shortening the action potential; however, absence of shortening is normally found in LQTs. It is believed that adrenergic stimulation (e.g., exercise, excitement) can increase the risk of sudden death in individuals with LQTs due to an increase in the activity of I_CaL channels.
The re-opening of I_{cal} channels during the plateau phase of the cardiac AP has been demonstrated to cause EADs, which is the trigger of TdP (Modell and Lehmann, 2006).

6. Drug dosage—plasma and tissue levels

Generally, dose dependency exists in relation to incidence of TdP; the higher the dose, the higher the incidence of TdP incidence (Weissenburger et al., 1991) except for quinidine (Vos et al., 2001). Another important contributor is co-administration of drugs that interfere with the first-pass metabolism of torsadogens. A delayed or slowed clearance of the drug can create higher plasma or tissue levels than anticipated, based on the dosage of the drug used (e.g., co-administration of macrolide antibiotics and azole antifungal drugs that inhibit cytochrome P4503a4 (CYP3A4)) (Vos et al., 2001).

Indices for proarrhythmic activity

Currently, the lengthening of QTc interval is the surrogate marker for drug-induced TdP. However, several studies (Antzelevitch and Shimizu, 2002; Belardinelli et al., 2003; Franz et al., 1992; Hondeghem et al., 2001a; Hondeghem et al., 2001b; Milberg et al., 2002; Milberg et al., 2004; Poelzing and Rosenbaum, 2004, Thomsen et al., 2004; Yap and Camm, 2003) have shown that QTc prolongation is not a good marker because similar prolongation of QTc intervals may have distinct arrhythmogenic outcomes (Thomsen et al., 2004). The clarification of the electrophysiological events that
underlie TdP may provide a useful surrogate marker for the identification of drugs with
the potential of causing TdP. Hence, other parameters have been suggested to be more
valuable and crucial in assessing arrhythmogenicity of NCE [e.g., TRIaD, beat-to-beat
variability, transmural (spatial) dispersion of repolarization (TDR), etc.] (Berger et al.,
1997; Fossa et al., 2002; Gbadebo et al., 2002; Hondeghem et al., 2001a; Hondeghem
et al., 2001b; Thomsen et al., 2004; Zabel and Malik, 2002; van Opstal et al., 2002).

1. Lengthening of APD and QTc interval

Excessive prolongation of the QTc interval may lead to the development of TdP.
Therefore, at present, the best available surrogate marker of the risk of TdP is QTc
interval prolongation even though it is not a perfect marker. The CPMP document
recommended that the QT interval increases from baseline in individual patients of <30
ms are unlikely to represent risk of inducing TdP, 30-60 ms are more likely to represent
a drug effect and >60 ms raise clear concerns about the potential risk of inducing TdP
(Sides, 2002).

Prolongation of the QT interval in telemetered dogs and primates has a high
predictive value for QT interval prolongation in man; however, prolongation of QTc
interval has a weak correlation with TdP incidence in humans (Hoffmann and Warner,
2006). Recently, Thomsen et al. (2004) have shown that there is little relationship
between the magnitude of the QT interval and the incidence of TdP. This finding is also
supported by the fact that Ziprasidone prolongs QTc to a greater extent than haloperidol,
risperidone or olanzapine, but ziprasidone has not been associated with TdP (Taylor,
2003).
2. Increase TRIaD elements

TRIaD is a term coined by Hondeghem and represents triangulation (T), reverse-use-dependence (R) and instability (I) (high variability of APD, beat-to-beat) and dispersion (D) of the AP (Hondeghem et al., 2001a; Hondeghem et al., 2003; Valentin et al., 2004).

**Triangulation (T)** is derived from a qualitative assessment of the AP. In the presence of certain drugs that delay repolarization, the typical square shape of the AP becomes more triangular in appearance. Constitutively, this is defined as the repolarization time from APD$_{30}$ to APD$_{90}$ expressed in ms (Figure 1.11), which reflects the rate of repolarization (Phase 3) of the AP. It is believable that blocking the repolarizing currents during Phase 3 slows repolarization and causes triangulation, leading to a change of repolarization and finally to triggered events such as EADs, TdP, re-entry, and ultimately ventricular fibrillation (Valentin et al., 2004). Unlike oscillations during phase 3, oscillations during the plateau (Phase 2) are inherently less arrhythmogenic because they fall in the absolute refractory period (Valentin et al., 2004).

Valentin and colleagues (2004) suggested several reasons why the slowing of phase 3 repolarization might be a cause for concern.

1. Increasing the time spent in the voltage window for the calcium current can trigger early EADs during the repolarization (January et al., 1991). These oscillations could be suppressed by calcium channel blockers (Mason et al., 1983).

2. Increasing the time in the voltage window for the sodium current can similarly yield late EADs (Katzung et al., 1975). These oscillations could be suppressed by sodium channel blockers (Mason et al., 1983).
3. During the final part of repolarization, the recovery of sodium channels from inactivation provides more time for the currents to trigger EADs, and if they are propagated, they propagate more slowly because they fall in the relative refractory period of conductile tissue which then conducts more slowly and allows for reentrant loops.

4. Many potassium channels are open at the end (late Phase 3) of the AP and early in diastole (phase 4) leading to a clamp of the membrane potential closer to the potassium equilibrium potential. This reduces tissue impedance, rendering less likely activation due to current flow between cells at different potentials. For the above reason, it is expected that prolongation of APD by triangulation will be more supportive of the occurrence of TdP than prolongation of APD by extension of the plateau.

**Reverse-use dependency** reflects the fact that for certain drugs that delay repolarization, the prolongation of the AP is greater at low heart rate (long cycle lengths) compared with high heart rates (short cycle lengths). Therefore, it increases the likelihood that TdP occurs at low heart rates (Valentin et al., 2004). In the SCREENIT, reverse-use dependence is measured as the difference between APD$_{60}$ and APD$_{90}$ at different cycle lengths.

**Instability** reflects the beat to beat variability in the duration of the APD. Increased instability is seen as an increased difference in the duration of successive APs. The variability of APD has been ascribed to stochastic variation in the slowly inactivating sodium current, the delayed rectifier current, intra-cellular calcium transients, and reduced cellular coupling (Valentin et al., 2004). Interestingly, recent data demonstrated that increases in instability are frequently detected before (e.g., at lower concentrations or
earlier time points) increases in triangulation or reverse-use dependency are observed (Hondeghem et al., 2003). Typically, instability can be illustrated by plotting the $\text{APD}_{60}$ of a beat against the $\text{APD}_{60}$ of the preceding beat. This constitutes a Poincaré plot, which is the easiest way to rapidly differentiate drugs with different torsadogenic propensity. Hence, if the Poincaré plot indicates increased variability, the proarrhythmic potential is great; however, if the Poincaré is negative, it remains important to analyze the reverse-use dependency and triangulation of the AP (Valentin et al., 2004). Hondeghem and coworkers (2001a) reported that the most important parameter relating to proarrhythmia appears to be instability because compounds that induced instability tend to be more torsadogenic, whereas agents that did not exhibit instability tended to be less so.
Figure 1.11. Schematic representation of monophasic action potentials illustrating the concept of triangulation. Triangulation is defined as relative prolongation of the repolarization time from APD$_{30}$ to APD$_{90}$. (Courtesy of Valentin et al., 2004).
3. Blocking of hERG assay

As mentioned previously, the hERG gene encodes the pore-forming subunit of the hERG or I_{Kr} channels. Inhibition of the hERG current leads to prolongation of AP and consequently lengthening of QTc interval and the generation of potentially fatal arrhythmias. The vast majority of the work has involved electrophysiological measurements of currents from single cells, and this approach remains the “gold standard” for testing hERG channel physiology (Witchel et al., 2002). These electrophysiological recordings are obtained when in the voltage clamp mode. Until recently, most ion-channel studies have been performed using isolated single cells usually from cardiac myocytes in which the channels of interest were naturally present with auxiliary subunits or intracellular factors that modulate channel physiology. On the other hand, the use of isolated myocytes poses technical difficulties, including the need to isolate fresh myocytes on each experimental day and possible overlap of the current of interest with other ionic currents (Fenichel et al., 2004). More recently, the conventional hERG patch clamp has been performed by using one of these cells: isolated ventricular myocytes, atrial cell lines, neuroblastoma cell lines. But the majority of studies employ cells which have the hERG channel introduced into them using molecular biological methods, known as “heterologous expression”, such as: transfected human embryonic kidney-293 cells (HEK 293), Chinese Hamster Ovary (CHO) cells, or Xenopus oocytes expressing high levels of functional hERG. The conventional whole-cell patch clamp configuration is used to record membrane currents in single cells. Cells are continuously superfused with a bath solution and measurements are made either at room temperature or 37°C.
In order to record hERG tail currents, the cells are clamped at a holding potential of -80 mV. Then the cell receives depolarizing step voltages at or above 0 mV (e.g., +30 mV) for long enough to activate the channel (4 to 5 seconds), followed by a repolarization to a voltage (e.g., -50 mV) at which two conditions are met: the channel recovers from inactivation, and there is sufficient electrochemical force to drive K\(^+\) ions through the channel (Witchel et al., 2002). The current flow through the hERG channel is recorded as a tail current. The amplitude of the tail current reflects the activity of the hERG channel and is measured as the difference between the peak of the current (measured just after repolarization) and the steady-state current measured at -50 mV. Then, a concentration response curve occurs. The inhibitory concentration (IC\(_{50}\)) is compared to the effective concentration (EC\(_{50}\)) to determine a cardiac safety index (Cavero et al., 2000). Clinically relevant blockade is generally accepted to occur when > 20% inhibition of the I\(_{Kr}\) channel is observed at anticipated therapeutic plasma or myocardial tissue concentration of parent drug or metabolite(s) (Sides, 2002). A ratio of IC\(_{50}\) to EC\(_{50}\) of 1:10 to 1:100 is required based upon the seriousness of the disease for which the drug is indicated (i.e., cancer versus rhinorrhea) and the novelty of the drug.

It has been shown that the degree of I\(_{Kr}\) blockade has proven to be an imperfect predictor of torsadogenicity, failing to distinguish verapamil (IC\(_{50} = 143\) nM) and loratadine (IC\(_{50} = 173\) nM) from terfenadine (IC\(_{50} = 150\) nM) and quinidine (IC\(_{50} = 0.2–1.0\) µM at normal potassium levels). Verapamil is a moderately potent I\(_{Kr}\) blocker and thus might be expected at ordinary concentrations to prolong repolarization. However, verapamil blocks I\(_{CaL}\) channels with similar potency. The usual effect of verapamil is to
shorten repolarization, and has not been associated with tachyarrhythmias (Fenichel et al., 2004). IC$_{50}$ for I$_{Kr}$ is less significant for drugs that affect many channels that might possess opposing physiological effects.

4. Beat-to-beat variability of the QT interval

Temporal instability of the repolarization phase of the action potential is described as heterogeneity of the action potential duration from one cycle to the next cycle. In view of the predictive value of *in vitro* instability of the action potential duration, Hondeghem and colleagues (2003) reported that the instability of repolarization was found to be a reliable predictor of the proarrhythmic risk of hERG channel blocking agents demonstrated in isolated rabbit hearts (Hondeghem et al., 2001a, 2003; Hondeghem and Hoffmann, 2003). Interestingly, the evaluation of *in vivo* instability of the QT interval also adds useful information to the proarrhythmic risk assessment of QT interval prolonging agents. The variability of the QT duration was measured in a beat-to-beat electrocardiographic analysis (Schneider et al., 2005). To perform beat-to-beat analysis, QT values of many consecutive cycles (i.e., 30, 100, 300 cycles) were recorded and listed in an excel sheet. Poincaré plot with QT$_n$ versus QT$_{n+1}$ values (ms) were prepared for each of the two beat-to-beat analysis times. Much work has been done to quantify variability of the Poincaré QT plot (Schneider et al., 2005; Thomsen et al., 2004; van der Linde et al., 2005) and the methods are list below.
4.1 Thomsen and colleagues (2004) proposed a formula for measuring short-term variability (STV) and long-term variability (LTV) adopted from investigations of heart rate variability (Brennan et al., 2001) using Holter monitoring of humans over several hours. The steady changes over time tend to follow the diagonal and sudden changes result in a deviation from the diagonal. In order to examine the beat-to-beat variability of QT interval, Poincaré plots were generated by plotting each duration in millisecond (QT_n) against the former value (QT_{n+1}) for 30 consecutive beats of sinus origin. STV is the mean orthogonal distance from the diagonal to the points of Poincaré plot whereas LTV is the average distance to the mean of the parameter parallel to the diagonal.

\[
STV = \frac{\sum |D_{n+1} - D_n|}{\sqrt{2}}
\]

\[
LTV = \frac{\sum |D_{n+1} + D_n - 2D_{\text{mean}}|}{\sqrt{2}}
\]

D = the duration of QT (ms)

In the study of Thomsen et al. (2004), the predictive value of different repolarization parameters (e.g., QT, QTc, LV MAPD, QT plot area, LV plot area, QTVI_{30}, instability_{30}), including beat-to-beat variability of repolarization was compared in dogs with chronic AV block anesthetized with halothane to which d-sotalol was administered. TdP occurrence was associated with an increase in STV of the left ventricular MAPD when the dogs were infused with d-sotalol whereas STV remains unchanged when the dogs were treated with amiodarone. The authors concluded that STV could be a
new parameter to predict drug-induced TdP in patients. Moreover, an increase in beat-to-beat variability of repolarization has been shown in dogs with chronic AV block that died suddenly (Thomsen et al., 2005).

In the study of Schneider et al (2005), the same quantification method of Poincaré plot was used to measure QT interval variability in conscious telemetry dog; however, STV was named as $P$-width and LTV was named as $P$-length. In this study, 100 cardiac cycles were used to calculated $P$-width and $P$-length. The authors found that proarrhythmic agents, but not non-proarrhythmic agents, increase temporal dispersion of cardiac repolarization.

4.2 The index of QT instability, a novel mathematical model for quantification of the beat-to-beat variability of the QT interval, was proposed by van der Linde and colleagues (2005). In this method, the QT$_{n+1}$ was plotted as a function of QT$_n$ in a Poincaré plot (van der Linde et al., 2005). An ECG tracing of 30 consecutive beats, without either artifacts or ectopic beats, was chosen for this analysis. The width of Poincaré plot is a measurement of the short-term instability (Figure 1.12), the length of the plot is a measurement of long-term instability (LTI), and a width- and length-dependent parameter is an indicator for the total instability (TI). This analysis composed of 4 steps as follows:
a) Calculated the center of gravity (cg)

Center of gravity (cg):

\[ cg(x) = \frac{\sum_{i=m}^{m+29} (QT_i)}{30} \]

\[ cg(y) = \frac{\sum_{i=m+1}^{m+30} (QT_i)}{30} \]

b) Calculated the Total instability (TI) over the 30 points

Total instability (TI):

\[ TI_n = \sqrt{[cg(x)-QT_n]^2 + [cg(y)-QT_{n+1}]} \]

\[ TI = M(TI_n) \]

c) Calculated rotation of -45° around the origin of cg

Rotated cg(x):

\[ R_{cg}(x) = [\cos \theta \times cg(x)] - [\sin \theta \times cg(y)] \]

Rotated cg(y):

\[ R_{cg}(y) = [\sin \theta \times cg(x)] + [\cos \theta \times cg(y)] \]
\textit{d) Calculated LTI and STI}

Long-Term instability: \[ LTI_n = \left| R_{cg}(x) - [(\cos \theta \times QT_{n+1}) - (\sin \theta \times QT_n)] \right| \]

\[ LTI = M(LTI_n) \]

Short-Term instability: \[ STI_n = \left| R_{cg}(y) - [(\sin \theta \times QT_{n+1}) + (\cos \theta \times QT_n)] \right| \]

\[ STI = M(STI_n) \]

Whereas:
- \( n \) = Number of the beat from 1 to 29
- \( m \) = The first beat of a selected epoch
- \( cg(x) \) = x-coordinate of center of gravity
- \( cg(y) \) = y-coordinate of center of gravity
- \( M \) = Median over 30 beats
- \( \theta = -\pi/4 \)

In the study of \textit{van der Linde} et al. (2005), dofetilide administration to anesthetized dogs prolongs ventricular repolarization, concomitantly increases beat-to-beat QT instability and induces EADs. The authors suggested that the use of short-term, long-term, and total instability parameters in this anesthetized dog model shows the ability to identify clear potential for cardiovascular safety assessment (\textit{van der Linde} et al., 2005).
4.3 The QT variability index (QTVI) is another way to express beat-to-beat variability of QT interval. This method was first used by Berger and colleagues (1997) in patient with dilated cardiomyopathy. A normalized QT variability index from 256 second epochs was derived according to the equation:

\[
\text{QTVI} = \log_{10} \left[ \frac{(QT_v/QT_m^2)/(HR_v/HR_m^2)} \right]
\]

\[\begin{align*}
QT_v &= \text{variance of QT interval (ms)} \\
QT_m &= \text{mean of QT interval (ms)} \\
HR_v &= \text{variance of QT interval (bpm)} \\
HR_m &= \text{mean of heart rate (bpm)}
\end{align*}\]

4.4 QT instability_{30} (QTI_{30}) was first used in the study of Thomsen et al. (2004) for measuring beat-to-beat instability of QT interval obtained from 30 consecutive beats originated from sinus node in the canine heart; however, this formula was adopted from Hondeghem and colleagues (2001b) who originally used it for measuring instability of action potential duration at 60% of depolarization (APD_{60}) in isolated perfused pacing rabbit heart, the SCREENIT system. In order to calculate QTI_{30}, the QT interval (ms) was sorted by linear interpolation, and then the median, the upper and the lower 25% values were computed. An instability index was obtained by computing the difference between the upper and lower quartile estimates in milliseconds.

\[\text{QTI}_{30} = 75^{th} \text{ percentile} - 25^{th} \text{ percentile}\]
Figure 1.12. Quantification of the instability visualized in the Poincaré plot is based on the distances of a cluster of 30 QT intervals to the center of gravity. (Courtesy of van der Linde et al., 2005)
5. Increase Tpeak-Tend/TDR

Transmural dispersion of repolarization (TDR) is the difference of repolarization across the ventricular wall...also known as spatial dispersion of repolarization (Belardinelli et al., 2003). Generally the T wave is the result of repolarization gradients within the ventricles (Yan and Antzelevitch, 1998). That is, the transmembrane action potentials recorded from the right ventricle are usually longer than those from the left; APs from the apical regions are generally longer than those recorded near the base; the duration of epicardial APs is longer than endocardial APs. The differences in configurations and durations of action potentials determine durations of refractory periods, and are due to the variable expressions and densities of ionic currents in the different cell types of myocardium. In every species tested, including dogs, rabbits, guinea pigs, pigs, and humans, the myocardium has been found to include three different tissues, roughly, layered within the endocardium, a middle region (the M cells), and the epicardium. The three layers are histologically indistinguishable, but electrophysiologically distinct, again based upon durations of action potentials. Among many other differences, the repolarizing current $I_{Ks}$ is important in the epicardium and endocardium, but much less so in the M cells, and once depolarization is well under way, the depolarizing current $I_{Na}$ tapers off rapidly in epicardium and endocardium, but it lingers, opposing the repolarization currents, in M cells (Fenichel et al., 2004).

The transmural differences in action-potential durations are normally no more than a few ms, but drug effects can change the relationships. With relatively low $I_{Ks}$ activity, the repolarization of M cells is critically dependent on $I_{Kr}$. When $I_{Kr}$ is blocked,
repolarization is delayed everywhere, but much more in the M cells than in those of the epicardium or endocardium. As a result, not only is the QT interval prolonged, but the TDR is prolonged to an even greater degree (Fenichel et al., 2004).

