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A cardiomyocyte-based biosensor for antiarrhythmic drug evaluation by simultaneously monitoring cell growth and beating

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ABSTRACT

Drug-induced cardiotoxicity greatly endangers the human health and results in resource waste. Also, it is a leading attribution to drug withdrawal and late-stage attrition in pharmaceutical industry. In the study, a dual function cardiomyocyte-based biosensor was introduced for rapid drug evaluation with xCELLigence RTCA Cardio system. The cardiomyocyte-based biosensor can monitor the cardiomyocyte growth and beating status simultaneously under the drug effects. Two typical cardiovascular drug, verapamil and flecainide were selected as treatment agents to test the performance of this biosensor. The experiment results showed that the performance of cardiomyocyte-based biosensor verified the basic drug effects by beating status and also tested the drug cytotoxicity by the cell index curves of cardiomyocyte growth. Based on the advanced sensor detection technology and cell culture technology, this cardiomyocyte-based biosensor will be a utility platform for the drug preclinical assessment.

1. Introduction

Early and effective drug screening methods are demanded in the field of biotechnology and pharmaceutical industry for predicting drug-induced cardiotoxicity and reducing late-stage drug attrition (Kim et al., 2011; Natarajan et al., 2011; Xiao et al., 2010). Cardiotoxicity accounts for about one third of safety-based withdrawn pharmaceuticals (Lawrence et al., 2008). Earlier and broader screening is a validated approach to improve cardiovascular safety as demonstrated with human Ether-a-go-go-related gene (hERG) screening to reduce drug-induced arrhythmia (Braam et al., 2010; Giorgi et al., 2010; Moss and Kass, 2005). An urgent need was demanded for the novel *in vitro* assays to address other mechanism researches of cardiovascular function, including contractility, heart rate, toxicity, hypertrophy, and non hERG arrhythmia. Therefore, advanced *in vitro* systems for predicting drug-induced cardiotoxicity are meaningful in the pharmaceutical and biotechnology industries to decrease late-stage drug attrition.

Cell-based biosensor takes cells as the sensitive component to sense the physiological microenvironment, which is also an effective approach to achieve the drug preclinical assessing (Corcoran and Rechnitz, 1985; Pancrazio et al., 1999; Stenger et al., 2001; Ziegler, 2000). Compared to *in vivo* animal method, *in vitro* cell-based biosensor method can reflect the pharmacological effects sensitively in a shorter term. Electrical cell-substrate impedance sensing (ECIS) was common cell-based biosensor technology, which was first proposed by (Giaever and Keese, 1984). In the last two decades, ECIS had been successfully applied to a number of biological assays, such as cell growth, cell proliferation, cell migration and invasion, cytotoxicity (Keese et al., 2002; Luong et al., 2001; Opp et al., 2009; Xiao et al., 2002). Real time cell analysis (RTCA) is also the xCELLigence RTCA Cardio system developed by ACEA and Roche. The temporal resolution of RTCA was 12.9 ms for each well of 16-well sensor or 96-well sensor with the multiple A/D convertors. There are other similar ECIS detection system, such as Bionas 9600 adcon reader (Ceriotti et al., 2007; Thakur et al., 2012) and automated multi-well setup (Wegener et al., 2004), which can both detect the multi-well cell electrical impedance simultaneously. Bionas 9600 adcon reader has total scanning of the 96-well plate in less than 15 s. And the actual automated multi-well setup has a temporal resolution of 12 min in a 12-well sensor. The temporal resolution was most important for recording the relatively intact cardiomyocytes beating signals. However, both of them have a low temporal resolution that hampers to monitor the rapid changing impedance signals, such as cardiomyocyte beating signals. RTCA technology is an advanced ECIS technology that significantly explores the high sample rate and high throughput detection (Kammermann et al., 2011; Said and Aykut, 2011; Smout et al., 2009). Therefore, it can record the rapid changing impedance signals and facilitate to monitor the cardiomyocytes beating status. RTCA technology provides an efficient approach to establish a high performance cardiomyocyte-based biosensor.

