In vivo recording of adult zebrafish electrocardiogram and assessment of drug-induced QT prolongation

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In vivo recording of adult zebrafish electrocardiogram and assessment of drug-induced QT prolongation. Am J Physiol Heart Circ Physiol 291: H269–H273, 2006. First published February 17, 2006; doi:10.1152/ajpheart.00960.2005.—In the last decade the zebrafish has become a major model organism for the study of development and organogenesis. To maximize the experimental utility of this organism, it will be important to establish methods for adult phenotyping. We previously proposed that the embryonic zebrafish may be useful in high-throughput screening for drug-induced cardiotoxicity. We now describe a method for the reproducible recording of the adult zebrafish ECG and illustrate its application in the investigation of QT-prolonging drugs. Zebrafish ECGs were obtained by inserting two needle electrodes through the ventral epidermis. Fish were perfused orally, and motion artifacts were eliminated with a paralytic dose of µ-conotoxin GIIIB. Test compounds were delivered via the perfusion system. Without a means of hydration and oxygenation, the fish succumb rapidly. The use of a perfusion system allowed stable recording for >6 h. Baseline conduction intervals were as follows: PR, 66 ms (SD 14); QRS, 34 ms (SD 11); QT, 242 ms (SD 54); and R-R, 398 ms (SD 77). The known QT-prolonging agents astemizole, haloperidol, pimozide, and terfenadine caused corrected QT increases of 18% (SD 9), 16% (SD 11), 17% (SD 9), and 11% (SD 6), respectively. The control drugs clonidine, penicillin and propranolol did not prolong the corrected QT interval. In conclusion, perfusion and muscular paralysis allows stable, low-noise recording of zebrafish ECGs. Agents known to cause QT prolongation in humans caused QT prolongation in fish in each case. The development of rigorous tools for the phenotyping of adult zebrafish will complement the high-throughput assays currently under development for embryonic and larval fish.

METHODS

Perfusion and ECG recording. Adult TübingenAB zebrafish (2–4 cm long, with a mass of 0.5–0.75 g, with hearts 2–3 mm in length and weighing <5 mg) were utilized for all experiments. For the recordings, the fish were paralyzed with a 1.2 nmol/g intraperitoneal injection of µ-conotoxin GIIIB (CTX). Paralyzed zebrafish were placed in a damp sponge, and a 1-mm perfusion needle was placed into the oral cavity of each zebrafish. The fish were then orally perfused with 10 mM HEPES (pH 7.5) in E3 solution (containing 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, and 0.33 mM MgSO4) at a rate of 10 μl/min. A 29-gauge needle electrode (ADInstruments, Colorado Springs, CO) was inserted in the ventral midline directly between the pectoral fins to a depth of 1 mm. A second electrode was introduced in the ventral midline, two-thirds of the body length from the head, and positioned at a depth of 1 mm.

The ECG signal was amplified (A-M systems 1700 differential amplifier, Carlsborg, WA), filtered at 0.1–500 Hz and 60 Hz (notch), digitized (Digidata A/D 1322A, Axon Instruments, Union City, CA), and then recorded in 1-s sweeps at an acquisition rate of 2,000 Hz with digital filtering at 0.5–50 Hz (Clampex, Axon Instruments). Off-line processing was performed with Clampfit (Axon Instruments). For QT

repolarization; arrhythmia

The zebrafish has emerged as a major model organism in the last decade, as a result of its external development, transparency during organogenesis, and tractable diploid genome (7). Initial studies using reverse genetics led to the identification of multiple mutants affecting the specification of cell types and the formation of tissues and organs (4). The ability to manipulate gene expression during early embryogenesis has led to increasing use of the fish as a platform for the evaluation of gene function (5). The development of the fish as a comprehensive tool for physiological and pharmacological studies will require the characterization of adult phenotypes, enabling the systematic study of structure and function from the single cell stage to adulthood.

Recently, Milan’s laboratory (12) described the use of the embryonic zebrafish in the study of drug-induced QT prolongation. In recent years, unanticipated QT prolongation and the associated risks of the potentially lethal arrhythmia, Torsades de Pointes, have led to the postmarketing withdrawal of multiple medications from the US market. Because drug-induced QT prolongation is difficult to predict, it has become the focus of considerable regulatory scrutiny as well as pharmaceutical and academic research (9).