It was demonstrated that the increase in dispersion of ventricular repolarization serves as a substrate for re-entrant ventricular tachycardia (Antzelevitch and Shimizu, 2002). An increase in TDR seems to play an important role in determining whether an EAD propagates transmurally to induce an ectopic beat, R-on-T extrasystole and/or TdP. These data point to transmural re-entry as a mechanism for the maintenance of TdP and to a transmurally conducted EAD as the initiating mechanism. The findings of Yan et al. (2001) suggest that an increase in TDR not only facilitates transmural propagation of EADs but importantly seems to contribute to the maintenance of TdP. This is consistent with the clinical observation that patients with congenital Long QT Syndromes have longer QTend-QTpeak intervals and therefore an increased TDR (Lubinski et al., 1998). Yan et al. (2001), and suggests that TDR is better correlated with TdP in humans than the length of the QT interval (Lawrence et al., 2005).

One method for evaluation of TDR is to measure the difference in duration between the peak of T wave to the end of T wave so called “T\text{peak}-T\text{end}” (Antzelevitch, 2001b). According to Yan and Antzelevitch (1998), the duration of the epicardial action potential determines the peak of the T-wave whereas the duration of the M cell action potential, determines the end of the QT interval (Figure 1.13). An interesting finding originating from their studies is the T\text{peak}-T\text{end} interval encompasses all the ventricular APDs, clearly illustrating that the T\text{peak}-T\text{end} interval is a direct measure of TDR. Yan and colleagues (2001) demonstrated that APs from epicardium, M cells, and
endocardium prolong when exposed in the canine wedge to dl-sotalol; however, the M cell AP prolongs more than either the epicardial or endocardial APs, thus widening the T wave. The exaggerated prolongation of the M cell AP causes a greater separation of APDs between all cell types, with the greater separation of repolarization times being between epicardial and M cells. Greater separation between the APDs across the wall of the ventricular myocardium results in an increase in TDR. The dl-sotalol induced increase in dispersion of repolarization is accompanied by a corresponding increase in the T_peak-T_end interval and therefore an increase TDR. In clinical studies, T_peak-T_end/QT has been shown to be a reliable predictor for TdP in patients with long QT syndromes (Yamaguchi et al., 2003). These researchers measured the T_peak-T_end interval divided by the QT interval in lead V5. A T_peak-T_end/QT value of 0.28 ms appeared to discriminate between patients who developed TdP and those who did not (Antzelevitch, 2005).
Figure 1.13. Schematic illustrating of Tpeak-Tend as an indicator of transmural dispersion of repolarization. A) Action potentials from epicardial (Epi), midmyocardial (M), and endocardial (Endo) sites were simultaneously recorded using floating glass microelectrodes. A transmural ECG was recorded concurrently. B) Action potentials from epicardium (Epi), midmyocardium (M), and subendocardial Purkinje fibers were recorded simultaneously together with a transmural ECG. Notice that repolarization of epicardium is coincident with the peak of the T wave of the ECG. The endocardial APD is intermediate and the repolarization of M cells is coincident with the end of the T wave of the ECG. (Courtesy of Yan and Antzelevitch, 1998)
In vitro and in vivo models for predicting drug-induced arrhythmias in humans

Over recent years a number of models have been developed that specifically look at the ability of these electrophysiological events, either singly or in combination, to predict TdP in humans.

1. SCREENIT (a Langendorff-perfused isolated rabbit heart preparation)

The model consists of a paced Langendorff-perfused rabbit heart from which monophasic action potentials (MAPs) are recorded. This system was developed by Hondeghem in 1994. It is a computerized system that is fully automated and reports the concentration-dependent electrophysiological effects of drugs. Measurements are made in functioning perfused hearts from adult female New Zealand white rabbits.

After removing the heart from the rabbit, the heart is washed in normal Tyrodes buffer. Under a dissecting microscope, the right atrium is opened and the interatrial septum is dissected until the central fibrous body is exposed. The His bundle is cut, and a pair of stimulating electrodes is sutured to each side of the distal His bundle. A recording electrode is advanced until it reaches the left ventricular subendocardium of the septum. The isolated heart is then transferred to the experimental station, where it is perfused at a constant pressure of about 80-cm water with a bicarbonate buffer. A reference electrode and an epicardial recording electrode are positioned on the left ventricular epicardium. The reference electrode is perfused at about 1 ml/min with isotonic KCl, enriched with
1.8 mM CaCl₂, and grounded. Because the cells at the reference electrode are only depolarized, but not damaged (as with pressure or suction electrodes), stable MAP recording are expected for the duration of the experiment (<4 hr). Once the electrodes are positioned, Screenit is a fully automated system.

Thereafter, the threshold stimulation current is determined. The heart is then stimulated until the APD₆₀ has not varied by more than 10 ms from the median for at least 20 min. If the preparation has been accepted, Screenit executes the defined experimental program, consisting of two protocols. Brief protocols are executed every minute and large protocols are executed at 10 (end of the control period) and 60 min (end of highest drug concentration tested) in the experiment.

In this model, MAP recording electrodes are placed on epicardial and endocardial ventricular surfaces to record TRIaD elements, APD₁₀₋₉₀, conduction velocity, and proarrhythmic events such as EADs.

The SCREENIT model and the TRIaD concept has been tested in three validations with the aim to determine if the model could discriminate between pro-and antiarrhythmic drugs and therefore to determine the predictivity of this model to TdP in man. Two of these validations have already been published (Hondeghem and Hoffman, 2003; Hondeghem et al., 2003).

Advantages

1. The similarity between the rabbit and human ERG indicates a high degree of homology between the two proteins (99%). The amino acid residues responsible for drug binding are identical between both species (Valentin et al., 2004).
2. Female rabbits are usually used because there is evidence that this sex may be more sensitive to the development of TdP in rabbits, as well as in humans (Abi-Gerges et al., 2004; Ebert et al., 1998; Lu et al., 2001a). The QT intervals in untreated male and female rabbit do not differ (unlike in human beings in whom QT is slightly longer in females than in males, however when exposed to drugs which lengthen QT, QT lengthens more in females than in males (Lu et al., 2001b).

Limitations

1. The rabbit has little $I_{Ks}$ activity (Nattel, 1999) so that the main repolarizing current in this species is $I_{Kr}$. Thus if a potential torsadogen is hazardous because of its effects on $I_{Ks}$, the preparation will not be sensitive.

2. Isolated hearts do not metabolize the drug; hence, the potential effects of metabolites are not detected.

3. The hearts are devoid of innervation, and it is known that drug effects on innervation may affect physiological effects.

4. The exposure times are relatively short (<20 min), thus, pharmacokinetic equilibrium might not be reached for some drugs (e.g., sotalol).

5. The drug binding to tissue may be different in a protein-free buffer than in a protein-rich environment in vivo, which may eventually affect the drug concentration at the active site, particularly, for drugs with slow association/dissociation protein binding kinetics.
2. The arterially-perfused left ventricular wedge preparation

The arterially-perfused left ventricular wedge preparation (Figure 1.14) was originally developed in the laboratory of Antzelevitch (Yan and Antzelevitch, 1998). This model records a pseudo electrocardiogram and three transmembrane action potentials recorded simultaneously. Antzelevitch’s group has identified three distinct regions within the layers of the myocardium that are electrically and pharmacologically distinct: endocardium, mid-myocardium, epicardium (Antzelevitch, 2001a; Antzelevitch and Shimizu, 2002). Simultaneous recordings of action potentials from each of these cell layer can provide information of the relative differences in APD and repolarization characteristics between the layers and thus provides a measure of dispersion across the wall of the ventricle (TDR)—a key factor in precipitating arrhythmias (Antzelevitch, 2001a; Antzelevich and Shimizu, 2002).

In this model, repolarization of the epicardial cell is coincident with the peak of the T-wave, and repolarization of the M cell is temporally aligned with the end of the T-wave. The APD of endocardial cells is usually intermediate. The M cell action potential is longer than that of cells in either of the neighboring regions. This characteristic of M cells is thought to be due to a smaller $I_{Ks}$ current and a larger, sustained sodium current and sodium-calcium exchange current (Antzelevitch and Shimizu, 2002).

The pivotal finding from the left ventricular wedge preparation is that augmentation of the natural heterogeneity by an increase in TDR is a consequence of the preferential prolongation of the action potential in the M cell, leading to TDP.
Advantages

Simultaneously recording of transmembrane action potentials (epicardium, M cells, and endocardium) and a transmural pseudo electrocardiogram can be performed at the same time. TDR index can be achieved from this model by using $T_{\text{peak}} - T_{\text{end}}$.

Limitations

Like other models, a wide concentration of a compound needs to be explore when assessing the proarrhythmia propensity (i.e., high dose of some compounds may attenuate APD while it may react differently at lower dose). Furthermore, this model is technically challenging, and requires a relatively long time for stabilization.
Figure 1.14. Schematic illustration of the arterially perfused left ventricular wedge preparation. The wedge is perfused through a branch of the left descending coronary artery and stimulated from the endocardial surface. Action potentials are simultaneously recorded from epicardial (Epi), midmyocardial (M), and endocardial (Endo) sites using floating microelectrodes and a transmural pseudo ECG is recorded concurrently. (Courtesy of Yan et al., 1998)
3. The anesthetized methoxamine-sensitized rabbit heart

The methoxamine-sensitized rabbit model was developed in the 1990s by Carlsson and colleagues (Carlsson et al., 1990) and has been used extensively (Akita et al., 2004; Brooks et al., 2000; Chiba et al., 2004; Johansson and Carlsson, 2001; Lu et al., 2004; Verduyn et al., 2001b). Alpha-chloralose-anesthetized rabbits are pretreated with methoxamine, the $\alpha_1$-agonist. These animals then have an increased sensitivity to the repolarization-altering effects of some drugs, especially those that are relatively pure $I_{Kr}$ blockers, for example dofetilide and $dl$-sotalol. Methoxamine predisposes the myocardium to reproducibly develop TdP-like arrhythmias. There are many possible mechanisms to explain why $\alpha_1$-adrenoceptor stimulation increases the susceptibility of drug-induced TdP; however, these mechanisms are not clear. The first potential mechanism is that $\alpha_1$-adrenoceptor stimulation increases intracellular $Ca^{2+}$ concentration through inositol triphosphate (IP3) and diacylglycerol (DAG), which are considered to be proarrhythmic (Carlsson et al., 1993). Another mechanism is reflex bradycardia due to activation of the baroreceptor reflex and elevation of parasympathetic efferent traffic, following an increase in blood pressure produced by $\alpha_1$-adrenoceptor stimulation. The last contributing factor to the increased propensity for TdP in $\alpha_1$-adrenoceptor agonist-sensitized rabbit is that $\alpha_1$-adrenoceptor agonist per se delays ventricular repolarization and reduces the repolarization reserve (Carlsson, 2006). However, it was demonstrated that intravenous administration of one of the $\alpha_1$-adrenoceptor agonist, methoxamine, alone in rabbit did not prolonged epicardial monophasic action potential duration (Carlsson et al., 1990) and QTc interval (Brooks et al., 2000; Buchanan et al., 1993).
Advantages

The \( \alpha_1 \)-adrenoceptor agonist sensitized rabbit is inexpensive, easily produced, reproducible, and sensitive. This model continues to be used extensively and extends our understanding of the mechanisms of class III antiarrhythmic agents (Lawrence et al., 2005) because there has generally been a good correlation between data generated from this model and clinical data.

Limitations

This model is limited to assessment of the proarrhythmic proclivity of class III antiarrhythmic agents (\( I_{Kr} \) blockers) due to the fact that incidence of TdP is higher when examining potent \( I_{Kr} \) blockers (Carlsson, 2006), whereas the incidence is either low or nonexistent when challenged with quinolone antibiotics (Akita et al., 2004; Anderson et al., 2001), antihistamines (Beatch, 1996), antiarrhythmic class IA (Lu et al., 2000), and non-selective \( I_{Kr} \) blockers (Brooks et al., 2000; Lu et al., 2000).

4. The chronically atrioventricular (A-V) block dog model

The chronic atrioventricular (A-V) blocked dog model was first used by Vos and colleagues (1995) to study the torsadogenic effects of repolarization-prolonging drugs. It has also been used, recently, by Japanese investigators in monkeys. In this model the A-V node is ablated by radio frequency and/or injection of formaldehyde, and then ventricular pacing electrodes are put in place to prevent a lethal bradycardia. The dogs are followed for 2-4 months to allow for bradycardia-induced ventricular remodeling. Susceptibility to TdP can be increased further by using furosemide to induce hypokalemia.
**Advantages**

Studies are generally conducted on conscious animals, so a main advantage of this model is that all routes of drug administration are possible (Lawrence et al., 2005). Although dog metabolism may vary from that of humans (e.g., procainamide).

**Limitations**

The chronically AVB dogs represent only a small population of persons who have are at risk factor for TdP induction. Additionally, all dogs required 2-4 months for cardiac remodeling before they may be used for drug testing.

**Conclusion**

Identifying the proarrhythmic potentials of drugs is important to identify potential risk of TdP in humans. The discrepancy between QT interval prolongation and proarrhythmia is a significant concern for clinicians, pharmaceutical companies and regulatory authorities. Several proarrhythmia models exist and are well characterized. Undoubtedly, no single assay and/or proarrhythmia model is sufficient to predict the risk of TdP in humans, highlighting the complexity of the issue and the need for proarrhythmia models that encompass most if not all of the predisposing factors. Several new surrogate parameters predicting TdP and sudden death have been proposed. Currently, the TRIaD elements including beat-to-beat variability of QT interval trend to be an excellent biomarker for risk of TdP and have been validated in many proarrhythmic models. Up to date, the principal evidence linked to the induction of TdP is the
development of EADs, which have been identified in several models as being primary to the induction of TdP. It has been shown that the factors that leading to EADs are triangulation of the APs and dispersion of APD, specifically temporal dispersion (instability of the APD also called alternans) and transmural dispersion of repolarization. The latter appears to be more substantial in predicting the reentrant arrhythmia following the initial EAD. Further research in this area may provide better predictive power for identification of the proarrhythmic drug.

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ASSESSMENT OF DRUG-INDUCED QT INTERVAL PROLONGATION
IN CONSCIOUS RABBITS

ABSTRACT

Introduction: Most preclinical trials designed to identify potential torsadogenicity test only for surrogates of torsade de pointes, most commonly prolongation of the heart rate corrected QT interval (QTc). This study was conducted to determine which correction method best accounts for the effects of changes in the RR interval on the QT interval of conscious rabbits. This study was also conducted to validate the use of conscious, sling-trained rabbits to assess the QTc interval, and to evaluate the reliability and accuracy of this preparation in predicting drug-induced QTc prolongation in humans.
Methods: ECGs were recorded via bipolar transthoracic ECG leads in 7 conscious rabbits previously trained to rest quietly in slings. The heart rate was slowed with 2.0 mg/kg zatebradine to assess the effects of heart rate on the QT interval. The same ECG and sling preparation was used to evaluate the effects in of three drugs known to be torsadogenic in humans (cisapride, dofetilide and haloperidol), two drugs known to be non-torsadogenic in humans (propranolol and enalaprilat) and a control article (vehicle). All of the test articles were administered intravenously to 4 rabbits, and both RR and QT intervals were measured and the corrected QT values were calculated by an investigator blinded to the test article, utilizing our own algorithm (QTc=QT/(RR)^0.72) which permitted the least dependency of QTc on RR interval.

Results: The following regression equations were obtained relating QT to RR: QT=2.4RR^0.72, r^2=0.79, with RR intervals varying between 210 and 350 ms. QTc lengthened significantly in all conscious rabbits given intravenous cisapride, dofetilide or haloperidol (p < 0.05), and QTc did not change with DMSO (vehicle control), propranolol or enalaprilat.

Discussion: Results indicate that a bipolar transthoracic ECG recorded in conscious, sling-trained rabbits may provide an easy and economical methodology useful in predicting QTc lengthening of novel pharmacological entities.
INTRODUCTION

No potential pharmacological agent is studied in clinical trials without first evaluating its potential to lengthen QTc, a known sentinel for predicting torsadogenicity in humans. Anesthetics used in preclinical testing on intact animals either alter QTc, or alter the relationship between QTc and the test article to be evaluated (Hamlin et al., 2003a). Furthermore, meaningful preclinical tests of potential therapeutic articles that affect ventricular repolarization by altering specific ion channels must be conducted on species that possess the same spectrum of ion channels known to be present in humans. The rabbit has been shown to be one such species (Kaab and Nabauer, 2001). Finally, in order to determine whether a test article lengthens ventricular repolarization directly or merely because it lengthens RR interval (i.e., slows heart rate), the method of correcting the QT interval for RR interval must produce QTc intervals that are as independent of RR interval as possible (Bednar et al., 2001). Previous authors have proposed an equation, QTcL=QT-0.704(RR-250), to correct QT for RR interval in rabbits anesthetized with sodium pentobarbital (Batey and Coker, 2002), and these authors claim that an existing equation, QTc=QT-0.175(RR-300), developed from rabbits anesthetized with methohexitone sodium and alpha chloralose, did not produce a QTc independent of RR interval (Carlsson et al., 1993). We have demonstrated previously that drugs that prolong QTc intervals in humans also prolong QTc intervals in conscious guinea pigs (Hamlin et al., 2003a), but conscious rabbit models for assessment of QT liability have not been reported.
The purposes of this study were (1) to validate a method of correcting QT for RR interval that produces, in rabbits, a QTc nearly independent of RR interval, and (2) to use this validated method to determine whether the conscious rabbit may be a useful sentinel to predict QTc lengthening in humans.

MATERIALS AND METHODS

Approvals

This study was approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC) of the Ohio State University.

Animal preparation and ECG recording

A total of 31 rabbits were used. All weighed between 2.2 and 3.2 kg. Four males and 3 females were used to assess the relationship between RR interval and QT interval and for calculation of the rate-corrected QT interval (QTc). Twenty-four additional rabbits, distributed equally by sex, received test articles. Each of 6 test articles (cisapride, DMSO (vehicle control), dofetilide, enalaprilat, haloperidol, propranolol) were given to 4 rabbits each.

After clipping the hair from the ventral region of the thorax, rabbits were placed without chemical restraint in a ventrally recumbent position in a comfortable, padded sling. The sling is fitted with copper plates, which “sandwich” the ventro-cranial aspect
of the thorax, such that a bipolar transthoracic electrocardiogram between points rV2 (right, 4th intercostal space at the costochondral juncture) and V2 (left, 5th intercostal space at the costochondral juncture) can be obtained. The electrodes are made with a central hole so that electrode paste can be applied from outside the sling directly onto the rabbit-electrode interface to minimize impedance between the electrodes and the skin without disturbing the rabbit’s position in the sling. To obtain the ECG recordings, the right and left arm electrodes of the electrocardiograph were attached to the right and left sided sling electrodes, the electrocardiograph was switched to limb lead I, and a bipolar transthoracic ECG was obtained on a Biopac MP100 Data Acquisition Unit (Biopac Systems, Inc., Santa Barbara, CA). The high pass filter was set at 0.01 Hz and the low pass filter at 1 kHz, and signals were sampled at 2 kHz. Tracings were obtained for 15 to 60 seconds while the rabbits were conscious and quiet.

To examine the effect of RR interval on QT interval, rabbits were given 2.0 mg/kg of zatebradine (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT), a funny channel (I_{f}) blocker that reduces heart rate without direct effects on other electrophysiological parameters (Goethals et al., 1993; Hamlin et al., 2003b). ECG tracings were obtained at numerous RR intervals generated in response to zatebradine, and these measurements were used to generate curves of QT versus RR interval.
Data analysis

ECG intervals from beats originating from the sino-atrial node (sinus rhythms) and displayed at 100 mm/sec were measured manually using on-screen cursors, taking the mean of at least 12 consecutive cardiac cycles at each time point. The QT interval was measured from the onset of Q wave to the end of T wave, including U wave if present (Batey and Coker, 2002). Because the conscious, quiet rabbit has a relatively slow heart rate, the T wave was always over prior to the onset of the next P wave, and the end of the T wave was always easily identifiable (Figure 1). Furthermore, because all rabbits had regular sinus rhythms, no interval measurements were excluded because of arrhythmias.