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In the study, we employed the primary neonatal rats' cardiomyocytes to build a cardiomyocyte-based biosensor and applied the RTCA technology to monitoring the status of cardiomyocyte growth and beating simultaneously in the short term and in the long term. Cardiovascular drugs were used to test the performance of cardiomyocyte-based biosensor. The methods and experimental results are detailed in the following section.

2. Experimental and methods

2.1. Detection principle of a dual function cardiomyocyte-based biosensor

Cardiomyocyte-based biosensor applied the real time cell analysis (RTCA) technology to record the impedance of interdigitated electrodes (IDEs) to noninvasively quantify cardiomyocytes status in real time. A low voltage alternating current (AC) signal leads to the generation of an electric field between the IDEs, which interacts with the ionic environment of culture medium in the wells. Based on the IDEs, the electric field signals are differentially modulated by the number of cells covering the electrodes, the strength of cell attachment, and the morphology of cells, etc. (Fig. 1(a)) Beyond that, the biosensor can also monitor the cell morphology and adhesion that are induced by the rhythmic modulation of cardiomyocytes contraction and relaxation.(Fig. 1(b)). In these two stages, the cell morphology, cell–cell adhesion and cell-substrate adhesion are different, which induce the impedance change.

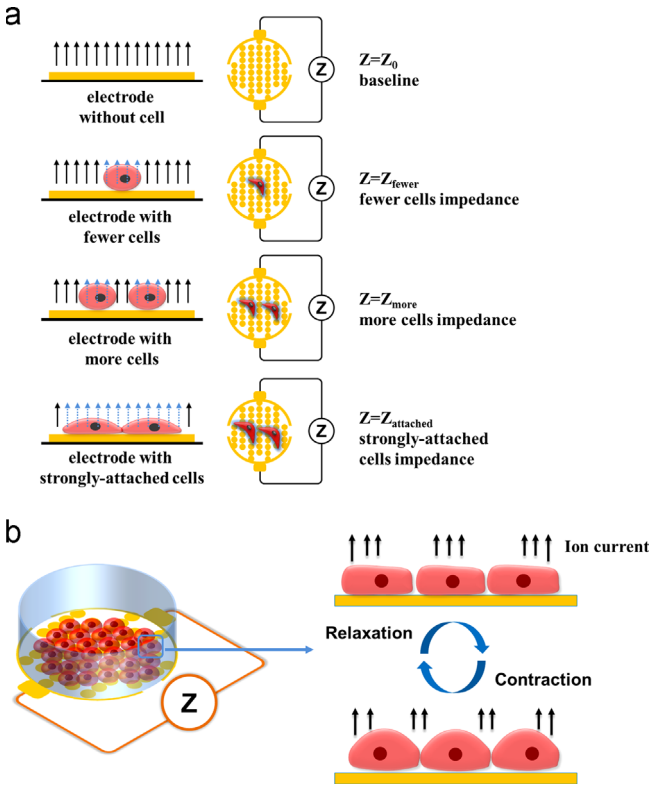

Fig. 1. Detection principle of a dual function cardiomyocyte-based biosensor. (a) The impedance signal Z is generated by the application of AC signal, which creates an ion current between the electrodes. The interaction of cells with the electrodes blocks the current and generates the impedance signal Z, which is proportional to the number of cells covering the electrode and the morphologic and adhesive characteristics of cells. The impedance signal represents in the form of cell index, which is a ratio of the change in impedance to background impedance. (b) The cardiomyocytes beating signal is based on the rhythmic changes of cell attachment and morphology due to contraction and relaxation of cardiomyocytes, which induces the fluctuation of impedance signal. (Reprinted with permission from website of ACEA Bioscience Inc.).