Repolarization of the cardiac myocyte requires the normal function of channels, receptors, and cytoskeletal proteins. Further complexity arises from regional heterogeneity within the heart. Drug effects on repolarization are often difficult to predict because many of the offending agents only cause repolarization abnormalities in combination with other drugs. Such interactions may be pharmacokinetic or pharmacodynamic in nature (1). Individual variation at a genetic level appears to affect the propensity to develop drug-induced arrhythmias and contributes to the difficulty in predicting this adverse effect in simple in vitro systems (19).

Animal models of repolarization toxicity have proven difficult to develop, largely as a result of inter species variation and limited understanding of the regulation of membrane ion currents. Milan’s laboratory (12) previously observed that zebrafish embryos consistently exhibit bradycardia on exposure to drugs known to cause QT prolongation in humans. We developed a method for the reproducible recording of the adult zebrafish ECG and employed this technique to study the effects of several QT-prolonging drugs on zebrafish cardiac repolarization.
measurements, the recordings were randomized and interpreted blind to the experimental conditions. Two independent and blinded investigators confirmed all QT measurements. Fewer than 5% of measurements were repeated based on disagreements over the QT measurement. For drug studies, the signal stability was critical for reliable measurements. Any recordings that showed loss of T-wave signal >50% over time were discarded (<30% of recordings). To correlate the electrical observations with cardiac mechanical events, video microscopy of the beating heart, synchronized with ECG recording, was employed. A high-speed CCD camera (Fastcam PCI 500, Photron USA, San Diego, CA) was synchronized with electrical recording by pulsing a light-emitting diode in the optical field and recording the electrical stimulus artifact. A photograph of the experimental setup is provided (Fig. 1).

**Statistical analysis.** Normally distributed values are displayed as means (SD). The means of normally distributed continuous variables were compared using unpaired two-tailed t-tests. P values ≤ 0.05 were considered significant. QT intervals were plotted against the associated R-R intervals and fit to the equation $QT = QTc \times \frac{R-R}{9251}$ by the method of least squares. The resulting equation was used to correct the observed QT interval for variations in heart rate.

**Drug administration.** Drugs were diluted from DMSO stocks to final concentrations between $10^{-5}$ and $10^{-3}$ M in E3. Baseline R-R and QT values were obtained after 10 min of perfusion with carrier alone. Each drug was then perfused until a stable plateau of R-R and QT intervals was attained. Recordings were acceptable if the T-wave amplitude was ≥25 μV and did not deteriorate by >50% during the recording.

The study was approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital and was conducted in accordance with the Animal Welfare Act.

**RESULTS**

**Optimization of zebrafish ECG recording.** Initial attempts at recording the zebrafish ECG were limited by both hypoxia and gill motion. Nonoxygenated zebrafish typically developed bradycardia, heart block, and asystole within 30 min (Fig. 2, squares). We therefore developed a perfusion system that permitted continuous electrocardiographic recording outside of the normal aqueous environment for up to 6 h. Recordings from perfused fish did not exhibit significant R-R interval variation (Fig. 2, circles).

Although this perfusion system allowed longer recordings to be obtained, the signals were contaminated by electromyographic noise, especially from gill motion (Fig. 3A). Signal averaging allowed reduction of this noise, but the resultant signals remained too contaminated to reliably measure cardiac repolarization (Fig. 3B). Optimal noise reduction was obtained with the intraperitoneal injection of CTX, which resulted in skeletal muscle paralysis (Fig. 3C).

Filter settings were employed that allowed the widest bandwidth possible without compromising the signal-to-noise ratio.
A high-pass cutoff value of 0.5 Hz was required to eliminate excessive baseline wander. This filter might be expected to truncate the end of the T wave. To address this concern, we performed duplicate measurements in five fish, varying the high-pass cutoff value. We found that reducing the cutoff frequency from 0.5 to 0.1 Hz resulted in a 1.5% increase in the measured QT interval \((P = 0.2)\). Based on this small change, and the higher percentage of successful recordings with the higher value, we chose a cutoff of 0.5 Hz for the remainder of the recordings. T-wave morphologies are not identical among fish. Subtle changes in the electrode placement or the orientation of the heart are likely responsible for observed differences.