The RR interval and the QT interval of the following QRS-T complex were measured at all recorded heart rates, and plots of QT versus RR intervals were constructed. Using Sigma Plot (SPSS Inc., Chicago, IL), the equation relating QT to RR interval and its regression coefficient ($r^2$) were calculated using a simple regression model of the generic form $QT = QT = \beta \times RR^\alpha$. QT was corrected for the preceding RR interval using the formula, $QTc = QT/RR^\alpha$. In addition, the QT intervals were corrected for the preceding RR intervals using common exponential (Bazett, Fridericia) (Bazett, 1920; Fridericia, 1920) and linear (Carlsson, Liverpool) equations (Carlsson et al., 1993; Batey and Coker, 2002). Plots were made of QTc versus RR interval, and regression lines and $r^2$ were calculated. These plots were made to determine which method(s) for the conscious rabbits produced the least dependence of QTc on RR.
Validation of the QTc formula

In a separate study, groups of four conscious rabbits resting quietly in slings were each given one of the following compounds intravenously over 10 minutes in a marginal ear vein: 2 mg/kg cisapride, 20 µg/kg dofetilide, 0.5 mg/kg haloperidol, 0.5 mg/kg propranolol, 0.5 mg/kg enalaprilat, or 0.1 ml/kg DMSO. All doses were selected from the literature (Bohmer et al., 1986; Carlsson et al., 1990; Madwed and Winquist, 1994; Poisson et al., 2000; Wu et al., 2003). Bipolar transthoracic ECGs were obtained before dosing and every 5 minutes during dosing and for 20 minutes after each compound had been administered. QT and RR interval measurements were made on at least 12 consecutive cardiac cycles at measurement time, and the average of 12 cycles was recorded for each measurement. Significant differences between test articles and vehicle were sought by a one-way ANOVA with repeated measures design on time. When indicated by a significant F-statistic, specific means were compared by the Tukey post-hoc test. Plots were made of the RR, QT and QTc interval differences from baseline versus time.

RESULTS

High quality electrocardiograms were obtained from all of the rabbits used all phases of the study (Figure 2.1). A plot of QT- versus RR interval for the seven conscious rabbits that received zatebradine is shown (Figure 2.2). The relationship between QT and RR interval for both sexes, as well as for males and females separately, respectively are: QT = 2.4 (RR)$^{0.72}$, having an $r^2$ of 0.79; QT = 2.4 (RR)$^{0.72}$, having an $r^2$ of 0.81; QT = 2.5
(RR)\(^{0.72}\), having an \(r^2\) of 0.79, respectively. This demonstrates that 79% of the variability in QT can be explained by the RR interval, and the relationship between the two is highly significant \((p < 0.001)\). Graphs made for each sex independently (Figure 2.3) demonstrate the absence of a significant effect of gender on the relationship between RR and QT or QTc intervals. Plots of QTc versus RR interval using each of the 5 methods of correction for RR interval are shown, with their slopes and \(r^2\) (Figure 2.4). With our equation, plotting rate-corrected QT interval (QTc-QTest (QTcQTest)) against RR interval produces a regression line with a slope of zero, indicating that this correction removes the influence of heart rate. For this reason, all QT intervals in the rests of the study were corrected according to this QTest formula. All other potential correction equations demonstrated at least some dependence of QTc on RR interval, with positive or negative slopes and an \(r^2\), although relatively small, different from zero. Correction factors varied in independence as follows: QTest < Bazett < Carlsson ≈ Fridericia < Liverpool, having \(r^2\) of, respectively, 0.00, 0.26, 0.50, 0.52, 0.75.

Plots of RR (Figure 2.5), QT (Figure 2.6) and QTc (Figure 2.7) versus Time (min) after injection are shown for groups of 4 rabbits each exposed to the test articles known to, and known not to lengthen QTc. All test articles except enalaprilat either lengthened (cisapride, dofetilide, haloperidol, and propranolol) or tended to lengthen (DMSO) RR interval when compared to RR intervals obtained at baseline. Enalaprilat, propranolol, and DMSO caused no QTc prolongation. QTc prolonged significantly in response to
Figure 2.1. Examples of Bipolar, transthoracic ECG in conscious rabbits which were receiving (from top panel to bottom panel) propranolol, haloperidol, dofetilide and baseline ECG. Notice the absence of baseline artifacts, the ease of identification of the onset of QRS and the end of T.
Figure 2.2. Plot of QT (ms) duration versus the preceding RR (ms) interval for conscious rabbits (4 males and 3 females) receiving zatebradine. The line of regression and its equation and $r^2$ are shown. Notice that the RR intervals varied from approximately 210 ms (a heart rate of 286 beat per minute) to 346 ms (a heart rate of 173 beat per minute). Each data point is an average of 12 consecutive cardiac cycles from a different rabbit.
Figure 2.3. Plots of RR, QT and QTc duration (ms) versus time (min) for rabbits that received zatebradine. Notice that zatebradine significantly lengthened RR and QT interval compared to baseline but there is no difference between male and female. Each data point is the average of 12 consecutive cardiac cycles. An asterisk indicates a difference between baseline and a post-dosing measurement. One asterisk indicates a $p < 0.05$, two asterisks indicate a $p < 0.01$, and three asterisks indicate a $p < 0.001$. 

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Figure 2.4. Plots of QTc, their slopes, and $r^2$, using many equations for removing influence of heart rate, versus RR (ms) interval for all conscious rabbits. Notice that the slope for the QTest equation is zero with an $r^2$ of 0.00. Each data point is an average of 12 consecutive cardiac cycles from a different conscious rabbit.
Figure 2.5. Plots of mean and SEM of values of changes between baseline and values at each time after dosing of 3 test articles known to lengthen QTc and 2 negative controls known not to lengthen QTc, and for the DMSO vehicle. Each data point is the average of 12 consecutive cardiac cycles. Notice that all test articles other than enalaprilat and DMSO lengthened RR interval. Each mean is the mean of four rabbits receiving each test article. Doses of test articles were in mg/kg except indicated: cisapride (2), dofetilide (20 µg/kg), haloperidol (0.5), propranolol (0.5), Enalaprilat (0.5) and DMSO (0.1 ml/kg). An asterisk indicates when a difference changed with statistical significance from baseline. One asterisk indicates a $p < 0.05$ and two asterisks indicate a $p < 0.01$. 
Figure 2.6. Plots of Change in QT (ms) between baseline and times post-dosing for 3 test articles known to lengthen QT and 2 negative controls known not to lengthen QT, and for the DMSO vehicle. Each data point is the average of 12 consecutive cardiac cycles. Notice the lengthening of QT for the three test article but not for the negative controls and vehicle. Each mean is the mean of four rabbits receiving each test article. Doses of test articles were in mg/kg except indicated: cisapride (2), dofetilide (20 µg/kg), haloperidol (0.5), propranolol (0.5), Enalaprilat (0.5) and DMSO (0.1 ml/kg). An asterisk indicates when a difference changed with statistical significance from baseline. One asterisk indicates a $p < 0.05$, and three asterisks indicate a $p < 0.001$. 

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Figure 2.7. Plots of difference between baseline and values at each time post-dosing for 3 test articles known to lengthen QTc and 2 negative controls known not to lengthen QTc, and for the DMSO vehicle. Each data point is the average of 12 consecutive cardiac cycles. Notice the lengthening of QTc for the three test article but not for the negative controls and vehicle. Each mean is the mean of four rabbits receiving each test article. Doses of test articles were in mg/kg except indicated: cisapride (2), dofetilide (20 µg/kg), haloperidol (0.5), propranolol (0.5), Enalaprilat (0.5) and DMSO (0.1 ml/kg). An asterisk indicates when a difference changed with statistical significance from baseline. One asterisk indicates a $p < 0.05$, two asterisks indicate a $p < 0.01$, and three asterisks indicate a $p < 0.001$. 
injections of cisapride, haloperidol and dofetilide. Most of the QTc prolongation had already occurred within 5 minutes after these test articles were injected, however QTc prolongation due to cisapride peaked at the 15 minute recording.

DISCUSSION

The purposes of this study, (1) to validate a method of correcting QT for RR interval that produces, in rabbits, a QTc nearly independent of RR interval, and (2) to use this validated method to determine whether the conscious rabbit may be a useful sentinel to predict QTc lengthening in humans were achieved. It was demonstrated that QTc prolongation occurred in this model in response to all three test articles known to lengthen QTc in humans, and QTc failed to prolong in conscious rabbits in response to all three test articles known to not lengthen QTc in humans. This study demonstrates that a single bipolar, transthoracic ECG, from which RR and QT may be measured easily, can be obtained from a conscious rabbit placed in a comfortable sling, and that a relationship between QT and RR can be successfully modeled by a power plot relationship.

Heart rates in the conscious rabbits used during this study varied between 170 and almost 300 beats per minute, a wide range that we utilized to confirm the dependence of QT on the preceding RR interval (QT=2.4RR^{0.72}, r^2=0.79), and to demonstrate that QTc could be independent of RR using the equation QTc=QT/(RR)^{0.72}. In the present study, Bazett, Carlsson, Fridericia, and Liverpool QT correction factors failed to correct adequately for the influence of heart rate in conscious rabbits. However, failure in this experiment should not imply that the QTc formulas would be equally applicable to the
data of other studies, since there may be significant individual differences in QT/RR patterns (Malik, 2002). This is in agreement with the finding of Batey and Coker (2002) who reported that the Carlsson’s correction formula (Carlsson et al., 1993) for anesthetized rabbits did not correct data of rabbits in their study.

This study also demonstrated the potential utility of the conscious rabbit for detecting the liability of test articles to lengthen QTc in humans. Three test articles of different pharmacological classes (pure antiarrhythmic class III, GI motility agents, and antipsychotics) known to lengthen human QT intervals also lengthened this parameter in the rabbits; and 2 test articles, also of different pharmacological classes (antiarrhythmic class II and ACE inhibitor) as well as DMSO, known not to lengthen QT interval in humans, did not lengthen QT interval in this study. Based solely upon these 6 test articles, sensitivity and specificity would appear to be 1.0 for the detection of QT liability in humans -- this does not imply that sensitivity and specificity would remain 1.0 for a greater number of test articles, nor does it imply that sensitivity and specificity achieved from conscious rabbits would necessarily differ from those obtained from rabbits anesthetized with any number of pre-anesthetic/anesthetic combinations, or in other models. Equally high sensitivities and specificities have been obtained using 50 test articles in the isolated, perfused guinea pig heart (Hamlin et al., 2004).

There are, however, several advantages of using the conscious rabbit to test for drug-induced prolongation of QTc. (1) Results are free of any possible interference from an anesthetic regimen. (2) Animals can be studied repeatedly without risk of death by anesthesia. (3) Recordings using the bipolar transthoracic electrocardiogram
permit easy and accurate measurements of the beginning of the QRS complex and the end of the T wave. (4) Uniform electrode placement can be accomplished without discomfort to the rabbit. (5) The rabbit heart shares with humans all of the transmembrane ion channels specific for controlling ventricular repolarization (Kaab and Nabauer, 2001). (6) A QTc formula now exists for conscious rabbits that appear to completely correct the QT for the effects of heart rate, even at extremely short and long RR intervals.

An interesting finding in this study was that correcting the QT interval for the preceding RR interval was actually unnecessary to achieve high sensitivity and specificity. Examination of Figures 5 and 6 shows that the additional information gained from QT correction does not change the conclusions regarding QT liability. This result is clearly limited to these 6 test articles, however, and it is clearly possible that test articles exist for which correction of the QT interval for the RR interval may be necessary.
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CHAPTER 3

USE OF A FAILING RABBIT HEART AS A MODEL TO PREDICT TORSADOGenicITY

ABSTRACT

Humans with underlying cardiovascular disease are at greater risk than humans with normal hearts for developing torsade de pointes (TdP) following exposure to some drugs that prolong ventricular repolarization. This study was designed to test the hypothesis that rabbits with ischemic myocardial failure are at similarly increased risk of developing QTc prolongation and torsade de pointes following exposure to escalating doses of drugs whose capacity to induce TdP is known in humans. Coronary artery ligation was performed in 28 rabbits, causing significant ($p<0.05$) reduction in left ventricular shortening fraction and systolic myocardial dysfunction 4 weeks after ligation in all operated animals compared to 38 normal, non-operated controls. All studies were performed on rabbits anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg).
Rabbits were exposed to escalating doses of amiodarone (3, 10, 30 mg/kg/10min), cisapride (0.10, 0.25, 0.50 mg/kg/10min), clofilium (0.1, 0.2, 0.4 mg/kg/10min), dofetilide (0.005, 0.01, 0.02, 0.04 mg/kg/10min), quinidine (3, 10, 30 mg/kg/10 min), and verapamil (0.25, 0.5, 1.0 mg/kg/10min). A greater percentage of rabbits with failing hearts developed TdP following intravenous infusion of escalating doses of dofetilide (85%), clofilium (100%), or cisapride (50%) than did normal rabbits exposed to the same drug protocol (20%, 33% and 0%, respectively). None of the rabbits in either group developed TdP when exposed to escalating doses of amiodarone, verapamil, or quinidine. Two out of 4 test articles lengthened QTc more in rabbits with myocardial failure than in normals, and TdP occurred in 13 out of 28 rabbits with myocardial failure as opposed to only 4 out of 38 rabbits with normal myocardial function.

INTRODUCTION

In humans, heart disease appears to be an independent risk factor for development of torsade de pointes (TdP) (Buja et al., 1993; Kannel and Sorlie, 1981) and other life-threatening arrhythmias. Recently, TdP was produced in dogs (Kozhevnikov et al., 2002; Sugiyama et al., 2002; Verduyn et al., 1997; Vos et al., 1995) and rabbits (Tsuji et al., 2002) with long-standing complete AV block, whereas in normal animals it can be produced only in the setting of dramatic alterations in plasma electrolytes (Fabritz et al., 2003) or by administering multiple drugs (Batey and Coker, 2002; Carlsson et al., 1990). Currently, most preclinical trials performed to assess the torsadogenicity of potentially therapeutic compounds test only surrogates of TdP, most commonly QTc prolongation.
Prolongation of QTc is a common effect of certain cardiovascular and non-cardiovascular drugs (Belardinelli et al., 2003). Not all drugs that prolong the QT interval are associated with an increased risk for TdP (Redfern et al., 2003). The mechanism of TdP development may involve altered calcium kinetics, possibly resulting from calmodulin kinase II-phosphorylation of ryanodine channels (Verduyn et al., 1995). This finding is supported by the fact that a calcium-calmodulin inhibitor, W-7, prevents TdP development in the setting of QTc lengthening induced by a torsadogenic drug without actually shortening the QTc interval (Gbadebo et al., 2002; Mazur et al., 1999). A model capable of developing TdP might thus permit more accurate and direct assessment of a compound’s torsadogenic potential, rather than relying on the results of a surrogate test for TdP, such as prolongation of QTc, to identify risk. The hypotheses of this study were:

(1) QTc lengthens more in failing hearts than in non-failing hearts in response to articles that lengthen QTc. (2) Failing hearts develop TdP more often than do non-failing hearts.

This paper describes the effect of a coronary ligation model of myocardial ischemia leading to heart failure in rabbits, and documents the differential effects of amiodarone, cisapride, clofilium, dofetilide, quinidine, and verapamil on QTc and torsadogenicity and that this rabbit model of left ventricular myocardial failure manifests a more torsadogenic substrate normal rabbits without heart failure.
MATERIALS AND METHODS

This study was approved by the ILACUC of the Ohio State University and QTest Labs, Inc. A total of 71 rabbits were used (31 in the coronary ligation / myocardial failure group, 38 in the non-operated control group, and 2 in the sham-operated control group). All rabbits weighed between 2.5 and 3.5 kg. All animal procedures were conducted in accordance with guidelines published in the Guide for the Care and Use of Laboratory Animals. Before commencing the main experiments, pilot studies with dofetilide were performed to establish that there was no difference in QTc interval prolongation and TdP induction between normal rabbits and sham-operated rabbits. In both cases, dofetilide (0.04 mg/kg/10 min) lengthened the QTc interval (371ms versus 357ms, respectively, no statistical difference by t-test, \( p=0.33 \)) and failed to cause torsade de pontes in either group of animals.

Surgical Procedure

Thirty one male New Zealand White rabbits were anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg) administered intramuscularly. Animals received 100% oxygen (at a rate of 400-600 ml/min) and isoflurane (at the rate of 0.5-1.0%) through a loose-fitting face mask designed for small animals. Surgery was performed by using a modification of the method of Fujita et al. (2004). Rabbits were place in dorsal recumbency, and surgical anesthesia was confirmed by the absence of the pedal reflex. Standard limb lead ECGs were obtained during the period of surgery. Enrofloxacin (12 mg/kg) was given intramuscularly prior to surgery. The skin over the sternum was denuded of hair and prepared aseptically. The incision line was infiltrated with 2%
lidocaine. A midline incision (approximately 3-4 cm) was made in the skin, and the sternum was split with a #10 scalpel blade, being careful to remain precisely on the midline so that injury to the parietal pleura was avoided. The sternum was gently retracted so that the pericardial sac could be observed, and the sac was incised to reveal the surface of the right and left ventricles and their respective coronary arteries. Both the left anterior descending and a major apical branch of the left circumflex artery were ligated at a level midway between the origin of the major apical branch and the cardiac apex. Ligatures were performed using 4/0 monofilament polypropylene suture. Production of myocardial ischemia was confirmed by ST-segment elevation on the ECG and observation of grossly visible regional cyanosis of the myocardial surface. Of the 31 rabbits subjected to this procedure, 3 died immediately following coronary ligation from ventricular fibrillation. In the remaining 28 rabbits, the pericardial sac was left open, but the sternum was closed with three simple interrupted sutures of 3/0 monofilament polydioxanone suture. Muscle layers were closed with simple interrupted sutures of 4/0 nylon, and the skin was stapled. All 28 rabbits that survived the surgery were given 0.03 mg/kg buprenorphine IM TID and 12 mg/kg enrofloxacin IM BID for 3 days postoperatively.

Echocardiographic assessment of left ventricular function

Echocardiographic examination was performed under light ketamine/xylazine sedation (15 mg/kg and 3 mg/kg respectively) on the day prior to coronary ligation and again 4 weeks after surgery. Each rabbit was placed in right lateral recumbency, with an area of the right hemithorax denuded to allow echocardiographic images to be obtained
from the dependent right hemithorax. Imaging was performed using an Aloka SSD-1400 Echocardiographic System (Aloka America, Wallingford, CT) with a 5 MHz transducer. Echocardiographic recordings included a simultaneously recorded electrocardiogram (ECG), and all raw data was captured digitally for off-line measurement. Left ventricular structure and function were assessed by evaluation of standard 2-dimensional and M-mode imaging planes (Schiller et al., 1989). Measurement of left ventricle wall thickness and internal ventricular dimensions during systole and diastole were made from M-mode images obtained from the standard right parasternal short axis view at a level just beneath the mitral valve, with the M-mode cursor directed between the papillary muscles. These measurements subsequently were used to calculate the shortening fraction (SF). A reduction in shortening fraction of 15% or more from the baseline preoperative value was required for entry into the myocardial failure group for the study. All echocardiographic images were acquired and analyzed by a single experienced operator. Mean ± SEM was calculated for shortening fraction, left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD), left ventricular end-diastolic wall thickness (LVEDWT), and left ventricular end-systolic wall thickness (LVESWT). Values obtained before and 4 weeks after surgery were compared utilizing a paired $t$-test.