The instrument we used was the xCELLigence RTCA Cardio system, and it was developed by ACEA and Roche. The biosensor plate contains microtiter wells, which are integrated with gold microelectrodes in the bottom (Fig. 2). An AC sinusoidal signal (20 mV RMS @10 kHz) is applied onto the sensors, and the ion current signals were amplified and filtered before the A/D conversion. Finally, impedance signal Z value can be calculated by the original data. An AC voltage of 20 mV RMS is applied onto the each well including the lead rather than IDEs, so the IDEs lead impedance Rs on the sensor chip cannot be ignored, typically, 30Ω–80 Ω, and the well impedance Rw was 15 Ω. The 3–6 mV RMS is applied onto the IDEs. This voltage range was also tested for determining the non-invasive measurement. The AC voltage was applied onto some wells in the whole experiment, while AC voltage was applied onto other wells once every 1 h. The cardiomyocytes growth and beating was similar between the two groups. Therefore, the AC voltage range is available for non-invasive measurements. In the xCELLigence RTCA Cardio system, MCU can operate system simultaneously and continuously to measure and calculate the impedance signals. RTCA system measures impedance signal, processes and calculates the data by converting impedance value into a cell index (CI) value. CI is an arbitrary unit which is a ratio $(Z_X–Z_0)/Z_0$ of the well impedance change $Z_X–Z_0$ to well background impedance Z_0. CI value is influenced by a complex mixture factors such as cell growth, proliferation, cell–cell contact and cell-substrate adhesion. Therefore, it can be used to reflect cell viability, number, morphology, and adhesion degree in the cell-based impedance assay. The RTCA system has a multi-well fast data acquisition rate, which allows high temporal resolution for recording cardiomyocytes rhythmic beating. Therefore, impedance sensor detection technology can also be applied for monitoring cardiomyocytes beating status. Since impedance measurement is non-invasive, the millisecond data acquisition rate can be combined with longer-term monitoring to study both the short-term and long-term compound effects on cardiomyocytes growth and beating.

2.2. Cell culture procedure

The sensor plate was coated with 0.1% gelatin overnight in 4 °C refrigerator. Rats are sterilized by 75% alcohol. The rat chest wall was cut and heart was isolate by scissors. Hearts from neonatal rats should be rapidly excised, rinsed in ice-cold Dulbecco's modified Eagle medium (DMEM), and washed to remove blood and debris. Atriums were removed from the isolated hearts. Ventricles are left

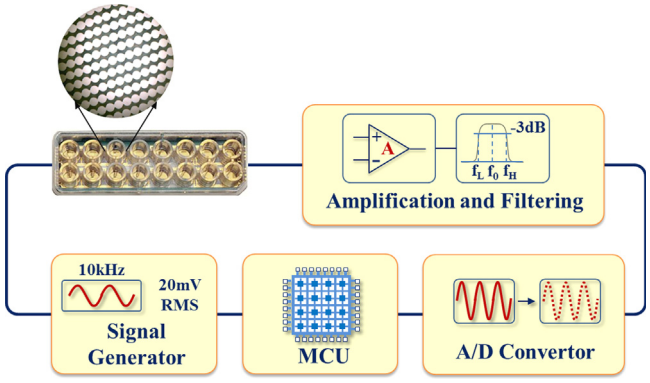

Fig. 2. The schematic of RTCA detection system including signal generator, amplification and filtering, MCU, and AD Convertor module. Due to fast data acquisition rate of the RTCA system, the cardiomyocytes beating can be monitored with high temporal resolution.

and moved into a bottle with 2 ml Hanks balanced salt solution (HBSS) inside. Then ventricles are shredded into tissue fragments (1 mm^3). And then HBSS was removed. Tissue fragments are digested by stepwise trypsin and collagenaseII. Each step needs 10 min, and bottle was shaken every 5 min. The fragments were blown using a glass pipette. Supernatant was aspirated and moved into another tube with 10% Fetal Bovine Serum (FBS) DMEM to stop digestion. Cell suspension was centrifuged with 800 rpm for 5 min, and then the suspension was removed. 4 ml DMEM (10%FBS) was added to re-suspend the cells. Cells were derived with a filter. And then cells are cultured in sensor plate (15,000 cells/well) and are maintained at 37 °C in a humidified incubator with 5% CO_2. The medium was changed every 24 h. It is suitable to record the cellular impedance signal 2 h later after each medium replacement. Usually, about 40 h later after cells addition into sensor plate, the cells could be used for compound treatment.