**Basic intervals.** The combination of continuous perfusion and paralysis resulted in reproducible electrocardiographic recordings (Fig. 3C). Using this system, we were able to obtain satisfactory recordings in 80% of zebrafish tested. Synchronized video microscopy and electrocardiography were employed to demonstrate the correlation of electrical and mechanical events (supplemental movie may be found at http://ajpheart.physiology.org/cgi/content/full/00960.2005/DC1). The movie demonstrates that the recorded P wave corresponds to atrial contraction and the recorded QRS complex to ventricular contraction. The mean heart rate of the adult male zebrafish is 151 ± 30 beats/min, and the baseline intervals are as indicated in Table 1. Administration of CTX resulted in no significant effects on heart rate, QRS, or QT intervals; however, there was an increase in the PR interval from 52 (SD 19) to 66 ms (SD 14) \((P = 0.03)\). Of note, the sodium channel blocking agent tetrodotoxin also was tested as a paralytic agent, but at concentrations sufficient for paralysis, it resulted in 2:1 heart block, bradyarrhythmias, and ultimately asystole within 10 min of administration (data not shown).

The zebrafish QT and R-R data were obtained from 110 fish. Each point in Fig. 4 is a three-beat average of QT and R-R for a given fish. The data were best fit by the equation QT = QTc \(\times R-R^{0.05}\) with a QTc of 634 \((R^2 = 0.84)\) (Fig. 4). The simpler linear equation QT = 634 \(\times R-R\), which provided a nearly equivalent fit \((R^2 = 0.83)\), was employed for all subsequent analyses of zebrafish QT intervals.

**Drug effects.** Zebrafish were perfused with seven drugs, four of which are known to prolong the QT interval in humans (astemizole, haloperidol, pimozide, and terfenadine), and three control drugs that do not prolong the QT interval (clonidine, penicillin, and propranolol). Exposure to the known QT-prolonging drugs resulted in an increase in the QTc in each case as follows: astemizole, 18% (SD 9), \(P = 0.009\); haloperidol, 16% (SD 11), \(P = 0.019\); pimozide, 17% (SD 9), \(P = 0.005\); and terfenadine, 11% (SD 6), \(P = 0.015\). Drugs not known to prolong cardiac repolarization resulted in no statistically significant change in the QTc interval: clonidine, -1% (SD 6), \(P = 0.5\); penicillin, -1% (SD 2), \(P = 0.46\); and propranolol, -6% (SD 6), \(P = 0.054\). These results are summarized in Fig. 5. The R-R intervals increased for each drug as follows: astemizole, 16% (SD 6), \(P = 0.006\); haloperidol, 38% (SD 14), \(P = 0.004\); pimozide, 9% (SD 13), \(P = 0.19\); terfenadine, 18% (SD 15), \(P = 0.07\); clonidine, 9% (SD 11), \(P = 0.11\); penicillin, 4% (SD 6), \(P = 0.12\); and propranolol, 19% (SD 18), \(P = 0.04\). Drug-induced QT prolongation was dose dependent as shown for astemizole (Fig. 6) with a sigmoidal dose-response relationship.

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Table 1. Baseline electrocardiographic intervals

<table>
<thead>
<tr>
<th>Males</th>
<th>n</th>
<th>PR</th>
<th>QRS</th>
<th>QT</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(−) CTX, ms 5</td>
<td>52 (SD 19)</td>
<td>26 (SD 3)</td>
<td>229 (SD 11)</td>
<td>348 (SD 43)</td>
<td></td>
</tr>
<tr>
<td>(+) CTX, ms 58–110</td>
<td>66 (SD 14)</td>
<td>34 (SD 11)</td>
<td>242 (SD 54)</td>
<td>398 (SD 77)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means (SD); n, number of fish. Baseline electrocardiographic intervals of the adult male zebrafish in the presence (+) or absence (−) of μ-conotoxin GIIIB (CTX).

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1 Simultaneous video microscopy and electrocardiographic recording is played at 1/10th normal speed. Ventricle is seen in foreground to bottom left of image, and atrium is posterior and to the right. Correlation of electrical recording with mechanical events is possible with this video recording and demonstrates that smaller P wave corresponds to atrial contraction, whereas the QRS-T complex corresponds to ventricular contraction.
response curve. Typical QT prolongation responses are shown in Fig. 7 for the QT-prolonging drugs tested.