**Experimental protocol**

Animals were anesthetized with a combination of ketamine and xylazine (35- and 5 mg/kg IM). The right marginal ear vein was cannulated for IV administration of drug. Rabbits were placed in dorsal recumbency. The right and left thoracic limb electrodes were attached to the right and left hemithoraces, the electrocardiograph was switched to
limb lead I, and a bipolar transthoracic ECG was obtained on a Biopac MP100 Data Acquisition Unit (Biopac Systems, Inc., Santa Barbara, CA). The high pass filter was set at 0.01 Hz and the low pass filter at 1 kHz, and signals were digitally sampled at a frequency of 2 kHz. Anesthesia was maintained as necessary by administration of further doses of ketamine and xylazine (20 and 3 mg/kg IM). All rabbits were allowed at least 10 min stabilization before starting any drug infusion. All drugs were infused intravenously over a period of 10 minutes, with a 20 minute interval between doses. The infusion was stopped as soon as TdP started. The doses of drugs tested in this study were amiodarone (3, 10, and 30 mg/kg; control group n=6, myocardial failure group n=4), cisapride (0.1, 0.25, 0.50 mg/kg; control group n=4, myocardial failure group n=4), clofilium (0.1, 0.2, 0.4 mg/kg control group n=6, myocardial failure group n=5), dofetilide (0.005, 0.01, 0.02, and 0.04 mg/kg control group n=10, myocardial failure group n=7), quinidine (3, 10, and 30 mg/kg control group n=6, myocardial failure group n=6), and verapamil (0.25, 0.50, and 1.0 mg/kg control group n=6, myocardial failure group n=6). The doses of each drug were chosen according to those used in previously published rabbit studies by other research groups (Batey and Coker, 2002; Carlsson et al., 1997; Farkas et al., 2002; Lu et al., 2000). RR and QT intervals were measured 15 min. after each drug dose had been infused. The QT interval was corrected for heart rate by dividing the QT interval by the cube root of the preceding RR interval (Fridericia, 1920). Measurements were made from at least 12 consecutive cardiac cycles, and the average was used. The ECG intervals were measured in QRS complexes that originated from the sinoatrial node; however, at some points ECG intervals could not be obtained from all animal because of marked
arrhythmia. The QT interval was defined as the time between the first deviation from the isoelectric line during the terminal phase of the PR segment until the end of the T wave. TdP was defined as a polymorphic ventricular tachycardia where clear twisting of the QRS complexes around the isoelectric axis was seen.

Drugs

Amiodarone was prepared by diluting commercial amiodarone for injection (450 mg amiodarone in 9 ml of diluent) with 9 ml of 0.9% NaCl to a final concentration of 25 mg/ml. Cisapride was dissolved in 10% acetic acid to form a stock concentration of 10 mg/ml. Clofilium tosylate was dissolved in 0.9% NaCl solution to form a stock concentration of 3 mg/ml. Dofetilide was dissolved in 0.9% NaCl with help of 0.1 M hydrochloric acid to form a stock concentration of 0.1 mg/ml. Quinidine (stock concentration of 30 mg/ml) and verapamil (stock concentration of 2 mg/ml) were also dissolved in 0.9% NaCl.

Statistics

Values are expressed as mean ± SEM. Fisher’s exact tests were used to compare the incidence of TdP between groups. A probability value of $p<0.05$ was considered to be significant.
RESULTS

*Effects of coronary ligation on echocardiogram:*

Coronary ligation, followed by a 4 week healing period, provided a reproducible model of left ventricular systolic dysfunction in this study. Effects of myocardial infarction on regional wall motion and regional and global geometry were clearly detected 4 wks after coronary ligation. Hypokinesis was observed at the posterior and lateral walls of the left ventricle. Figure 3.1 shows typical M-mode echocardiograms of the left ventricle before and 4 weeks after ligation of the coronary arteries. In all 28 surviving rabbits in the infarction group, left ventricular end diastolic diameter (LVIDd) increased significantly (mean increase 11.8%, $p<0.01$), as did end-systolic diameter (LVIDs, mean increase 19.2%; $p<0.01$) and the difference between LVIDd and LVIDs (shortening fraction, see Table 3.1). Mean shortening fraction decreased 14.8% ($p<0.05$), from 30.41% before infarction to 25.90% afterward.

*Effects of coronary ligation on electrocardiograms:*

No significant differences were observed in heart rate, QT, and QTc between ECGs recorded in the 28 rabbits prior to coronary ligation, immediately following coronary ligation, and 4 wks after coronary ligation (Table 3.2). Immediately following coronary ligation, ST segment deviation (Figure 3.2) and a variety of ventricular ectopic beats were observed (i.e., ventricular premature beats and ventricular tachycardia).
Arrhythmia incidence

Four weeks following coronary ligation, no spontaneous arrhythmias (without provocation from drugs) were observed in the surgical ligation / myocardial failure group, and none were observed in the control group at any time. TdP (Figure 3.3) was induced by drug administration in 4 of the 38 rabbits in the control group (10.5%). Of these 4 rabbits, TdP was produced by dofetilide in two rabbits and clofilium in two rabbits. In contrast, TdP was produced in 13 (>46%; *p*=0.001) of the 28 rabbits in the myocardial failure group. Torsade de pointes was induced by cisapride, clofilium and dofetilide (Figure 3.4). The incidence of this arrhythmia was significantly higher in the myocardial failure group compared to the normal control group in response to clofilium (*p*<0.05, 100% [5 of 5 rabbits] of the myocardial failure group affected at the 1st or 2nd dose level, versus 33% [2 of 6] of controls at the 2nd dose level) and dofetilide (*p*<0.05). Cisapride induced TdP only in rabbits with myocardial failure (50%; 2 of 4). The occurrence of TdP in response to dofetilide was not directly proportional to dose. TdP occurred in 6 rabbits in the myocardial failure group overall (85%; 6 of 7), and was induced at the 3rd dose (0.02 mg/kg) in 3 of 6 rabbits in the myocardial failure group, as well as in 3 of 6 rabbits in that group at the 4th dose (0.04 mg/kg). TdP was induced only by the higher 4th dose of dofetilide (0.04 mg/kg) in 2 of the 10 rabbits (20%) in the normal (nonoperated) control group.
Effects of compounds on QTc

The plots of differences in QTc interval from baseline values versus each dose of each compound for both myocardial failure and non-failing control rabbits (Figure 3.5a-f) reveal that QTc lengthened more in rabbits in the myocardial failure group than in those in the control group in response to escalating doses of dofetilide (p<0.01) and quinidine (p<0.05). No significant differences in the effect of the other compounds on QTc interval were identified between the myocardial failure group and controls.
### Table 3.1. Measurements from 2-dimensional short-axis echocardiograms at baseline (before ligation), and 4 weeks post-ligation.

<table>
<thead>
<tr>
<th></th>
<th>Before ligation</th>
<th>4 wks post-ligation</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVIDd (cm)</td>
<td>1.35 ± 0.04</td>
<td>1.51 ± 0.05**</td>
</tr>
<tr>
<td>LVIDs (cm)</td>
<td>0.94 ± 0.03</td>
<td>1.12 ± 0.04**</td>
</tr>
<tr>
<td>LVPWd (cm)</td>
<td>0.27 ± 0.02</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>LVPWs (cm)</td>
<td>0.38 ± 0.01</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td>IVSd (cm)</td>
<td>0.22 ± 0.01</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>IVSs (cm)</td>
<td>0.29 ± 0.01</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>SF (%)</td>
<td>30.41 ± 0.94</td>
<td>25.90 ± 0.59*</td>
</tr>
<tr>
<td>EF (%)</td>
<td>60.04 ± 1.51</td>
<td>56.16 ± 0.69*</td>
</tr>
</tbody>
</table>

*p<0.05 vs baseline; **p<0.01 vs baseline.

Value are shown as mean ± SEM. LVIDd = Left ventricular internal diastolic diameter, LVIDs = Left ventricular internal systolic dimension, LVPWd = Left ventricular posterior wall diastolic dimension, LVPWs = Left ventricular posterior wall systolic dimension, IVSd = Interventricular septum diastolic dimension, IVSs = Interventricular septum systolic dimension, SF = Shortening fraction, EF = Ejection fraction.
<table>
<thead>
<tr>
<th></th>
<th>HR (bpm)</th>
<th>QT (ms)</th>
<th>QTc (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Surgery</td>
<td>175.0 ± 7.0</td>
<td>185.4 ± 6.9</td>
<td>262.8 ± 6.2</td>
</tr>
<tr>
<td>After LAD ligation</td>
<td>214.0 ± 8.0</td>
<td>168.3 ± 4.0</td>
<td>255.8 ± 5.2</td>
</tr>
<tr>
<td>4wks after surgery</td>
<td>166.0 ± 6.0</td>
<td>184.5 ± 4.6</td>
<td>258.4 ± 5.4</td>
</tr>
</tbody>
</table>

Table 3.2. Effects of coronary ligation on heat rate (HR), QT interval (ms), and QTc interval (ms) obtained from anesthetized rabbit at the time points before and after left anterior descending (LAD) ligation, and 4 weeks post surgery.
Figure 3.1. M-mode imaging of the left ventricular wall of a rabbit prior to coronary artery ligation (left) and 4 weeks post surgery (right) obtained from the standard right parasternal short axis view at a level just beneath the mitral valve, with the M-mode cursor directed between the papillary muscles. The wall thickness and internal ventricular dimensions during systole and diastole were measured (see cursors) which were used to calculate the shortening fraction (SF). Notice left ventricular chamber dilated after surgery.
Figure 3.2 Bipolar, transthoracic electrocardiograms from an anesthetized rabbit obtained prior to surgery (left), immediately after coronary ligation (middle), and 4 weeks post surgery (right). Notice that the J-point (ST segment) elevated dramatically after coronary artery ligation indicating massive myocardial ischemia, but that after evolution of the ischemia, the J-point was at isoelectric.
Figure 3.3. Effects of torsadogenic compounds (cisapride, clofilium, dofetilide, quinidine) and non-torsadogenic compounds (amiodarone, verapamil) on the incidence of torsade de pointes (TdP) in anesthetized rabbits with and without failing hearts. The numbers presented over the bar graph are the numbers of rabbits that had episodes of TdP/total number of rabbits in each group.
Figure 3.4. Bipolar, transthoracic ECGs obtained from 2 rabbits, the top trace from a normal rabbit and the bottom trace from a rabbit with a failing heart. Notice the rabbit with a failing heart developed torsade de pointes after intravenous infusion of 40 µg/kg dofetilide while the ECG from the healthy rabbit manifested only prolongation of QT.
Figure 3.5. Plots of difference of QTc values (ms) from baseline values versus each dose (mg/kg) of each compound for both myocardial failure and non-failing control rabbits. Notice that dofetilide and quinidine significantly lengthened QTc interval more in rabbits with myocardial failure than in non-failing control rabbits. Each data point is the average of 12 consecutive cardiac cycles. An asterisk indicates a difference between myocardial failure and non-failing control rabbits at the same dose. One asterisk indicates a $p < 0.05$, two asterisks indicate a $p < 0.01$. 
DISCUSSION

This study was designed to test the hypotheses that (1) for equivalent doses of torsadogenic compounds, QTc lengthens more in rabbits with failing hearts than in normal rabbits, and (2) rabbits with failing hearts are more prone to develop TdP than rabbits with normal hearts. Based on the results of this study, both hypotheses should be accepted, however, QTc prolonged more in rabbits with myocardial failure than in normal controls in response to cisapride, dofetilide, and quinidine, but not in response to clofilium.

Potential explanations of the pathophysiology that underpins these results remain speculative, but might include the effects of infarction and/or myocardial failure on calcium metabolism. These effects include dysfunction of channels/pumps specific for calcium cycling between the extracellular and intracellular space (e.g., calcium channel, I_{CaL}; sodium calcium exchanger, NCX; Ca-ATPase pump), and between the cytosol and sarcoplasmic reticulum (e.g., inositol 1,4,5-trisphosphate, IP3; ryanodine receptor Ca^{2+} channel; cardiac sarco/endoplasmic reticulum Ca^{2+}-ATPase, SERCA2). These channels/pumps may be affected by the degree of phosphorylation, possibly by calmodulin kinase II (CaMKII) activated by the augmented calcium-calmodulin complex (Anderson, 2006). Myocardial failure characterized by a structurally diseased heart in which the repolarizing currents are typically reduced in concert with diminished calcium handling capacity (Anderson, 2006), combined with delayed repolarization, may increase the free cytosolic calcium concentration, a factor that has been shown to be important in the induction of early afterdepolarizations and triggered activity (Qin et al., 1996).
CaMKII is upregulated in many animal models of cardiac failure (Anderson, 2006). Animals with myocardial failure may have impaired repolarization reserves (abbreviation of the duration for repolarization), a known risk of TdP in humans with failing heart patients (Roden and Yang, 2005).

Increased heterogeneity of ion channel physiology (Qin et al., 1996; Rozanski et al., 1998) is known to occur in the setting of myocardial failure. The importance of increased cardiac ion current heterogeneity and subsequently increased dispersion of repolarization and refractoriness across the left ventricular wall (i.e. transmural dispersion) to the potential genesis of TdP has been suggested in both clinical and experimental studies (Antzelevitch and Shimizu, 2002; Belardinelli et al., 2003; Lubinski et al., 1998). However, the exact relationship between dispersion of repolarization and TdP is not fully understood. Recent data suggest that spatial dispersion of ventricular repolarization provides the substrate for TdP, and early afterdepolarizations and the ectopic beats that result from them may provide the trigger (Belardinelli et al., 2003).

The lack of quinidine induced TdP in our experiments is in accord with the results of previous in vivo animal studies (Chezalviel-Guilbert et al., 1995; Farkas et al., 2002; Lu et al., 2000). One possible explanation is that the proarrhythmic activity of quinidine was blunted in this rabbit model compared to its effect in humans by the rabbit’s high heart rate (Farkas et al., 2002). Another potential explanation is that quinidine has multiple channel blocking properties (i.e., at lower concentration quinidine blocks I_{Kr} while it suppresses I_{Ks} and late I_{Na} at higher concentrations) and reduces transmural dispersion of ventricular repolarization (i.e., at higher concentration, quinidine produces a
further prolongation of the epicardial and endocardial action potentials while abbreviation of the action potential duration of M cells), a factor that has been shown to be responsible for TdP (Di Diego et al., 2003).

Intravenous amiodarone did not induce TdP in this study, a finding consistent with the findings of previous studies (Farkas et al., 2002; van Opstal et al., 2001). Amiodarone has the ability to inhibit a constellation of cardiac ionic currents (i.e., I_{Kr}, I_{Ks}, I_{Na}, I_{CaL}), resulting in little proarrhythmia (Farkas et al., 2002). Amiodarone also produces a greater prolongation of the action potential duration in the epicardium and endocardium, but less of an increase or decrease in the M cells, thereby reducing transmural dispersion of repolarization.

It appears that dofetilide, cisapride and clofilium are associated with the lowest 50% inhibitory concentration (IC50) for the human ether-a-go-go-related gene (hERG), and these compounds are more torsadogenic in failing hearts than in normals. The IC50 for amiodarone and quinidine are 0.7 µM and 0.4 µM, respectively; whereas the IC50 for clofilium, dofetilide and cisapride are 0.001 µM, 0.012 µM and 0.02 µM, respectively (Diaz et al., 2004; Kim et al., 2005).

In the current study, clofilium, dofetilide, and cisapride induced TdP. This agrees with the results of other investigators (Batey and Coker, 2002; Carlsson et al., 1990; Carlsson et al., 1997; Lu et al., 2000). These compounds block the hERG current at very low concentrations, causing lengthening of action potential duration and allowing a longer time for cellular membrane depolarization within a voltage window that allows for repetitive I_{CaL} channel re-opening. These effects initiate early afterdepolarizations (EAD),
the factor that has been shown to be the initiating mechanism of TdP (Anderson, 2006). Other *in vivo* and *in vitro* studies have shown that cisapride, clofilium and dofetilide increase transmural dispersion of repolarization, providing a substrate for TdP (Di Diego et al., 2003; Faivre et al., 1999; Wu et al., 2005).

The dosage of dofetilide that induced TdP in this study is in the range that evoked TdP in other in vivo rabbit studies, i.e. rabbits with chronic AV block (0.02 mg/kg) or $\alpha_1$-adrenoceptor stimulated rabbit (0.04 mg/kg) (Lu et al., 2000; Tsuji et al., 2006). The dofetilide concentration in this study was equal to or lower than that used to evoke TdP in isolated, perfused rabbit hearts stimulated with methoxamine and acetylcholine (0.1-0.7 µM) (D’Alonzo et al., 1999), based upon the concentration of unbound dofetilide (Smith et al., 1992). In contrast, an efficacious dose of dofetilide (0.75 µg/kg p.o.) in humans produces a plasma concentration of 2.26 ng/ml (approximately 0.005 µM). The concentration of dofetilide in the present study is approximately ten times higher than that effective human concentration (Tham et al., 1993).

More than 90% of rabbits operated upon to produce myocardial infarction survived the duration of the study. In both humans with spontaneous coronary occlusion and presumably in rabbits undergoing coronary ligation, the extent of resulting infarction may be highly variable (Cobb and Chu, 1988). Although not quantified by morphometry in this study, the extent of infarction produced by this anatomically defined technique appeared reasonably consistent from rabbit to rabbit. This model, however, is not a model of ischemic myocardial failure, since the rabbits manifested myocardial failure weeks after the ischemic event, when ischemic myocardium could be expected to be replaced by
fibrous tissue. This pathophysiology is supported by the finding of J-point deviation that persists for days after coronary ligation, but has completely resolved by the time the rabbits were exposed to test articles 4 weeks following coronary ligation.

The surgical procedure used in the study requires reasonable—but not exceptional—surgical skill and takes less than 1 hour. Within 12 hours after surgery, operated rabbits were jumping and eating normally. Although dramatic reductions in left ventricular shortening fraction indicated significant myocardial dysfunction, none of the 28 rabbits studied died from heart failure or manifested signs that might have indicated discomfort, thus we believe that this model is humane. No post-operative discomfort was observed, possibly because of the aggressive use of analgesics, and possibly because of the surgical approach (a midline sternotomy) combined with the small size of the incision. Because of the rabbits’ unique pleural anatomy, neither endotracheal intubation nor positive pressure ventilation was required (Fujita et al., 2004); and the rabbits breathed with what appeared to be a nearly normal tidal volume under anesthesia. It is important that the incision be made precisely on the midline so that no incursion is made into either the left or right thoracic cavities.

The rabbits that served as the control group for the group of operated rabbits were not sham-operated. In light of our pilot results (see methods) it is unlikely that a thoracotomy alone could account for the differences in susceptibility to TdP between the operated and non-operated rabbits.

The operated rabbits were not considered to be in heart failure because they did not show clinical signs (e.g., dyspnea, exercise intolerance), however based upon the
echocardiographic findings of left ventricular enlargement and reduction in left ventricular shortening fraction, it is clear that they had myocardial failure (Katz, 1993). This model appears to share at least some of the important properties of human heart diseases that predispose to the development of TdP.