3. Results and discussion

3.1. Cardiomyocyte-based biosensor for verapamil test

Verapamil is an L-type calcium channel blocker of the phenylalkylamine class. It has been used in the treatment of hypertension, angina pectoris, cardiac arrhythmia and cluster headaches. It is also an effective preventive medication for migraine. Verapamil has been used as a vasodilator during cryopreservation of blood vessels. It is a class IV antiarrhythmic, is more effective than digoxin in controlling ventricular rate and was approved by the US Food and Drug Administration (Srinivasan et al., 2011).

To evaluate verapamil effects, different concentrations (0.065, 0.13, 0.25, 0.50, 1.00, and 2.00 μM) of verapamil-based culture medium were prepared. The verapamil medium was added into the wells, when the cardiomyocytes beat rhythmically with almost the same rate in each well. From the beating status in Fig. 3(a), Verapamil reduced the cardiomyocyte beating rate significantly and showed dose- and time-dependent recovery in the long term. The high dose verapamil treatment induced the beating stop in the short term. Fig. 3(b) displayed the normalized CI of cell growth

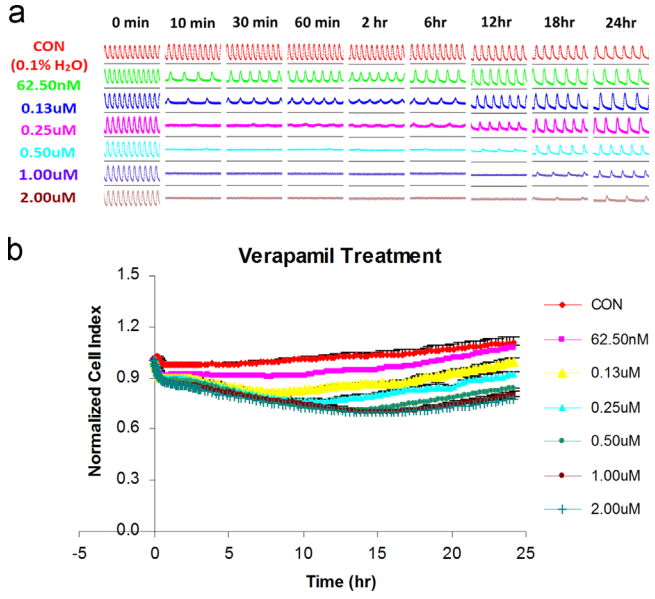

Fig. 3. Typical primary neonatal rat cardiomyocytes status in the presence of verapamil. (a) Snap shot of cardiomyocytes beating profile under the verapamil that induced the beating stop in the short term and showed dose and time-dependent recovery in the long term. (b) Cardiomyocytes impedance growth curve under the verapamil that showed dose-dependent inhibition effect for CI.

after the verapamil treatment. And the cardiomyocytes impedance growth showed dose-dependent inhibition effect under the verapamil for 24 h culture. The cardiomyocytes were cultured without compound additions as the control group in the whole experiments, and cells were not disturbed by the compound. Exponential growth stage is usually selected in the drug experiment, when CI values increase. Firstly, cells can freely grow in the well under the fresh medium condition including growth, proliferation, and adhesion. In the late stage, CI values increased due to the cell–cell contact strength. Therefore, CI values are increasing in the whole experiments.

The chronotropic effects of verapamil were obvious in statistical results. The left panels of Fig. 4 (a) and (b) show the cardiomyocyte beating rate for 1 h after the verapamil was added. And the right panels were cardiomyocyte beating status for the following 24 h. The beating rate and normalized beating rate in Fig. 4(a) and (b) presented that the beating rate significantly decreased to a low rate after the compound was added, and then beating rates slowly recovered with dose and time-dependence.

Verapamil reduces calcium influx to prolong atrioventricular node effective refractory period, and slows down the conduction rate, in order to reduce ventricular rate of patients with chronic atrial fibrillation, atrial flutter and paroxysmal supraventricular tachycardia frequency. Verapamil serves as a calcium antagonist that makes pharmacological effects by regulating calcium influx on the plasma of myocardial conduction cells, myocardial contractile cells and vascular smooth muscle cells, without changing the serum calcium concentration.

3.2. Cardiomyocyte-based biosensor for flecainide test

Flecainide is a class Ic antiarrhythmic agent used to prevent and treat tachyarrhythmias. It is used to treat a variety of cardiac arrhythmias including paroxysmal atrial fibrillation, paroxysmal supraventricular tachycardia, and ventricular tachycardia.