**DISCUSSION**

Over the last decade the zebrafish has become a major model organism for biomedical research. This emergence was pioneered in developmental biology laboratories (3) but has been consolidated by the acquisition of genetic and genomic resources, including the sequencing of the zebrafish genome. The zebrafish is particularly suited to the study of the cardiovascular system because the heart is easily visualized, and within 72 h of fertilization, there is a fully functional vascular tree (17). Although a number of cardiovascular mutants have been identified, the basic tools used to examine cardiac function in other model systems are not readily available in zebrafish due to the small size of the heart and the need for an aqueous environment. Stable ECGs previously have been recorded from large fish species including tuna, carp, and trout, and ex vivo cardiac preparations from many species have been studied (10, 14, 18).

To further the study of cardiac structure and function in the zebrafish, we have developed a recording system that will permit the rapid assessment of the adult zebrafish ECG. Through the use of continuous perfusion and skeletal muscle paralysis, we have overcome the technical limitations of hypoxia and gill motion. The zebrafish ECG obtained is highly reproducible, with a resting heart rate and conduction intervals similar to those observed in adult humans. The stability of the recordings also enables study of the effects of pharmacological agents, including those known to prolong the QT interval in humans. In the series of test drugs employed in this study, we found an excellent correlation between known QT effects in humans and observed QT effects on the zebrafish. We did note a trend of shortening of the QT interval with propranolol that did not quite reach statistical significance, but such effects have been observed in humans as well (6, 13, 16).

There is tremendous variation in the basal characteristics of cardiac electrophysiology across the vertebrate phyla. For example, resting heart rates range from the low teens to over 800 beats/min. Variations seen in the surface ECGs have been directly linked to interspecies differences in the contributions of multiple ionic currents to the cardiac action potential. Recent work (15) using both gene knockout and gene knock-in homologous recombination technologies has demonstrated that the full effects of ion channels extend beyond the regulation of transmembrane voltage to include other types of biological

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![Fig. 6. Administration of increasing doses of astemizole resulted in a dose-dependent lengthening of QTc interval. Data demonstrate a characteristic sigmoid-shaped dose-response curve.](image)

![Fig. 7. Sample tracings from representative fish. For each fish, baseline recording is shown above recording obtained after drug treatment. Dotted lines indicate QT interval, and QTc measurement is indicated for each tracing.](image)
signal. It is possible, therefore, that superficial similarities of or differences in electrocardiographic characteristics may not correlate well with the ultimate predictive utility of the species for human physiology and pharmacology.

Issues surrounding the undesired cardiac toxicities of new drugs remain challenging to patients, physicians, researchers, and regulatory agencies around the world (8). Indeed, of all the drugs recalled from the US market in the last 10 years, more were withdrawn due to concerns regarding QT prolongation than any other single cause. Among the issues that make drug-induced QT prolongation so difficult to predict are the low frequencies of clinical events, high rate of drug-drug interactions, high false positive rate of cellular-based HERG assays, and low throughput of whole organism models (11). There have been no fewer than six new assays entering into development or use in the past three years, which speaks not only to the importance of this problem but also to the lack of satisfaction with current techniques (2). In one such assay, we previously demonstrated that zebrafish embryos develop bradycardia in response to the application of drugs known to prolong the QT interval in humans. Although it was assumed that the bradycardia was due to rapidly activating delayed rectifier K+ current blockade and action potential prolongation, it was not possible to directly measure the zebrafish ECG at that time.

Limitations of the current adult model of drug-induced QT prolongation include the lack of aqueous solubility of all medications and relatively slow throughput. Nevertheless, when used in combination with a higher throughput technique, such as the embryonic zebrafish assay, this adult zebrafish model will allow confirmation of the repolarization effects of a drug by direct visualization of the QT interval. The methods described here add to the available assays for detecting drug-induced QT prolongation in a model with remarkably similar heart rate and repolarization time as humans. Recording the ECG of the adult zebrafish will not only permit the screening of QT-prolonging drugs but also will add to the phenotypic characterization of cardiovascular zebrafish mutants and potentially expand the panel of mutants to include those with isolated electrophysiological phenotypes.

In conclusion, we have demonstrated a novel system for recording the ECG of adult zebrafish and employed this technique to study the drug effects on cardiac repolarization. This simple system expands the current models available for the study of repolarization and will permit the further assessment of cardiovascular phenotypes in mutant and drug-treated zebrafish.

GRANTS
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