**Conclusions**

Our findings show that rabbits with failing myocardium are more susceptible to developing drug-induced TdP than rabbits without myocardial failure, and that lower doses of torsadogens are required to produce this effect in the setting of ischemic myocardial dysfunction. Cisapride, clofilium and dofetilide induced a higher incidence of TdP in rabbits with myocardial dysfunction, whereas amiodarone, quinidine and verapamil failed to evoke TdP. This rabbit model of ischemic myocardial failure may be more useful than current surrogates for predicting torsadogenic risk in man, since it tests directly for production of TdP rather than merely for lengthening QTc interval in response to drug administration.
REFERENCES


Cardiac remodeling after myocardial infarction is associated with increased risk of ventricular arrhythmias and susceptibility to proarrhythmic effects of torsadogenic drugs. The rabbit model of myocardial failure has been shown to be prone to develop torsades de pointes (TdP) when exposed to torsadogens. However, there are little data concerning the cellular basis of arrhythmias in this model. This study was aimed to investigate the \textit{in vivo} dispersion of repolarization and the myocyte action potential duration (APD$_{90}$) of the normal and myocardial failing rabbit heart in response to doxetilide. Comparison with normal conscious rabbits, rabbits with myocardial failing hearts had a slower heart rate (228 ± 10 vs 244 ± 10 bpm; $p=\text{ns}$), prolongation of QT
(p < 0.001) and QTc (p < 0.001) intervals (154.3 ± 4.5 vs 133.3 ± 2.4 ms and 280.9 ± 6.2 vs 243.4 ± 6.1 ms, respectively). Short-term variability was increased (p < 0.05) in the myocardial failure group (0.38 ± 0.02 ms) when compared with the normal conscious group (0.29 ± 0.01 ms) whereas long-term variability tended to increase (0.39 ± 0.03 vs 0.33 ± 0.02 ms; p=ns). Myocytes isolated from border zone of infarcted area could be separated into two groups: non-hypertrophic cells and hypertrophic cells with pathological parameters of EC coupling. APD<sub>90</sub> of hypertrophic failing myocytes (912 ± 44 ms) was prolonged (p < 0.01) compared with action potentials from the same area of normal rabbits (388 ± 19 ms). In normal myocytes, dofetilide increased APD<sub>90</sub> (p < 0.01) at 0.1 µM (from 388 ± 19 to 921 ± 68 ms) with evidence of EADs. In hypertrophic myocytes, dofetilide (1.0 µM) increased APD<sub>90</sub> 21 ± 8 % from baseline (p < 0.05) and EADs were observed both before and after applied dofetilide. The hypertrophic cell has smaller Ca<sup>2+</sup> transient amplitudes than normal myocyte. Dofetilide has no effect on Ca<sup>2+</sup> current and Ca<sup>2+</sup> transient amplitude. It can be concluded that (1) the re-entrant circuit from different APD profiles, (2) EAD triggered activity and (3) increased dispersion of repolarization are responsible for genesis of TdP in the failing rabbit heart.

**INTRODUCTION**

Cardiac remodeling after myocardial infarction is associated with increased risk of ventricular arrhythmias (Qin et al., 1996) and susceptibility to proarrhythmic effects of torsadogenic drugs (Kijtawornrat et al., 2006a). Electrical remodeling is known to be responsible for the lengthening of ventricular action potential duration.
(APD) which is found in both patients with and animal models of heart failure (Tomaselli and Marban, 1999). It was demonstrated in many studies (Qin et al., 1996; Rozanski et al., 1998) that changes in the APD resulted from alterations in the functional expression of depolarizing and repolarizing currents, especially down-regulation of several potassium currents (Tomaselli and Marban, 1999) and alterations in calcium homeostasis (i.e., changes in kinetics of the ryanodine channel and the sarcoplasmic reticulum calcium ATPase) (Kleiman and Houser, 1988). Unfortunately, the prolongation of the APD in ventricular myocytes and consequently lengthening of QT interval in the electrocardiograms is associated with life-threatening polymorphic ventricular tachycardia, torsades de pointes (TdP).

Enhanced spatial and temporal dispersion of repolarization across the ventricular wall was also found in the remodeling of animal and human hearts, and was showed to produce regional differences in the APD (Berger et al., 1997; Drouin et al., 1995; Lukas and Antzelevitch, 1993). Recent reports (Antzelevitch, 2005; Di Diego et al., 2003) showed that torsadogens can amplify the dispersion of repolarization not only across different regions of ventricle but also between different layers of ventricular wall (e.g., endocardium, M cells, Purkinje fiber, and epicardium). This regional inhomogeneity of repolarization is a major substrate for reentrant ventricular tachyarrhythmias (El-Sherif et al., 1996). The importance of increased cardiac ion current heterogeneity and subsequently increased dispersion of repolarization and refractoriness across the ventricular wall (i.e. transmural dispersion) to the potential genesis of TdP has been suggested in both clinical and experimental studies (Antzelevitch and Shimizu, 2002;
Belardinelli et al., 2003; Lubinski et al., 1998). However, the exact relationship between dispersion of repolarization and TdP is not fully understood. Recent data suggest that spatial and temporal dispersion of ventricular repolarization provides the substrate for TdP, whereas the trigger for TdP appears to be EAD-induced ectopic beats originated at the site were the action potential duration is long (Belardinelli et al., 2003; Vos et al., 2001).

The rabbit model of myocardial failure produced by ligation of coronary arteries has been shown by our laboratory (Kijtawornrat et al., 2006a) to be prone to develop TdP when exposed to torsadogens. However, there are little data concerning the cellular basis of arrhythmias in this model to support this explanation. The present study was aimed at investigating the in vivo dispersion of repolarization and the cellular electrophysiology (e.g., myocyte action potential, calcium transient, and calcium current) of the normal and myocardial failing rabbit heart in response to dofetilide, a pure class III antiarrhythmic drug that has been known to produce TdP in both humans and animals.

**MATERIALS AND METHODS**

This study was approved by the Institutional Laboratory Animal Care and Use Committees (ILACUCs) of both the Ohio State University and QTest Labs, LLC. All animal procedures were conducted in accordance with guidelines published in the *Guide for the Care and Use of Laboratory Animals.*
**In vivo experiment**

A total of 39 adult male New Zealand White rabbits were used. All weighed between 2.2 and 2.8 kg. The left anterior descending and a major descending branch of left circumflex coronary artery were ligated as described previously (Kijtawornrat et al., 2006a). Echocardiographic examination of left ventricular function was performed before and 4 weeks after coronary artery ligation. A reduction in shortening fraction >15% from the baseline preoperative value was required for entry into the study. The significant but relatively small (~15%) reduction in shortening fraction was sought so that rabbits would not die from heart failure, and because experience indicated that they were prone to develop TdP (Kijtawornrat et al., 2006a).

The following experiments were performed in 21 normal rabbits and 18 rabbits with myocardial failure. It should be emphasized that the rabbits with failing myocardia were not symptomatic (i.e., no shortness of breath, no apparent exercise incapacity, no inappetence), therefore although they had failing myocardia, they would not have been classified as being in heart failure. After clipping the hair from the ventral region of the thorax, rabbits from both the normal and the myocardial failure groups were placed without chemical restraint in a ventral recumbency in a comfortable, padded sling. The sling is fitted with copper plates, which “sandwich” the ventro-cranial aspect of the thorax, such that a bipolar transthoracic electrocardiogram between points rV2 (right, 4th intercostal space at the costochondral juncture) and V2 (left, 5th intercostal space at the costochondral juncture) can be obtained. The electrodes are made with a central hole so that electrode paste can be applied from outside the sling directly onto the rabbit-
electrode interface to minimize impedance between the electrodes and the skin without disturbing the rabbit’s position in the sling. To obtain the ECG recordings, the right and left arm electrodes of the electrocardiograph were attached to the right and left sided sling electrodes, the electrocardiograph was switched to limb lead I, and a bipolar transthoracic ECG was obtained on a Biopac MP100 Data Acquisition Unit (Biopac Systems, Inc., Santa Barbara, CA). The high pass filter was set at 0.01 Hz and the low pass filter at 1 kHz, and signals were sampled at 2 kHz. After 10 minutes stabilization period in the sling, tracings were obtained for 5 minutes while the rabbits were conscious and quiet.

Electrocardiograms were analyzed for heart rate and QT. The QT interval was defined as the time between the first deviation from the isoelectric line during the PR interval until the end of the T wave. The values for QT interval were corrected for heart rate using the equation: \( \text{QTc} = \frac{\text{QT}}{\text{RR}^{0.72}} \), which has been shown previously to be appropriate for these conscious rabbits in our laboratory (Kijtawornrat et al., 2006b). RR and QT durations were measured manually by using on screen cursors during the 5th minute of recording. Measurements were made for 30 consecutive cardiac cycles, and the average was used.

The temporal dispersion of repolarization was calculated from Poincaré plot as proposed by Thomsen et al (2004). Poincaré plots were drawn by plotting each QT interval (QT\(_{n+1}\)) against the former value (QT\(_n\)). The mean orthogonal distance from the diagonal to the points of the Poincaré plot was determined and referred to as short-term
variability (STV = \( \sum |D_{n+1} - D_n| / [30 \times \sqrt{2}] \), where D represents the duration of QT interval in ms). The average distance to the mean of the parameter parallel to the diagonal (LTV = \( \sum |D_{n+1} + D_n - 2D_{mean}| / [30 \times \sqrt{2}] \)) was regarded as long-term variability.

**In vitro experiment**

**Single cell isolation**

Ventricular myocytes were isolated by enzymatic dissociation from normal and myocardial failing hearts. The process used was a modified version of Mitra and Morad (1985). Briefly, rabbits were injected with heparin 300 i.u./kg intravenously and sacrificed with an overdose of ketamine (50 mg/kg) and Xylazine (8 mg/kg) in accordance with guidelines published in the *Guide for the Care and Use of Laboratory Animals*. The heart is rapidly removed and perfused with cold Minimum Essential Medium Eagle (MEM) solution containing (in g/L): 0.09366 MgCl₂, 0.4 KCl, 6.5 NaCl, 1.154 NaH₂PO₄, 0.126 L-Arginine.HCl, 0.0324 L-Cystine.2HCl, 0.292 L-Glutamine, 0.042 L-Histidine.HCl.H₂O, 0.052 L-Isoleucine, 0.052 L-Leucine, 0.0725 L-Lysine.HCl, 0.015 L-Methionine, 0.032 L-Phenylalanine, 0.048 L-Threonine, 0.01 L-Tryptophan, 0.05452 L-Tyrosine.2Na.2H₂O, 0.046 L-Valine, 0.001 Choline Chloride, 0.001 Folic acid, 0.002 myo-Inositol, 0.001 Niacinamide, 0.001 D-Pantothenic acid, 0.001 Pyridoxal.HCl, 0.0001 Riboflavin, 0.001 Thiamine.HCl, 2.0 Glucose, 0.011 Phenol Red.Na, 2.0 NaHCO₃ at pH 7.2 for 2-3 min then the heart was quickly mounted on a Langendorff perfusion apparatus with a constant pressure of approximately 70 cm H₂O above the coronary ostia. The isolated heart was retrogradely perfused for 10 min (at
37°C) with MEM solution to remove any remaining blood. Then perfusion was switch to nominally Ca\(^{2+}\)-free MEM solution with collagenase enzyme (60 units/ml) for 15 min. Following the digestion period, the heart is removed from the Langendorff column, and the left ventricle was minced into small pieces and incubated in MEM solution with fresh enzyme containing collagenase (50 units/ml) and albumin (20 mg/ml) for 10 minutes at 37°C while being agitated in a shaking water bath. Next, the solution containing isolated myocytes was transferred into polypropylene graduated tube and the rest of cardiac tissue was digested 3 times. In case of infarcted hearts, myocytes were selectively taken from a 2 to 3 mm rim of surviving myocardium surrounding the clearly demarcated scar (Litwin et al., 2000). Subsequently, the cells in the solution were filtered (approximately 100 μM mesh), resuspended with MEM solution containing albumin, and centrifuged at 60 g for 2 minutes. Then the supernatant was removed, the unfractured cells were resuspended with MEM solution containing albumin, the cells remained at room temperature and were allowed to precipitate for approximately 5 min. This process was repeated 3 times for cleaning purposes. Next the myocytes were resuspended in modified Tyrode’s solution containing (in mM): NaCl 140, MgCl\(_2\) 0.5, KCl 5.4, glucose 5.6, CaCl\(_2\) 1, HEPES 10; pH 7.3; 23-25°C) to clean the cells. Finally, the isolated myocytes were kept in Tyrode’s solution at room temperature for 20 min and used for whole-cell recording within 3-4 hours of isolation.
*Measurement of L-type Ca\textsuperscript{2+} currents (I_{Ca,L}) and action potential (AP) by Patch clamp technique*

Whole cell transmembrane ionic currents and changes in membrane potential were recorded with an Axopatch 200B amplifier (Axon Instruments, Union City, CA) and pClamp version 6.03 (software, Axon Instruments, Union City, CA). The pipettes, borosilicate with filament (Sutter Instrument Co., Novato, CA), were pulled on a P-97 Flaming-Brown Micropipette Puller (Sutter Instrument Co., Novato, CA).

A suspension of myocytes was placed into a bath chamber (~0.5 ml) on the microscope stage and allowed to settle for a few minutes in order to attach lightly to the glass bottom of the chamber. The inverted microscope, Olympus IX81 (Olympus Optical Co. Ltd, Tokyo, Japan) sits on a vibration-free table (Technical Manufacturing Corporation, Peabody, MA) in order to minimize mechanical vibration which could disrupt the seals. The pipettes were filled with a pipette solution (in mM: cecium aspartate 70, CsCl 50, ATP (Mg\textsuperscript{2+}) 3, NaCl 4, HEPES 5, fluo-3 potassium salt 0.05; pH 7.3) and then attached to a pipette holder with side port. A length of flexible tubing was attached to the side port for subsequent suction. The pipette holder was attached to the amplifier headstage, which was in turn mounted on a hydraulic micromanipulator (model MO-303 Narishige Co., Ltd.).

Subsequently, the pipette was brought down onto the cell at a fairly steep angle (>45°) to increase the probability of forming a tight membrane-glass seal. Once a gigahm seal was formed between the glass and cell membrane, capacitance transients were
generated by applying 5 mV voltage pulses to the pipette. An Axopatch 200B amplifier, pClamp software was used to deliver the voltage protocols and to acquire all the data, which were stored on a PC for subsequent analysis by the pClamp software.

The whole-cell configuration was subsequently attained by rupturing the patch of membrane within the pipette by “zapping” the membrane. This latter zapping is a feature of amplifiers which consists of a very short duration, high voltage (> 1V) pulse, which will often cause the dielectric breakdown of the small patch of membrane in the pipette tip. Attainment of the whole-cell mode is evident as a sudden dramatic increase in the duration of capacitance transient, due to charging of the entire cell membrane. Once access to the cell interior was obtained, several minutes were allowed to pass to enable cell dialysis and current levels to stabilize. Once the currents were stable, the experiments proceeded.

**Voltage Clamp**

Measurements of calcium current and action potential were performed at baseline and 3-5 min after application of 0.1 µM and/or 1.0 µM dofetilide into the bath solution. Myocytes were voltage-clamped using borosilicate micropipettes (resistance 1 to 2 MΩ). Voltage pulses of 400 ms duration were applied from a holding potential of -40 mV at 0.2 Hz. Signals were sampled at 5 kHz and filtered at 2 kHz. The use of room temperature, together with a low stimulation frequency, also minimizes the amount of spontaneous “rundown” of the current, a problem that often plagues recording of calcium current.
**Current Clamp**

Action potential waveforms were recorded from dispersed left ventricular cells from normal myocardium and from peri-infarcted regions of hearts in failure. Action potentials were recorded under steady-state conditions (1.0 Hz; 23-25°C) in current clamp mode.

**Confocal microscopy**

Cytosolic Ca\(^{2+}\) changes in patch-clamped myocytes were measured with an Olympus Laser Scanning Confocal System (LSM-GB200, Olympus Optical Co. Ltd, Tokyo, Japan) equipped with an Olympus 60X oil, 1.4 NA objective. Fluo-3 was excited at the 488-nm line by an argon laser, with emission collected through a 515-nm long-pass filter. Fluorescence images were recorded in the line-scan mode oriented along the long axis of the cell at the rate of 2 ms per line. Recordings were made simultaneously with either current clamp or voltage clamp by Olympus Fluoview 1.4a software (Olympus Optical Co. Ltd, Tokyo, Japan).

Dofetilide stock solution (100 µg/ml) was prepared by dissolved dofetilide 500 µg in 5 ml of sterile water for injection with a just sufficient quantity of 0.1 M HCl. Dofetilide was administered into bath solution to yield a concentration of 0.1 µM and 1.0 µM after baseline measurement.

Dofetilide was obtained from Pfizer (Pfizer, Groton, CT). All other reagents were from Sigma-Aldrich (Sigma, St Louis, MO).
**Statistical analysis**

Data are expressed as means ± SEM’s. Comparisons of RR, QT, QTc, STV and LTV parameters between normal rabbits and myocardial failure groups were performed with Student’s \( t \)-test. Comparisons of data from normal and myocardial failure myocytes were performed by using Student’s \( t \)-test whereas the comparisons of data between before and after dofetilide application were performed by using Student’s paired \( t \)-test. Statistical difference was acknowledged at \( p < 0.05 \).

**RESULTS**

*In vivo changes in echocardiograms and electrophysiology parameters*

After 4 weeks of coronary ligation, echocardiographic measurements revealed significant left ventricular chamber dilatation and systolic dysfunction in the rabbits with coronary ligation. Hypokinesis was observed at the posterior and lateral walls of the left ventricle. Compared with normal conscious rabbits, rabbits with myocardial failure had slower heart rates (228 ± 10 bpm vs 244 ± 10 bpm) but the difference did not achieve statistical significance. There was significant prolongation in the rabbits with myocardial failure of QT (\( p < 0.001 \)) and QTc (\( p < 0.001 \)) intervals (154.3 ± 4.5 ms vs 133.3 ± 2.4 ms and 280.9 ± 6.2 ms vs 243.4 ± 6.1 ms, respectively). Short-term variability (STV) in QT, an indicator of beat-to-beat variability, was significantly increased (\( p < 0.05 \)) in the myocardial failure group (0.38 ± 0.02 ms) when compared with normal conscious group (0.29 ± 0.01 ms) whereas long-term variability (LTV) tended to increase but the increase did not achieve statistical significance (0.39 ± 0.03 ms vs 0.33 ± 0.02 ms).
Figure 4.1 shows Poincaré plots of $QT_n$ versus $QT_{n+1}$ from one normal rabbit and one rabbit with myocardial failure. It can be observed that the STV, the mean orthogonal distance from the diagonal to the points of the Poincaré plot, and LTV, the average distance to the mean of the parameter parallel to the diagonal, were increased in the rabbit with myocardial failure.

Changes in myocyte morphology

Four weeks after coronary ligation, the infarcted area of the left ventricle showed obvious changes. There was a relatively clear border between infarcted and noninfarcted areas. The free wall of the infarcted area was very thin, whereas the noninfarcted left ventricular wall was much thicker than normal. Interestingly, cells isolated from non-infarcted area (myocardial failing heart) have biophysical properties similar to cells isolated from normal heart. On the other hands, cells isolated from border zone of infarcted area could be separated into two groups: non-hypertrophied cells and hypertrophied cells with pathological parameters of EC coupling (Figure 4.2). In the normal myocytes, the cell membrane capacitance was $137 \pm 11$ pF compared with $272 \pm 35$ pF in the myocardial hypertrophic myocytes, a significant increase ($p < 0.01$), and $151 \pm 18$ in cells from non-hypertrophy myocytes isolated from border zone of infarcted left ventricle.
Figure 4.1. Examples of beat-to-beat Poincare plots of QT duration in one cycle ($QT_n$) to its subsequent cycle ($QT_{n+1}$) in the conscious normal and myocardial failing rabbits (4 wks after coronary artery ligation). Thirty cycles of each animal were evaluated at the 5th min of baseline recording while remained conscious but quiet in the comfortable padded sling. STV is stand for short-term variability, which is the mean orthogonal distance from the diagonal to the points of the Poincare plot. LTV stands for long-term variability, which is the average distance to the mean of the parameter along the diagonal.
Figure 4.2. Images from confocal microscope (60X) of single ventricular myocyte isolated from the left ventricular wall of normal heart (a), and myocardial failing heart at the border of infarcted zone (b and c). It can be observed that the border of infarcted zone has two types of myocytes, hypertrophic (b) and non-hypertrophic (c) myocytes.