Similar experiments were carried out to evaluate effects of the flecainide, and different concentrations of flecainide (1.56, 3.13, 6.25, 12.50, 25.00, and 50.00 μM) were prepared. These flecainide mediums were injected into the cardiomyocyte culture wells, respectively, when the cultured cardiomyocytes beats rhythmically with an almost same rate in each well. From the results in Fig. 5(a), the cardiomyocytes beating was inhibited in the short term by flecainide treatment and presented dose- and time-dependent recovery in the long term. From the Fig. 5(b), normalized CI curves are similar after the flecainide treatment, so it indicated that the flecainide seldom affected the cardiomyocyte growth status.

The left panels of Fig. 6(a) and (b) were cardiomyocyte beating status derived from the original data for 1 h after the flecainide was added. And the right ones were cardiomyocyte beating status for the following 24 h. After the flecainide treatment, the beating rate increased under the low dose (1.56 and 3.13 μM), while beating was completely inhibited under the high dose and presented the time-dependent recovery in the long term. And the cardiomyocytes beating was faster after beating recovery under all the doses.

Flecainide works by blocking the Nav1.5 sodium channel in the heart to prolong of the cardiac action potential (Ramos and O'Leary, 2004). It slows cardiac conduction system and ventricular conduction, and significantly affects the His–Purkinje system. The flecainide effect on the ventricular myocardium causes decreased contractility of the muscle, which leads to a decrease in the ejection fraction. The flecainide effect on the sodium channels of the heart increases as the heart rate increases (Wang et al., 1993). Flecainide is potentially more useful to break a tachyarrhythmia than to prevent a bradyarrhythmia from occurring. The three experiment series are performed for the flecainide treatment. The results were similar with the Fig. 6. Other drug experiments were displayed in the supplementary material.