Changes in action potential configurations

The action potential durations from two different myocytes isolated from the border area of the infarcted zone of myocardial infarcted rabbit expressed differences in action potential configuration. The hypertrophic myocyte has a prominently longer APD$_{90}$ than the non-hypertrophic myocyte, and EADs were only observed from hypertrophic myocytes without any intervention (Figure 4.3). Action potential characteristics of single isolated myocardial failure myocytes were analyzed and
compared with isolated normal rabbit myocytes (Figure 4.4). Action potentials were analyzed at 0.5 Hz stimulation. Action potentials from hypertrophic failing myocytes (912 ± 44) were significantly prolonged ($p < 0.01$) compared with action potentials from the same area of normal rabbits (388 ± 19). Dofetilide was applied in current clamp mode for at least 3 minutes to achieve steady state before any measurements. In normal rabbit myocytes isolated from the left ventricular free wall, dofetilide increased APD$_{90}$ significantly ($p < 0.01$) at 0.1 µM (from 388 ± 19 to 921 ± 68 ms; Figure 4.4). Furthermore, early afterdepolarizations were observed in every myocyte after application of dofetilide (11 out of 11 cells). On the other hand, action potential durations of hypertrophic myocytes isolated from peri-infarcted zone of rabbit failing myocardium were not significantly prolonged in response to 0.1 µM dofetilide compared with baseline action potential (from 912 ± 44 to 922 ± 46 ms). However, when 1.0 µM dofetilide was applied to the bath solution, action potential durations increased significantly (21.8 %) from baseline ($p < 0.05$). Importantly, EADs were observed both before and after application of dofetilide into the bath solution.
Figure 4.3 Configurations of action potentials from two different myocytes isolated from the border of infarcted zone. It can be observed that two myocytes from the same area showed different properties (i.e., durations, stability of phase 2, the rate of change in phase 3, and EADs) of action potentials.
Figure 4.4 Effects of dofetilide on action potential duration in normal and failing myocardium. It can be observed that dofetilide increased APD$_{90}$ significantly in both normal (0.1 µM) and hypertrophic (1.0 µM) myocytes.
**Changes in calcium transient**

In the voltage clamp mode, myocytes isolated from the myocardial failure group exhibited smaller calcium transient amplitude (Figure 4.5; $p < 0.05$) when compared to normal myocytes. Dofetilide did not produce significant changes in calcium transient amplitude or decay time of fluorescence signal in both normal and failing myocardium.

**Changes in calcium current**

In the voltage clamp mode, myocytes isolated from the myocardial failure group exhibited minimal changes in calcium current amplitude (Figure 4.5) when compared to normal myocytes. Dofetilide did not exhibit significant changes of calcium current amplitude in either normal or failing myocardium.
Figure 4.5 Effects of dofetilide on calcium transients and calcium currents in normal and failing myocardium.
DISCUSSION

*Increase short-term variability together with prolongation of QT/QTc interval*

The primary aim of the present study was to compare liability of failing/hypertrophic myocardium to develop repolarization changes in conscious rabbits after 4 weeks of coronary ligation. Berger et al (1997), Hondeghem et al (2004), and Thomsen et al (2004) have showed that liability for repolarization is critical for predicting susceptibility to proarrhythmia in patients and to assess proarrhythmic potential of drugs. Previously, we demonstrated that TdP can be induced by escalating concentrations of torsadogenic compounds in rabbits with myocardial failure (Kjitawornrat et al., 2006a). In this study, we have shown that rabbits with myocardial failure have a higher STV of QT interval than normal rabbits. Thomsen and colleagues (2004) demonstrated that the higher the STV, the greater the likelihood for EADs, a known trigger for TdP. Therefore, an elevation of STV in our rabbit model might be responsible for enhanced susceptibility to arrhythmias. It could be possible that electrophysiological remodeling (e.g., slower heart rate, longer QT and QTc intervals) occurred after myocardial infarction induces changes in lability of repolarization, which can be expressed by measurement of beat-to-beat variability. Similar results have been reported in chronic AVB dogs in which enhanced variability of repolarization has been found to be related to sudden cardiac death (Thomsen et al., 2005). Furthermore, patients with ventricular disease experiencing sudden cardiac death have been reported to have an
increase in lability of repolarization (Atiga et al., 1998). Therefore, assessment of beat-to-beat variability together with QT/QTc interval could provide a better predictive value in patients who are susceptible for drug-induced arrhythmias.

**Remodeled myocytes and arrhythmia mechanisms**

With the help of patch clamp technology and laser-scan confocal imaging, we were able to measure Ca\(^{2+}\) transients at the same time with AP and Ca\(^{2+}\) current. Interestingly, two different action potential configurations were obtained from the failing myocardium. The hypertrophic cell has a marked prolongation of APD\(_{90}\) whereas the non-hypertrophic cell has the same APD\(_{90}\) as myocytes isolated from normal hearts. Hence, the results of the present study indicate that myocytes are remodeled after coronary arteries were ligated, and this remodeling could indicate a substrate to develop arrhythmias. Restivo et al (1990) reported that increased heterogeneity of APD in the remodeled hypertrophied ventricle can result in dispersion of refractoriness, a critical substrate for the development of circus movement reentrant tachyarrhythmias. Recently, Qin et al (1996) suggested that hypertrophy-induced increases in interstitial tissue with possible impairment of cellular coupling can also contribute to the occurrence of reentry. Possibly connexins may be important in the alterations of conductivity.

Another potential mechanism for ventricular tachyarrhythmias in the failing myocardium is triggered activity (EAD’s) (see Figure 4). In the present study, we demonstrated the development of single and repetitive EADs in isolated myocytes. Prolongation of APD is considered the initiating step for the development of EADs (Qin et al., 1996), and this can be supported by the finding of spontaneous generation of EAD’s in the hypertrophic cells of our study.


**Dofetilide increased APD\textsubscript{90} in rabbit ventricular myocytes**

Our results confirmed APD\textsubscript{90} prolongation in ventricular myocytes in response to dofetilide whether exposure is *in vivo* or *in vitro*. This result was consistent with previous work in human ventricular myocytes by Jost et al. (2005). The mechanism underlying this APD\textsubscript{90} prolongation was recently reported by Kamiya et al. (2006). The authors suggested that dofetilide reduced hERG currents because dofetilide was trapped in the central cavity of the hERG channel by the closure of the activation gate. They also suggested that three residues near the pore helix (Thr623, Ser624, and Val625) are the binding sites for dofetilide that block hERG in the open state. The reduction of I\textsubscript{Kr} currents resulted in prolongation of APD\textsubscript{90} and has been associated with EAD’s and polymorphic ventricular arrhythmias (Buchanan et al., 1993). It is interesting to note that APD\textsubscript{90} of normal rabbit myocytes is prolonged at lower concentrations (0.1 µM) of dofetilide, whereas APD\textsubscript{90} of hypertrophic myocytes was lengthened at higher concentration (1.0 µM). It is possible that densities of potassium channels, especially I\textsubscript{Kr} and I\textsubscript{Ks} channels, were downregulated during the process of remodeling after myocardial infarction. Tsuji et al (2006) and Volders et al (1999) have shown that both I\textsubscript{Kr} and I\textsubscript{Ks} were downregulated in the model of chronic AV block in both rabbit and dog. The down regulation of potassium channels in these models causes prominent delay in repolarization and spontaneous TdP. Therefore, the pronounced prolongation of APD\textsubscript{90} in the hypertrophic myocyte of our study may be partly due to downregulation of potassium channel. However, measurements of potassium currents and channel densities were not performed in this experiment.
Furthermore, the results of dofetilide in hypertrophic and non-hypertrophic myocytes in this study contradict to our previous results (Kijtawornrat et al., 2006a) obtained in anesthetized rabbits given escalating doses of dofetilide, in which dofetilide delayed repolarization more in rabbits with failing hearts than in normal rabbits given the same doses of dofetilide. The mechanism underlying such differences is not clear, but may be related to the fact that the heart, in vivo, is affected by innervation, circulating hormones, and metabolites which may alter responses differently than in isolated myocytes.

**Dofetilide-induced EADs in myocytes and spontaneous Ca\(^{2+}\) release from SR**

We have shown that dofetilide induces EADs in normal myocytes. It also increased frequency of Ca\(^{2+}\) sparks and spontaneous Ca\(^{2+}\) release from SR. This result is supported by the study of January and Riddle (1989) who showed that Ca\(^{2+}\) influx via \(I_{CaL}\) was responsible for EADs during prolonged action potentials, and the study of Wu et al. (2002) who showed that EADs may result from phosphorylation of calcium channels by CaMKII. This local increase of Ca\(^{2+}\) might induce more spontaneous release of Ca\(^{2+}\) via ryanodine channels, and therefore eventually premature Ca\(^{2+}\) transients (Guo et al., 2006).

**Dofetilide has no direct effect on \(I_{CaL}\) and Ca\(^{2+}\) transient amplitude**

Previous studies using this model (Litwin et al., 2000) have documented significant alterations in calcium transients and calcium currents in failing myocardium. In the present study we also found evidence of alteration in calcium transient amplitude, in which myocytes from failing myocardium have smaller Ca\(^{2+}\) transient
amplitudes than normal myocytes. The Ca\textsuperscript{2+} transient amplitudes were also measured before and after application of dofetilide in the voltage clamp mode. Surprisingly, the amplitude of the Ca\textsuperscript{2+} transients did not increase after dofetilide application. These data are consistent with previous observations by Srivastava et al. (2005) who measured Ca\textsuperscript{2+} transients in rabbit myocytes isolated from both neonates and adults.

As we expected, dofetilide did not affect Ca\textsuperscript{2+} current amplitude in either normal or failing myocardium. This result is consistent with the previous studies of normal rabbit myocytes (Paul et al., 2001; Srivastava et al., 2005).

It is not known if hypertrophic myocardium from etiologies other than infarction (e.g., hypertension, valvular disease, cardiomyopathy) might be equally prone to EADs and reentrant pathways; in particular how important the fibrosis of infarction and/or regional, residual ischemia might be.

**Conclusion:**

We concluded that myocardium from the failing rabbit heart has an elevated spatial dispersion of repolarization which is known to be a substrate for TdP. Moreover, myocytes are remodeled after myocardial infarction, produce triggered activity (EADs) and create reentrant circuits due to dispersion of repolarization (i.e., different in APD\textsubscript{90}) and possibly to islets of disease myocardium around which circus movements may occur.
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REFERENCES


CHAPTER 5

EFFECTS OF SARCOLEMMAL CALCIUM ENTRY BLOCKERS, SARCOPLASMIC RETICULUM CALCIUM RELEASE BLOCKER, AND SIGNALING PROTEIN BLOCKERS ON DOFETILIDE-INDUCED TORSADES DE POINTES IN CONSCIOUS RABBIT WITH FAILING HEART

ABSTRACT

QT interval prolongation may be antiarrhythmic, but also it may be associated with increased risk of arrhythmias, in particular torsades de pointes (TdP). Early afterdepolarizations (EADs) and transmural dispersion of repolarization have been known to serve as a physiological substrate and predictor for TdP. It is thought that abnormal calcium cycling is the proximate cause of EADs, and it is known that calcium cycling is abnormal in heart failure. However, the mechanisms for drug-induced TdP in heart failure are not well understood. The purpose of this study was to search for torsadogenic-modifying effects of verapamil, ryanodine, KB-R7943, W-7, KN-93, and
H-8 on ventricular premature depolarizations (VPD) and TdP in the conscious failing rabbit heart, a model known to be prone to develop TdP. Failing rabbit hearts were pretreated with propranolol followed by test articles before continuous infusion of dofetilide, used to induce TdP. In the vehicle control hearts, VPD and TdP were induced in all rabbits (8 of 8) and the onset of VPD and TdP were 3.6 ± 1.3 min and 10.3 ± 1.4 min, respectively. Intravenous infusion of dofetilide was associated with lengthening of RR, QT and QTc interval. Verapamil, ryanodine and H-8 significantly delayed onset of VPD ($p<0.05$) and suppressed occurrence of TdP ($p<0.01$). On the other hand, KB-R7943, W-7, and KN-93 accelerated onset of TdP. Blockade of L-type calcium channel, blockade of calcium release channel of sarcoplasmic reticulum, and blockade of protein kinase A prevent dofetilide-induced TdP, suggesting roles of intracellular calcium overload and calcium signaling pathway in drug-induced TdP. Also, our data indicates that blockade of sodium/calcium exchange (NCX), calmodulin, and calcium/calmodulin dependent protein kinase II (CaMKII) may favor the development of TdP in the setting of heart failure, and suggests potential new avenues for development of antiarrhythmics in the presence of heart failure.

INTRODUCTION

Prolongation of the action potential duration (APD), the result of a reduction in net repolarizing current or an increase in depolarizing current, translates to lengthening of the QTc interval on the electrocardiogram, the culprit marker for torsades de pointes
(TdP). It has been shown previously that early afterdepolarizations (EADs) and transmural dispersion of repolarization (TDR) are the candidates for causes of TdP (Antzelevitch, 2005; Belardinelli et al., 2003). While TDR serves as a substrate for the initial EADs and subsequent re-entrant tachycardia (Belardinelli et al., 2003), EADs, the oscillations of membrane potential during phase 2 and 3 of cardiac action potential, can trigger a new action potential (AP) and augment electrical heterogeneity in neighboring regions of myocardium. Thus, EADs can provide not only the trigger but also the substrate for the initiation and perpetuation of TdP (Volders et al., 2000).

It was suggested that EADs are associated with reactivation of long lasting calcium channels ($I_{\text{CaL}}$) in the setting of APD prolongation (January and Riddle, 1989). Nisoldipine, a Ca$^{2+}$ entry blocker, has been shown to attenuate clofilium-induced TdP in anesthetized rabbits (Carlsson et al., 1996). EADs are also linked to spontaneous releases of Ca$^{2+}$ from the sarcoplasmic reticulum (SR) calcium release channel (ryanodine channel). Antagonists tended to reduce the incidence of TdP but this reduction did not achieve statistical significance (Carlsson et al., 1996). Recently the sodium calcium exchange (NCX) was suggested to produce triggered arrhythmias in heart failure by prolonging the plateau phase of the AP thereby providing time for the reactivation of $I_{\text{CaL}}$ which then causes the EADs (Viswanathan and Rudy, 1999). The effects of NCX blocker on ventricular arrhythmias are still controversial; however, KB-R7943, a specific blocker of the reverse mode of NCX, has been shown to suppress arrhythmia in guinea pigs (Amran et al., 2004).
In the setting of heart failure, abnormalities in Ca\(^{2+}\) handling, reduction in cardiac contractility, and diminution in β-adrenergic receptor responsiveness due to an increase in circulating catecholamine were reported in both humans and animals (Ai et al., 2005; Antos et al., 2001; Kirchhefer et al., 1999). The underlying mechanisms of heart failure are still unclear; however, protein kinases (e.g., cAMP dependent protein kinase, PKA; calcium/calmodulin dependent protein kinase II, CaMKII) are known to regulate cardiac function and it is possible that altered activity of protein kinases are responsible for the disturbed Ca\(^{2+}\) homeostasis in heart failure (Kirchhefer et al., 1999). It was found that PKA activity and its protein level in cardiac muscle were significantly increased in genetically cardiomyopathic hamsters (Wang et al., 1999). Altered Ca\(^{2+}\) homeostasis in heart failure is also implicated in arrhythmogenesis (Pogwizd et al., 2001). Recent studies have shown that PKA and CaMKII signaling pathways are involved in arrhythmia induction, especially in the present of certain conditions such as prolongation of the action potential and cardiovascular remodeling (Ai et al., 2005; Anderson, 2006). Many studies demonstrated that the PKA inhibitor (H-8), the calmodulin antagonists (W-7), and the CaMKII inhibitor (KN-93) can suppress EADs and attenuate TdP (Anderson et al., 1998; Gbadebo et al., 2002; Mazur et al., 1999). This evidence implies that PKA inhibition, CaMKII inhibition and calmodulin antagonism could prevent TdP and therefore might be useful as novel antiarrhythmic drugs. However, these modalities have not been evaluated in a systematic manner in a uniform preparation. In addition, these compounds were not tested in the setting of HF, with increased adrenergic drive.
The purpose of this study was to search for torsadogenic-modifying effects of verapamil, ryanodine, KB-R7943, W-7, KN-93, and H-8 on ventricular premature depolarizations (VPD) and TdP in the conscious failing rabbit heart known to be prone to develop TdP. Our specific hypotheses are: 1) reduced sarcolemmal Ca\(^{2+}\) entry produced by verapamil and KB-R7943 prevent the development of TdP; 2) blocking of ryanodine receptor (RyR) of SR by ryanodine attenuates the incidence of TdP; 3) the calmodulin antagonist, PKA blocker, and the CaMKII inhibitor prevent the development of TdP but do not alter drug-induced lengthening of QTc. This study might provide understanding of the mechanisms of enhance arrhythmogenicity in hearts of patients with heart failure, and to develop novel strategies either therapeutic for or prophylaxis against development of TdP

**MATERIALS AND METHODS**

This study was approved by the *Institutional Laboratory Animal Care and Use Committees* (ILACUCs) of both the Ohio State University and QTest Labs, LLC. A total of 43 male rabbits were used. All weighed between 2.4 and 2.8 kg. All animal procedures were conducted in accordance with guidelines published in the *Guide for the Care and Use of Laboratory Animals*. The detail of producing heart failure in rabbit has been previously described (*Kijtawornrat et al.,* 2006a). Echocardiographic examination of left ventricular function was performed before and 4 weeks after coronary artery ligation. A reduction in shortening fraction of approximately 15 % from the baseline
preoperative value was required for the entry into the study. The significant but relatively small (~15%) reduction in shortening fraction was sought so that rabbits would not die from heart failure. All echocardiographic images were acquired and analyzed by a single experienced operator. Mean ± SEM was calculated for left ventricular shortening fraction (SF), left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD), left ventricular end-diastolic wall thickness (LVEDWT), and left ventricular end-systolic wall thickness (LVESWT). Values obtained before and 4 weeks after surgery were compared utilizing a paired t-test.