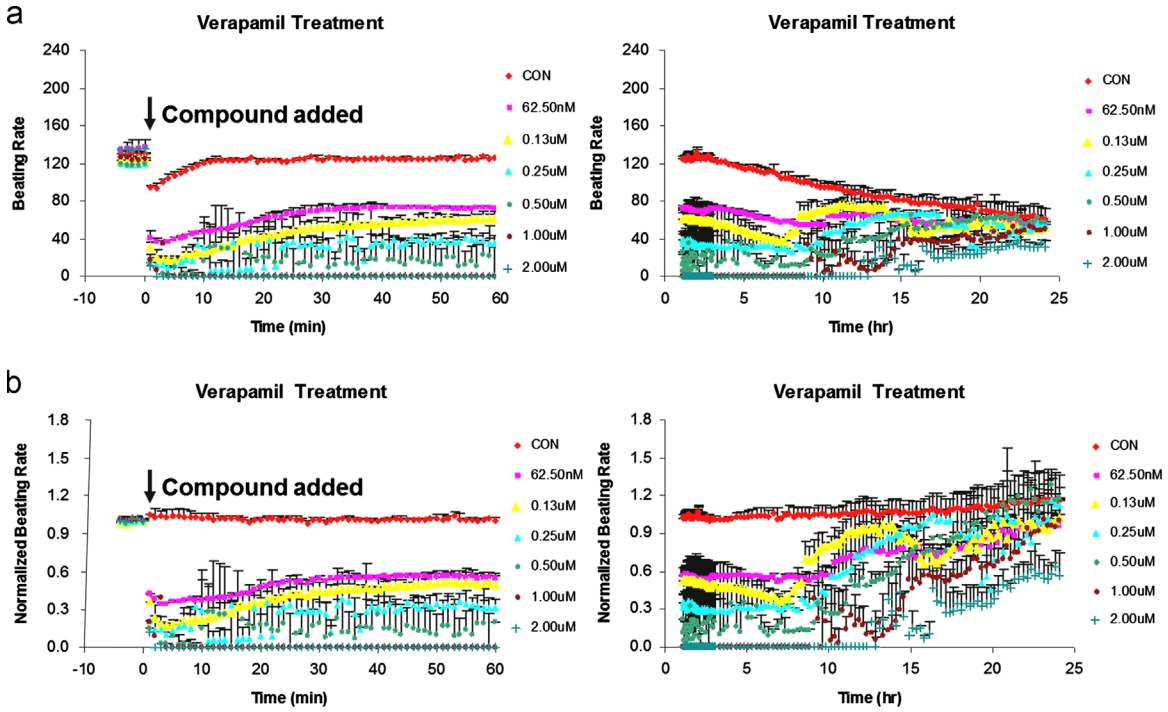

Fig. 4. The statistical results of cardiomyocytes beating status in the presence of verapamil. (a) and (b) Cardiomyocyte beating rate and normalized beating rate under the different verapamil doses. After the compound was added, the beating rate significantly decreased to a low rate and showed the dose and time-dependent recovery.

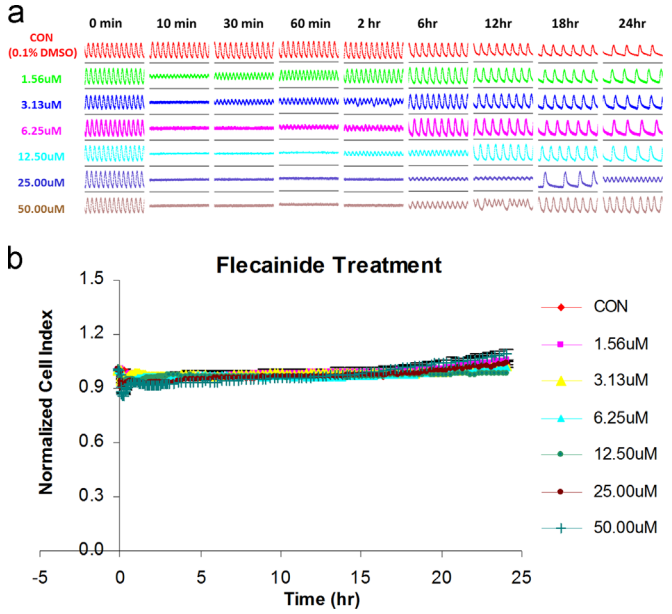

Fig. 5. Typical primary neonatal rat cardiomyocytes status in the presence of flecainide. (a) Snap shot of cardiomyocytes beating profile under the flecainide that induced the beating stop in the short term and showed dose and time-dependent recovery. (b) Cardiomyocytes impedance growth curve under the flecainide that showed dose-dependent inhibition effect for CI in the short term and then recovered CI in the long term.

The red dot curve was the control group in Fig. 4 and in Fig. 6. They were similar in the two experiments. The culture medium of control group was exchanged by the fresh medium without compound, while the culture medium of treatment groups was exchanged by the fresh medium with compound. Due to the culture medium exchange, cell status was slightly disturbed, so beating rate of the control group decreased as shown in both Fig. 4 and Fig. 6. And then the beating status recovers to the normal status. For the following 24 h, the beating rate gradually decreased, which was due to the insufficient nutrients in the culture medium. Besides, the coefficient of variation of beating rate status was in 5%, which indicated a good stability for the beating detection.

The signals induced by cardiomyocyte beating were detected by ECIS, and they were related to the cell morphology including cell–cell contact and cell-substrate adhesion. Other sensors, such as light-addressable potential sensor (LAPS), were used to monitor the beating status of mouse embryonic stem cells differentiation cardiomyocytes (Liu et al., 2011). LAPS has the characteristics of light addressability, which can record cell clusters at any desired position. The cardiomyocytes beating signal shapes recorded by ECIS and LAPS are similar. The ECIS signals was current or corresponding impedance signal, while the LAPS signals was photocurrent or corresponding surface potential. However, the LAPS needs the reference electrode and infrared LED to drive the sensor, so its detection system was more complex than the ECIS detection system. Besides, CMOS microelectrode array was fabricated to monitor the extracellular potential of embryonic chicken cardiomyocytes. (Heer et al., 2004) The CMOS microelectrode array was an advanced microelectrode array (MEA) with difficult design and fabrication processes. And it contained fully-integrated analog and digital circuits on a chip. So the device had on-chip signal filtering for improved signal-to-noise ratio (SNR), on-chip analog and digital conversion. CMOS microelectrode array was used to detect the extracellular potential of cardiomyocytes, which happened before the cardiomyocytes beating due to the mechanism of excitation–contraction coupling.