**Experimental protocol**

Thirty-nine surviving rabbits with failing hearts were randomized into 7 groups: 1) vehicle (n=8), 2) verapamil (n=5), 3) KB-R7943 (n=4), 4) ryanodine (n=5), 5) W-7 (n=8), 6) KN-93 (n=4), and 7) H-8 (n=5). Rabbits were placed in a padded sling which provided necessary but minimal restraint and permitted recording of a bipolar transthoracic electrocardiogram. The right and left thoracic limb electrodes are attached to the right and left hemithoraces, the electrocardiograph is switched to limb lead I, and a bipolar transthoracic ECG is obtained on a Biopac MP100 Data Acquisition Unit (Biopac Systems, Inc., Santa Barbara, CA). The high pass filter was set at 0.01 Hz and the low pass filter at 1 kHz, and signals were sampled at 2 kHz. Catheters were inserted into the right marginal ear veins and into the left central ear artery for infusion of drug and for recording of arterial blood pressure, respectively. All animals were allowed to stabilize in the sling for 10 minutes before start of any protocol.
After 10 min stabilization, all rabbits were pretreated with propranolol (0.5 mg/kg i.v.) in order to avoid reflex tachycardia (Carlsson et al., 1992). Ten minutes later, a continuous infusion of either vehicle (0.5 ml/kg), verapamil (0.3 mg/kg), KB-R7943 (3 mg/kg), ryanodine (10µg/kg), W-7 (50 µM/kg), KN-93 (0.1 µM/kg) or H-8 (10 µM/kg) was started and infused over a period of 10 min. Immediately after the end of infusion of vehicle or test articles, dofetilide was infused for at most 15 minutes at a rate of 0.04 mg/kg/min or until the first occurrence of TdP. Electrocardiograms were analyzed for heart rate, QT, and rhythm. The QT interval was defined as the time between the first deviation from the isoelectric line during the PR interval until the end of the T wave. The values for QT interval were corrected for heart rate using the equation: QTc = QT/(RR)^0.72, which has been shown previously to be appropriate for these conscious rabbits in our laboratory (Kijtawornrat et al., 2006b). RR and QT durations were measured manually by using on screen cursors during baseline, 10 minutes after propranolol injection, at the end of vehicle or treatment with test articles, and at 5 min of dofetilide infusion or before arrhythmias presented. Measurements were made for 30 consecutive cardiac cycles, and the average was used. The ECG intervals were measured in beats that originated from the sinoatrial node. Rhythm was classified as normal sinus rhythm, VPD or TdP. TdP was defined as a polymorphic ventricular tachycardia where clear twisting of the QRS complexes around the isoelectric axis was seen. Arterial blood pressure was continuously recorded for measurement of systolic blood pressure (SBP) and diastolic blood pressure (DBP). Mean arterial blood pressure (MBP) was calculated using the equation: MBP = DBP+1/3(SBP-DBP). At the end of the experiment, the rabbits were euthanatized with 50 mg/kg pentobarbital given intravenously.
**Drugs**

Dofetilide (Pfizer, Groton, CT) was prepared as a solution in pyrogen-free 0.9% NaCl acidified with 0.1 M hydrochloric acid, at a stock concentration of 0.1 mg/ml. Propranolol hydrochloride (Sigma, St. Louis, MO) was dissolved in 0.9% NaCl. Verapamil hydrochloride (Sigma, St. Louis, MO), W-7 and H-8 (Biomol international, Plymouth meeting, PA) were dissolved in 5% dextrose solution. KB-R7943 mesylate (Tocris bioscience, Ellisville, MO), ryanodine, and KN-93 (Biomol international, Plymouth meeting, PA) were dissolved in 0.1% DMSO. All drugs were freshly prepared each day. The dose of dofetilide and pretreated compounds were chosen according to previous publication (Amran et al., 2004; Anderson et al., 1998; Carlsson et al., 1996; Gbadebo et al., 2002; Kijtawornrat et al., 2006a; Mazur et al., 1999; Verduyn et al., 1995).

**Statistics**

Values are expressed as mean ± SEM and n indicates the number of animal. Student’s t test and one way Analysis of variance (ANOVA) were applied when appropriate. Post hoc comparisons were performed with Tukey’s test. Fisher’s exact tests were used to compare the incidence of VPD and TdP. A \( p < 0.05 \) was considered to be significant.
RESULTS

Effects of coronary ligation on echocardiogram:

Coronary ligation in the rabbit provided a reproducible model of left ventricular systolic dysfunction. Effects of myocardial infarction on wall motion and geometry were detected clearly 4 wks after coronary ligation. Hypokinesis was observed at the posterior and lateral walls of the left ventricle (LV). LV shortening fraction, the “gold standard” estimate of left ventricular function, decreases after coronary artery ligation from 32.64% to 25.21%, a 22.7% decrease ($p < 0.001$). Over the 4 wks after myocardial infarction, marked dilation in left ventricular systolic and diastolic chamber dimensions were observed. As a result, LVEDD and LVESD were significantly increased (46.2%; $p < 0.001$ and 63.7%; $p < 0.001$, respectively) whereas LVEDWT and LVESWT were insignificantly changed.

Induction of TdP

In the vehicle group, TdP developed in all rabbits after continuous infusion with 0.04 mg/kg/min of dofetilide. Rabbits developed bradycardia and prolongations of QT interval, VPD, and TdP after dofetilide administration (Figure 5.1). Figure 5.2 shows the types of dofetilide-induced ventricular arrhythmias, and Table 5.1 shows the time between dosing of dofetilide and onset of arrhythmia. VPD occurred in all rabbits given dofetilide (except for 1 rabbit treated with verapamil) whether or not they received vehicle (8 out of 8), verapamil (4 out of 5), KB-R7943 (4 out of 4), ryanodine (5 out of 5), W-7 (8 out of 8), KN-93 (4 out of 4), or H-8 (5 out of 5). TdP occurred in 8 out of 8
rabbits receiving vehicle and dofetilide. TdP did not occur in rabbits pretreated with verapamil (0 out of 5), and occurred only in 1 out of 5 rabbits receiving dofetilide and pretreated with either ryanodine or H-8. Rabbits pretreated with W-7 and KN-93 but not KB-R7943 development TdP less frequently (5 out of 8, 3 out of 4 and 4 out of 4, respectively). The time to the first VPD was significantly increased for verapamil, ryanodine, and H-8 treated animals compared with vehicle treated animal. Rabbits treated with KB-R7943, W-7, or KN-93 had a significantly shortened time to the development of first TdP when compared to vehicle treated animal.
Figure 5.1 Transthoracic electrocardiogram and evolutionary changes from rabbits receiving vehicle (0.1% DMSO at 0.5 ml/kg), verapamil (0.3 mg/kg), KB-R7943 (3 mg/kg), ryanodine (10µg/kg), W-7 (50 µM/kg), KN-93 (0.1 µM/kg) and H-8 (10 µM/kg) at baseline, after propranolol, after test articles, and at the first torsades de pointes (TdP) or after 15 min of continuous infusion of dofetilide (0.04 mg/kg/min). Notice that failing hearts exposed to KB-R7943, W-7, and KN-93 developed TdP. On the other hand, failing hearts exposed to verapamil, ryanodine, and H-8 did not develop TdP. Each segment of tracing is of 2.5 seconds.
Figure 5.2 Effects of pretreatment with vehicle (0.1% DMSO; 0.5 ml/kg), verapamil (0.3 mg/kg), KB-R7943 (3 mg/kg), ryanodine (10 µg/kg), W-7 (50 µM/kg), KN-93 (0.1 µM/kg) or H-8 (10 µM/kg) on the incidence of dofetilide-induced torsades de pointes (TdP) and ventricular premature depolarizations (VPD) in conscious rabbits with failing hearts. All rabbits were pretreated with propranolol (0.5 mg/kg) to avoid reflex tachycardia before beginning infusion of each test article. Differences in the incidence of TdP and VPD between the vehicle group and the test article groups were statistically evaluated by means of Fisher’s exact test. Notice that TdP inducibility is reduced with the calcium channel blocker (verapamil), sarcoplasmic reticulum calcium release channel blocker (ryanodine), and protein kinase A inhibitor (H-8). **p<0.01 compared to vehicle treated group.
<table>
<thead>
<tr>
<th>Pretreatment Compounds</th>
<th>Ventricular premature depolarizations</th>
<th>Torsades de Pointes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>3.6 ± 1.3</td>
<td>10.3 ± 1.4</td>
</tr>
<tr>
<td>Verapamil</td>
<td>11.7 ± 3.0*</td>
<td>NA</td>
</tr>
<tr>
<td>KB-R7943</td>
<td>2.1 ± 0.4</td>
<td>3.0 ± 0.3**</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>7.5 ± 1.3*</td>
<td>10.2a</td>
</tr>
<tr>
<td>W-7</td>
<td>4.1 ± 0.7</td>
<td>5.0 ± 1.8*</td>
</tr>
<tr>
<td>KN-93</td>
<td>1.7 ± 0.1</td>
<td>2.6 ± 0.6**</td>
</tr>
<tr>
<td>H-8</td>
<td>7.9 ± 1.6*</td>
<td>13.9a</td>
</tr>
</tbody>
</table>

Table 5.1 Effects of pretreatment compounds (vehicle, verapamil, KB-R7943, ryanodine, W-7, KN-93, and H-8) on the onset of arrhythmias (ventricular premature depolarizations and torsades de pointes) in conscious rabbit with failing heart. *p<0.05 vs vehicle, **p<0.01 vs vehicle. Values are shown as mean ± SEM. NA= no torsades de pointes has been induced after pretreatment with verapamil. a=SEM can not calculated since n=1.
The influence of verapamil, KB-R7943, Ryanodine, W-7, KN-93, and H-8 on electrocardiogram variables

Figures 5.3, 5.4 and 5.5 show plots of baseline adjusted RR interval, QT interval, and QTc interval for baseline, after propranolol, after vehicle or after each test article, and after dofetilide. Following propranolol, RR interval appeared to lengthen for all groups when compared to baseline; however, the lengthening was small and did not achieve statistical significance. KB-R7943 significantly lengthened RR interval when compared to baseline ($p<0.001$). Dofetilide significantly lengthened RR interval after vehicle ($p<0.05$) and verapamil ($p<0.05$) when compared to baseline of each group. QT appeared to lengthen whereas QTc appeared to shorten when rabbits were exposed to propranolol; however, this changed did not achieve statistical significant. Dofetilide produced not only dramatic lengthening of QT in all groups except for KB-R7943 but also prolonged QTc in all groups except KB-R7943 and verapamil. The QTc lengthened more in the groups receiving KN-93 ($p<0.01$) and H-8 ($p<0.01$) than in the group receiving vehicle. The QTc intervals are significantly shortened in verapamil treated group ($p<0.05$) receiving dofetilide compared to vehicle treated group.

The influence of verapamil, KB-R7943, Ryanodine, W-7, KN-93, and H-8 on mean arterial blood pressure

Figure 5.6 shows plots of baseline adjusted mean systemic arterial blood pressures (MBP) for each group of rabbits. MBP was not significantly changed in any group except rabbits with failing hearts that were pretreated with verapamil and H-8.
Rabbits pretreated with verapamil and H-8 had precipitously and significantly decreased MBP when compared to baseline values ($p<0.001$ and $p<0.01$, respectively). In those rabbits, MBP were also significantly decreased when compared to vehicle treated group at the same time ($p<0.05$ and $p<0.01$, respectively). In response to dofetilide, only the verapamil treated group had a significant decrease in MBP.
Figure 5.3 Plots of baseline adjusted RR interval for baseline, after propranolol, after pretreatment [i.e., vehicle (0.1% DMSO; 0.5ml/kg), verapamil (0.3 mg/kg), KB-R7943 (3 mg/kg), ryanodine (10µg/kg), W-7 (50 µM/kg), KN-93 (0.1 µM/kg) or H-8 (10 µM/kg)], and after dofetilide (0.04 mg/kg/min). All rabbits were pretreated with propranolol (0.5 mg/kg) to avoid reflex tachycardia before start infusion of each test article. Values shown are means ± SEM. *p<0.05 compared to baseline value of each test article.
Figure 5.4 Plots of baseline adjusted QT interval for baseline, after propranolol, after pretreatment [i.e., vehicle (0.1% DMSO; 0.5ml/kg), verapamil (0.3 mg/kg), KB-R7943 (3 mg/kg), ryanodine (10µg/kg), W-7 (50 µM/kg), KN-93 (0.1 µM/kg) or H-8 (10 µM/kg)], and after dofetilide (0.04 mg/kg/min). All rabbits were pretreated with propranolol (0.5 mg/kg) to avoid reflex tachycardia before start infusion of each test article. Values shown are means ± SEM. *p<0.05, and ***p<0.001 compared to baseline value of each test article.
Figure 5.5 Plots of baseline adjusted QTc interval for baseline, after propranolol, after pretreatment [i.e., vehicle (0.1% DMSO; 0.5ml/kg), verapamil (0.3 mg/kg), KB-R7943 (3 mg/kg), ryanodine (10µg/kg), W-7 (50 µM/kg), KN-93 (0.1 µM/kg) or H-8 (10 µM/kg)], and after dofetilide (0.04 mg/kg/min). All rabbits were pretreated with propranolol (0.5 mg/kg) to avoid reflex tachycardia before start infusion of each test article. QT was corrected for heart rate by the following equation: QTc = QT/(RR)^0.72. Values shown are means ± SEM. *p<0.05, **p<0.01, and ***p<0.001 compared to baseline value of each test article. ††p<0.01 compared with vehicle group at the same time point.
Figure 5.6 Plots of baseline adjusted mean arterial blood pressure (MBP) for baseline, after propranolol, after pretreatment [i.e., vehicle (0.1% DMSO; 0.5ml/kg), verapamil (0.3 mg/kg), KB-R7943 (3 mg/kg), ryanodine (10µg/kg), W-7 (50 µM/kg), KN-93 (0.1 µM/kg) or H-8 (10 µM/kg)], and after dofetilide (0.04 mg/kg/min). All rabbits were pretreated with propranolol (0.5 mg/kg) to avoid reflex tachycardia before start infusion of each test article. Values shown are means ± SEM. **p<0.01, and ***p<0.001 compared to baseline value of each test article. †p<0.05 and ††p<0.01 compared with vehicle group at the same time point.
DISCUSSION

In this study we explored the contribution of intracellular Ca\textsuperscript{2+} influxes through both I_{CaL} and reverse mode of NCX, SR Ca\textsuperscript{2+} release, calmodulin, CaMKII and PKA to the induction of TdP in the conscious rabbit with failing heart. The chemical compounds used for this objective were verapamil (an I_{CaL} blocker), KB-R7943 (a reverse mode of NCX blocker), ryanodine (a blocker of SR Ca\textsuperscript{2+} release channel), W-7 (a calmodulin antagonist), KN-93 (a CaMKII blocker), and H-8 (a PKA blocker). The failing rabbit heart has been shown to be prone to develop TdP, and we believe it is a more realistic (i.e., less contrived) model than either hearts with complete heart block or subjected to \(\alpha_1\)-agonists. That is, very few people with heart block develop TdP and very few people with TdP receive \(\alpha_1\)-agonists, whereas heart failure occurs in 5 million Americans and is a known torsadogenic substrate (Kijtawornrat et al., 2006a). Echocardiographic studies showed that the coronary ligation in rabbit produced cardiac dysfunction and marked left ventricular dilation consistent with the results of previous studies with this model (Pennock et al., 1997). More than 90% of rabbits operated upon to produce myocardial infarction survived the duration of the study (39 out of 43). No rabbit with a failing heart ever developed TdP unprovoked by torsadogenic articles. It is interesting to note that the heart failure present in these rabbits was relatively mild; no rabbits were symptomatic; no rabbit died of heart failure.
In this set of study, administration of dofetilide consistently caused prolongation of QTc followed by initiation of VPD and shortly leading to occurrence of TdP. Pretreatment with propranolol tends to prolong RR and QT interval but to shorten QTc interval. Pretreatment with vehicle has no significant effect on RR, QT and QTc intervals and did not affect induction of TdP by dofetilide.

*Prevention of dofetilide by verapamil, ryanodine, and H-8*

Recent data suggested that alterations in PKA, CaMKII, and calmodulin have been implicated in induction of EADs (Mazur et al., 1999). It is well known that afterdepolarizations, especially EADs, and reentry especially in regions of marked electrical inhomogeneity are responsible for the generation of TdP (Carlsson et al., 1996; Tan et al., 1995). Several interventions that prolong the APD may cause EADs, which can be differentiated into early EADs and late EADs. Early EADs are probably based on the Ca\(^{2+}\) entry through the I\(_{\text{CaL}}\) whereas late EADs seem to depend on an increased intracellular Ca\(^{2+}\) overload produced by SR (Verduyn et al., 1995). The present study shows that occurrence of dofetilide-induced TdP arrhythmias was prevented by administration of verapamil, ryanodine, and H-8. These compounds decrease intracellular Ca\(^{2+}\) influx, block SR Ca\(^{2+}\) release channel and block PKA activity, their inhibition of TdP point to the involvement of Ca mishandling (Ca\(^{2+}\) entry, Ca\(^{2+}\) overload) and/or increased PKA activation as the initiating mechanism of TdP. That the suppression of TdP in this study did not correlate with lengthening of QTc interval induced by dofetilide supports the contention that QTc is not a satisfactory culprit marker for TdP.
Verapamil

It has been demonstrated that the mechanism of TdP is multi-factorial and associated with triggered activity and spatial inhomogeneities of ion channel expression (Poelzing and Rosenbaum, 2005). The $I_{\text{CaL}}$ channel has been implicated as the charge carriers for the depolarizing current of EADs. The recovery and reactivation of inactivated $I_{\text{CaL}}$ channel may carry the inwardly directed depolarizing current (“window” current) of the EADs. In data from experimental preparations as well as from clinical subjects, numerous interventions blocking the $I_{\text{CaL}}$ channel have been demonstrated to suppress, effectively, EADs and TdP (January et al., 1988; Marban et al., 1986). Thus, the preventive effect of verapamil in our study supports the thought that $\text{Ca}^{2+}$ influx via $I_{\text{CaL}}$ channel plays a pivotal role in induction of TdP. The protective effect of verapamil against TdP induction in failing heart rabbit may be due to the direct effect of verapamil on $I_{\text{CaL}}$ channel or its effects on reduction of ventricular transmural dispersion of repolarization as reported by Milberg et al. (2005). Pretreatment with verapamil was also associated with reduction in MABP, with reduction of QTc interval, and with reduced torsadogenicity of dofetilide. This indicates that verapamil may influence the conditioning process for TdP (i.e., lengthening of QTc interval, elevation of MABP). However, Milberg and colleagues (2005) suggested that the effectiveness of an intervention to abbreviate the QT interval is not necessarily congruent with its efficacy to reduce the incidence of arrhythmogenesis. These data suggest that, in the setting of HF, $\text{Ca}^{2+}$ influx via $I_{\text{CaL}}$ is the major cause of TdP. This can be via many processes, for example the window current which causes late $\text{Ca}^{2+}$ influx leading to EAD.
increased Ca\(^{2+}\) influx will lead to SR Ca\(^{2+}\) overload, which may lead to spontaneous release leading to a Ca\(^{2+}\)-activated nonselective transient inward current (I\(_{\text{Na}}\)) (Houser, 2000; Marban et al., 1986).

**KB-R7943**

The sodium calcium exchange is an electrogenic pump and depends on Na\(^{+}\) and Ca\(^{2+}\) transmembrane gradients. Recent studies suggest that, in heart failure, stores of SR calcium decrease, release of calcium from SR to cytosol decreases, cytosolic calcium decreases, and inotropy decreases. However the NCX activity increases. This increased NCX activity tends to compensate for the decrease in SR calcium and to provide inotropic support (Armoundas et al., 2003; Bers and Despa, 2006). It has been shown by Weber et al (2002) that in failing human myocytes, there is a substantial Ca\(^{2+}\) entry through NCX as the AP prolonged. The increased activity of the NCX has been implicated in triggered arrhythmias (i.e., late EADs and DADs) in a number of conditions especially when the AP is prolonged and at membrane potentials negative to -35 mV, a potential at which activation of the I\(_{\text{CaL}}\) window current is unlikely (January et al., 1989; Sipido et al., 2006). Taken together, these concepts support the hypothesize that blocking of reverse mode of NCX might be an effective strategy to suppress TdP in the failing heart rabbit. Intravenous KB-R7943 has been suggested to suppress aconitine-induced arrhythmias in the anesthetized guinea pig (Amran et al., 2004). In the present study, KB-R7943 did not demonstrate inhibitory effect on dofetilide-induced TdP. On the other hand, KB-R7943 expedited the onset of VPD and TdP in our model. The mechanism(s) underlying this phenomenon is(are) still unclear. The dose of KB-R7943 used in
this study was based on positive findings in guinea pig study, and it is possible that these
doses were not appropriate for the rabbit. Furthermore, KB-R7943 significantly
prolonged RR interval in this failing heart rabbit which could precipitate the effects of
dofetilide to induced arrhythmias. KB-R7943 does not inhibit the forward mode of NCX,
which may contribute to I_{ii} with SR Ca^{2+} overload, and cannot be discounted in our
model.