A cardiomyocyte-based biosensor was usually used to assess drug effects by monitoring the beating status. However, drug effect for other cell status was seldom regarded. It was significant to study the cardiomyocyte drug (e.g. antiarrhythmics) by beating status, But the basic cell status was also important to determine the drug-induced cardiotoxicity. By the RTCA technology, the drug-induced cardiotoxicity can be tested by cell growth and beating simultaneously and easily.

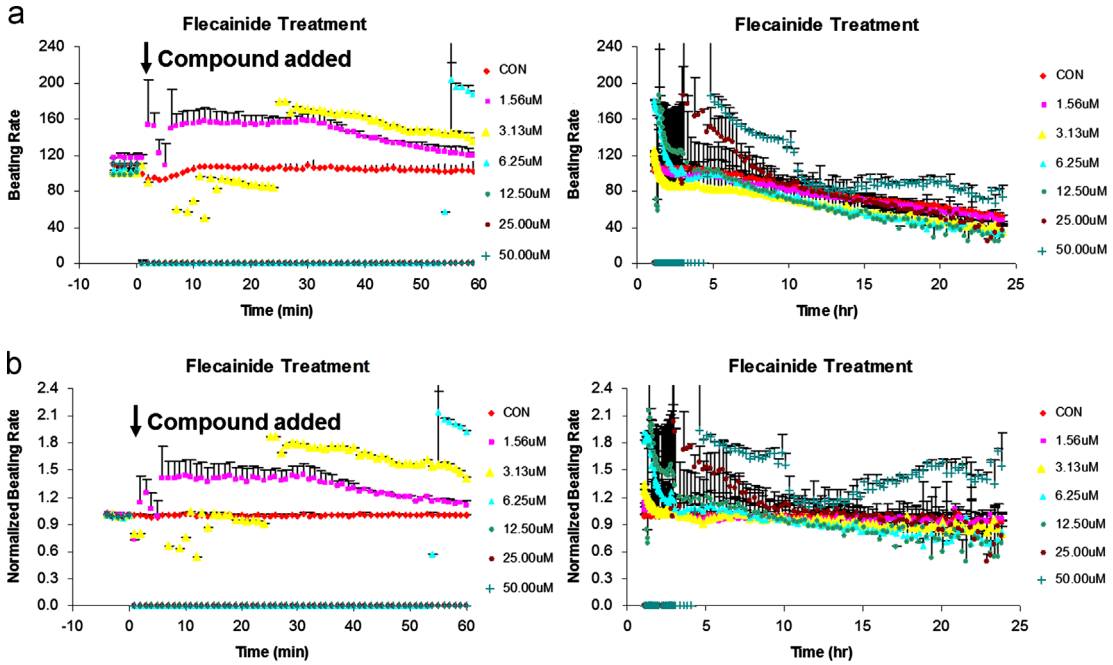

Fig. 6. The statistical results of cardiomyocytes beating status in the presence of flecainide. (a) and (b) Cardiomyocyte beating rate and normalized beating rate under the different flecainide doses. Flecainide induced the beating stop in the short term. Beating rate showed dose and time-dependent recovery in the long term, and the beating rate increased after the compound added.

4. Conclusions

In this study, we present a cardiomyocyte-based biosensor simultaneously monitoring cardiomyocyte growth and beating status. CI values are calculated by impedance values, which are influenced by a mixture of influences including the cardiomyocyte growth, proliferation, and adhesion status. The cardiomyocyte beating status was also monitored in parallel in order to reflect the drug effects on the cardiomyocytes. The performance of cardiomyocyte-based biosensor was tested by drug experiments. The results prove that cardiomyocyte-based biosensor can evaluate drug effect by cardiomyocyte growth and beating simultaneously in the short term and in the long term. With the improvement of sensor technology and cell culture, the cardiomyocyte-based biosensor will be a promising tool to preclinical drug screening in the field of pharmaceuticals and medicine.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.04.039.

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