**Ryanodine**

A substantial amount of evidence suggests that the abnormal regulation of
intracellular Ca^{2+} by the SR (i.e., calcium overload) is an underlying mechanism for late
EADs, since ryanodine abolished these EADs (Volders et al., 2000). In the present study,
calcium overload was manipulated by blocking the Ca^{2+} release channel of the SR with
ryanodine, resulting in significant delayed onset of VPD and attenuation of dofetilide-
induced TdP, suggesting a role for SR Ca^{2+} release and Ca^{2+} overload in induction of TdP
in failing heart rabbit. However, we did not measure Ca^{2+} sparks and Ca^{2+} transient, an
indication of Ca^{2+} overload, in dofetilide-induced TdP. Similarly, in a dog model of
pacing-induced TdP, Verduyn and coworkers (1995) showed that pacing-induced TdP
could be prevented when the dogs were given ryanodine. Thus, Ca^{2+} leak from the SR is
an important pathway for inducing TdP.
It is well known that certain signaling pathways such as β-adrenergic stimulation augment PKA activity, and an increased expression of CaMKII can phosphorylate I_{Ca,L} channel, SR Ca^{2+} release channels, and phospholamban (PLB) \((\text{Anderson, 2006})\). Experimental data demonstrated that phosphorylation of these channels increases the likelihood of EADs and ventricular arrhythmias \((\text{Mazur et al., 1999; Priori and Corr, 1990})\). The CaMKII target proteins and the proteins dually phosphorylated by PKA are overlap. Recently, \(\text{Anderson (2006)}\) suggested that PKA phosphorylation of I_{Ca,L}, PLB, and RyR increase intracellular Ca^{2+} and subsequently activate CaMKII, causing development of afterdepolarizations. In our study, H-8, a PKA inhibitor, delays onset of VPD and inhibits dofetilide-induced TdP suggested that, at least in our conscious model of rabbits with heart failure, PKA activation has a proarrhythmic effect and PKA inhibition could be used as a target for antiarrhythmic drug development. Our results are also in agreement with the results of Mazur et al. \(\text{(1999)}\) who demonstrated preventive effect of PKA inhibitor on rabbit model of TdP.

Interestingly, that infusion of PKA inhibitor delays the onset of VPD and suppresses TdP without shortening QTc interval induced by dofetilide, is consistent with other studies \(\text{(Bers, 2005; Mazur et al., 1999)}\) support the concept that prolongation of action potential duration is not sufficient to initiate arrhythmias and lengthening of QTc is not a good surrogate marker of TdP.

In contrast to PKA, CaMKII activity increases in response to elevated intracellular Ca^{2+}, and subsequently augments incidence of afterdepolarizations. The contribution of CaMKII inhibition to TdP has previously been examined in isolated
rabbit heart (Anderson et al., 1998). From that study, KN-93 demonstrated a preventive effect against EADs induction. However, that study was performed in normal hearts, not in failing hearts. In the present study, KN-93 was used to block CaMKII. There was a trend for KN-93 to prevent the dofetilide-induced TdP; however, this was not significant. In addition, KN-93 expedited the onset of TdP. To the best of our knowledge, no one has previously study the systemic effect of CaMKII inhibitor against TdP in failing hearts. The possible explanations could be 1) a potential obstacle of systemic kinase inhibition for therapy of cardiac arrhythmias due to the ubiquitous nature of CaMKII; 2) The cell membrane permeability data together with effective intracellular concentration of KN-93 is unknown; and 3) inadequate data is present on specificity of KN-93 on CaMKII as well as side effects when administered to systemic. It is known that CaMKII can increase $I_{\text{caL}}$; however, the major effect of CaMKII is to sensitize RyR opening (Maier et al., 2003). Thus, with CaMKII inhibition, SR $\text{Ca}^{2+}$ load dramatically increases. This increased SR $\text{Ca}^{2+}$ load, in the presence of PKA activation, may lead to the increased onset of TdP. Thus, in the setting of heart failure, although CaMKII seems to play a role in decreasing TdP, the PKA phosphorylation of the L-type $\text{Ca}^{2+}$ channel seems to be the major pathway.

W-7

Calmodulin is a ubiquitous $\text{Ca}^{2+}$ binding protein, which can activate enzymes (i.e., CaMKII) and regulate ion channel activity (Anderson, 2006). Increase activity of calmodulin after prolongation of repolarization has been implicated as a cause of afterdepolarizations in many studies, and the calmodulin antagonist (W-7) has been demonstrated to prevent TdP initiated by co-administration of methoxamine and
clofilium in anesthetized rabbit (Gbadebo et al., 2002; Mazur et al., 1999). Unexpectedly, our results show that W-7 expedites the onset of TdP and can not protect dofetilide-induced TdP in conscious rabbit with heart failure. It might be possible that the altered kinetics of calmodulin in heart failure is different from that in normal hearts, which in turn may have consequences for the induction of VPD and TdP. Also, it is known that calmodulin can inhibit RyR openings (Xu and Meissner, 2004). In the presence of W-7, RyR openings should increase which could lead to the increase prevalence and early onset of TdP.

In conclusion, the present experiments in failing hearts indicate that calcium entry via I_{CaL} channel, calcium overload by SR Ca^{2+} release, and blocking of PKA may play a major role in the appearance of TdP suggested by the preventive effect of verapamil, ryanodine and H-8. Furthermore, these findings may have implications for the clinical therapy of TdP and for the development of effective antiarrhythmic agents. The differences in these results compared to those of Gbadebo et al (2002) and Mazur et al (1999) may be explained by differences in failing versus non-failing hearts.
REFERENCES


Torsades de pointes (TdP) is a fatally polymorphic ventricular tachycardia characterized by a distinctive pattern of undulating QRS complexes that twist around the isoelectric line. TdP is usually self terminating or can subsequently degenerate into ventricular fibrillation, syncope, and sudden death. TdP has been associated with QT interval prolongation of the electrocardiogram; therefore, QT interval has come to be recognized as a surrogate marker for the risk of TdP.

QT interval, the duration from beginning of QRS complex to the end of T wave on the electrocardiogram, reflects the time of ventricular depolarization and repolarization. Prolongation of QT interval can result from genetic mutation (inherited long QT syndrome) or from drug induction (acquired long QT syndrome). Inherited long QT syndrome results from mutation of genes that encode cardiac ion channels (i.e., LQT1: KCNQ1 gene codes for I_{Ks}; LQT2: KCNH2 (hERG) gene codes for I_{Kr}; LQT3:
SCN5A gene codes for $I_{\text{Na}}$; LQT5: KCNE1 gene codes for $\beta$-subunit MinK; LQT6: KCNE2 gene codes for $\beta$-subunit MiRP1 and etc.). Currently, there are two forms of inherited long QT syndrome; Romano-Ward syndrome (an autosomal dominant form of LQTS that is not associated with deafness) and Jervell and Lange-Nielsen syndrome (an autosomal recessive form of LQTS with associated congenital deafness). On the other hand, acquired long QT syndrome results from drugs that block repolarizing currents or enhance depolarizing currents, interfere with trafficking of ion channel protein to the cell membrane, or drug-drug interaction. A large number of drugs (both cardiovascular and non-cardiovascular drugs) have been reported to produce prolongation of QTc interval and/or TdP, mostly by blocking of rapidly activated delayed rectifier potassium current ($I_{Kr}$). Therefore, international guidelines have been developed to harmonize both the preclinical and clinical studies for the evaluation of drug-induced TdP (e.g., ICH-S7A, ICH-S7B, and ICH-E14). Over the past few years, the guidelines concerning cardiac repolarization have been widely debated. Contemporary preclinical in vitro and in vivo methods (SCREENIT, wedge preparation, dog with chronic AV block, methoxamine-treated rabbit) as well as biomarkers for proarrhythmias (e.g., prolongation of QTc interval, increase TRIaD elements, blocking of hERG current, increase beat-to-beat variability of repolarization, and increase transmural dispersion of repolarization defined by increase $T_{\text{peak}}$-$T_{\text{end}}$) have been imperfect in predicting drug-induced TdP in humans. It is clear that relevant biomarkers together with appropriate models are needed to assess the arrhythmic risk of new chemical entities.
Moreover, elucidation of mechanisms underlying genesis of TdP is considered necessary. Currently, an EAD-induced extrasystole is believed to be responsible for the premature beat that initiates TdP, but the maintenance of the arrhythmia is generally thought to be due to a re-entrant mechanism. Amplified electrical heterogeneity principally in the form of transmural dispersion of repolarization by 1) agents that reduce net repolarizing currents, 2) ion channel mutations or 3) ventricular remodeling (e.g., hypertrophic and dilated cardiomyopathy) were proposed to be responsible for creating vulnerable windows for the development of re-entry.

The goal of the present dissertation is to establish an appropriate *in vivo* animal model to predict TdP in humans and to evaluate mechanism(s) underlying TdP in this model. It is believed that the proarrhythmic experimental models mimicking predisposed patients with cardiac pathologies (e.g., heart failure, chronic AV block) would be more appropriate for evaluation of drug-induced TdP since normally drugs are not given to healthy persons. The rabbit with myocardial failing heart has been developed as a model to predict TdP in humans.

The first step was to evaluate the relationship between QT and RR (the reciprocal of heart rate) in conscious rabbits and formulate the QTc formula. This is essential because QT interval changes inversely with heart rate. The next step was to challenge the QTc formula with torsadogenic and non-torsadogenic compounds. The primary study was conducted to determine which correction method best accounts for the effects of changes in the RR interval on the QT interval of conscious rabbits. It was also conducted to validate the use of conscious, sling-trained rabbits to assess the QTc interval, and to
evaluate the reliability and accuracy of this preparation in predicting drug-induced QTc prolongation in humans. In order to do that, ECGs were recorded from bipolar transthoracic leads in 7 conscious rabbits previously trained to rest quietly in slings. The heart rate was slowed with 2.0 mg/kg zatebradine (an \( I_f \) channel blocker) to assess the effects of heart rate on the QT interval. The same ECG and sling preparation was used to evaluate the effects in of three drugs known to be torsadogenic in humans (cisapride, dofetilide and haloperidol), two drugs known to be non-torsadogenic in humans (propranolol and enalaprilat) and a control article (vehicle). All of the test articles were administered intravenously to 4 rabbits, and both RR and QT intervals were measured and the corrected QT values were calculated by an investigator blinded to the test article, utilizing our own algorithm (QTc = QT/(RR)^{0.72}) which permitted the least dependency of QTc on RR interval. The following regression equations were obtained relating QT to RR: QT=2.4RR^{0.72}, r^2=0.79, p < 0.001, with RR intervals varying between 210 and 350 ms. In the present study, Bazett, Carlsson, Fridericia, and Liverpool QT correction factors failed to correct adequately for the influence of heart rate in conscious rabbits. However, failure in this experiment should not imply that the QTc formulas would be equally applicable to the data of other studies. It was demonstrated that QTc prolongation occurred in conscious rabbit in response to all three test articles known to lengthen QTc in humans (cisapride, dofetilide, and haloperidol), and QTc failed to prolong in conscious rabbits in response to all three test articles known to not lengthen QTc in humans (propranolol, enalaprilat, and DMSO). It was also demonstrates that a single bipolar, transthoracic ECG, from which RR and QT may be measured easily, can be obtained from a conscious rabbit placed in a comfortable sling, and that a relationship
between QT and RR can be successfully modeled by a power plot relationship. These results indicate that 1) a bipolar transthoracic ECG recorded in conscious, sling-trained rabbits may provide an easy and economical methodology useful in predicting QTc lengthening of novel pharmacological entities, and 2) our QTc formula \( \text{QTc} = \frac{\text{QT}}{(\text{RR})^{0.72}} \) is best fit for assessing compounds that lengthening repolarization in conscious rabbit.

Secondly, an animal model, rabbit with myocardial failure, that is believed to be appropriate to predict torsadogenic potential of NCE in humans was created and validated with drugs known to be torsadogenic or non-torsadogenic in humans. Since humans with underlying cardiovascular disease are at greater risk than humans with normal hearts for developing TdP following exposure to some drugs that prolong ventricular repolarization, the second study was designed to test the hypothesis that rabbits with ischemic myocardial failure are at similarly increased risk of developing QTc prolongation and TdP following exposure to escalating doses of drugs whose capacity to induce TdP is known in humans. In order to do this coronary artery ligation was performed in 28 rabbits, causing significant \( (p < 0.05) \) reduction in left ventricular shortening fraction and systolic myocardial dysfunction 4 weeks after ligation in all operated animals compared to 38 normal, non-operated controls. All studies were performed on rabbits anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg). Rabbits were exposed to escalating doses of amiodarone (3, 10, 30 mg/kg/10min), cisapride (0.10, 0.25, 0.50 mg/kg/10min), clofilium (0.1, 0.2, 0.4 mg/kg/10min), dofetilide (0.005, 0.01, 0.02, 0.04 mg/kg/10min), quinidine (3, 10, 30 mg/kg/10 min), and verapamil (0.25, 0.5, 1.0 mg/kg/10min). Rabbits
with failing myocardium are more susceptible to developing drug-induced TdP than rabbits without myocardial failure, and that lower doses of torsadogens are required to produce this effect in the setting of ischemic myocardial dysfunction. Cisapride (50%), clofilium (100%) and dofetilide (85%) induced a higher incidence of TdP in rabbits with myocardial dysfunction, whereas amiodarone, quinidine and verapamil failed to evoke TdP. Two out of 4 test articles lengthened QTc more in rabbits with myocardial failure than in normals, and TdP occurred in 13 out of 28 rabbits with myocardial failure as opposed to only 4 out of 38 rabbits with normal myocardial function. These results suggested that this rabbit model of ischemic myocardial failure may be more useful than current surrogates for predicting torsadogenic risk in man, since it tests directly for production of TdP rather than merely for lengthening QTc interval in response to drug administration.

There are limited data concerning the cellular basis of arrhythmias in the rabbit with myocardial failure (i.e., Why myocardial failure rabbits develop TdP more often than normal rabbits?). It has suggested that cardiac remodeling after myocardial infarction is associated with increased risk of ventricular arrhythmias and susceptibility to proarrhythmic effects of torsadogenic drugs. The third study was aimed at investigating the \textit{in vivo} dispersion of repolarization and the action potential duration (APD_{90}) of myocytes from normal and myocardial failing rabbit hearts in response to dofetilide. Comparison with normal conscious rabbits, rabbits with myocardial failing hearts had a slower heart rate (228 ± 10 vs 244 ± 10 bpm; \( p=\text{ns} \)), and prolongation of QT (\( p < 0.001 \)) and QTc (\( p < 0.001 \)) intervals (154.3 ± 4.5 vs 133.3 ± 2.4 ms and 280.9 ± 6.2 vs 243.4 ± 6.1 ms, respectively). Short-term variability was increased (\( p < 0.05 \)) in the
myocardial failure group (0.38 ± 0.02 ms) when compared with the normal conscious group (0.29 ± 0.01 ms) whereas long-term variability tended to increase (0.39 ± 0.03 vs 0.33 ± 0.02 ms; \( p=\text{ns} \)). Myocytes isolated from the border zone of infarcted area could be separated into two groups: non-hypertrophic cells and hypertrophic cells with pathological parameters of EC coupling. APD\(_{90}\) of hypertrophic failing myocytes (912 ± 44 ms) was prolonged (\( p < 0.01 \)) compared with action potentials from the same area of normal rabbits (388 ± 19 ms). In normal myocytes, dofetilide increased APD\(_{90}\) (\( p < 0.01 \)) at 0.1 µM (from 388 ± 19 to 921 ± 68 ms) with evidence of EADs. In hypertrophic myocytes, dofetilide (1.0 µM) increased APD\(_{90}\) 21 ± 8% from baseline (\( p < 0.05 \)) and EADs were observed both before and after application of dofetilide. The hypertrophic cell has smaller amplitudes of Ca\(^{2+}\) transients when compared with normal myocytes. Dofetilide has no effect on Ca\(^{2+}\) current and amplitudes of Ca\(^{2+}\) transients. It can be concluded that myocardium from the failing rabbit heart has an elevated spatial dispersion of repolarization which is known to be a substrate for TdP. Moreover, myocytes are remodeled after myocardial infarction, produce triggered activity (EAD’s) and create reentrant circuits due to dispersion of repolarization (i.e., different in APD\(_{90}\)) and possibly to islets of disease myocardium around which circus movements may occur.

The mechanisms for drug-induced TdP in heart failure (particularly involvement of calcium homeostasis) are not well understood. Early afterdepolarizations and transmural dispersion of repolarization have been known to serve as a physiological substrate and predictor for TdP. It is thought that abnormal calcium cycling is the proximate cause of EADs, and it is known that calcium cycling is abnormal in heart failure. The purpose of the final study was to search for torsadogenic-modifying
effects of verapamil (an \(I_{\text{CaL}}\) channel blocker), ryanodine (a ryanodine channel blocker), KB-R7943 (a reverse mode of NCX \(Ca^{2+}\) channel blocker), W-7 (a calmodulin antagonist), KN-93 (a CaMKII blocker), and H-8 (a PKA blocker) on ventricular premature depolarizations (VPD) and TdP in the conscious failing rabbit heart, a model known to be prone to develop TdP. Failing rabbit hearts were pretreated with propranolol followed by test articles before continuous infusion of dofetilide, used to induce TdP. In the vehicle control hearts, VPD and TdP were induced in all rabbits (8 of 8) and the onset of VPD and TdP were 3.6 ± 1.3 min and 10.3 ± 1.4 min, respectively. Intravenous infusion of dofetilide was associated with lengthening of RR, QT and QTc intervals. Verapamil, ryanodine and H-8 significantly delayed onset of VPD \((p<0.05)\) and suppressed occurrence of TdP \((p < 0.01)\). On the other hand, KB-R7943, W-7, and KN-93 accelerated onset of TdP. Blockade of L-type calcium channel, blockade of the calcium release channel of sarcoplasmic reticulum, and blockade of protein kinase A prevent dofetilide-induced TdP, suggesting roles of intracellular calcium overload and calcium signaling pathways in drug-induced TdP. Also, the data indicates that blockade of sodium/calcium exchange (NCX), calmodulin, and calcium/calmodulin dependent protein kinase II (CaMKII) may favor the development of TdP in the setting of heart failure, and suggests potential new avenues for development of antiarrhythmics in the presence of heart failure. The differences in these results compared to those of Gbadebo et al. (2002) and Mazur et al. (1999) may be explained by differences in failing versus non-failing hearts.
Although no single in vitro or in vivo model can predict with absolute accuracy which drugs will produce TdP in humans, evaluation of the electrophysiologic events underlying TdP, including lengthening of the QT interval, generation of VPD and TdP, increased short-term variability of QT interval (an indication of TDR), is useful for identifying drugs having the potential to cause TdP. The rabbit with myocardial failing heart is particularly useful in this regard.

It is clear that the current animal models which test only for lengthening of QT interval is not enough. Other appropriate models must be developed in order to more accurately assign clinical risk. Rabbit with myocardial failing heart is the model of choice. The findings of this study represent only the preliminary step in the long process of validation for in vivo animal model to predict drug-induced TdP in humans. In the future, this model needs to challenge with more torsadogenic and non-torsadogenic compounds in order to clarify specificity and sensitivity.

It has been shown in this study that alteration of calcium homeostasis in the setting of heart failure has tremendous effects on genesis of TdP. Therefore, the upcoming study should be emphasis on many aspects of calcium alteration, the vulnerable substrate for inducing TdP in patient with heart disease, in order to identify the patient at risk in the setting of heart disease and to search for new therapeutic strategies.
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