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xCELLigence System

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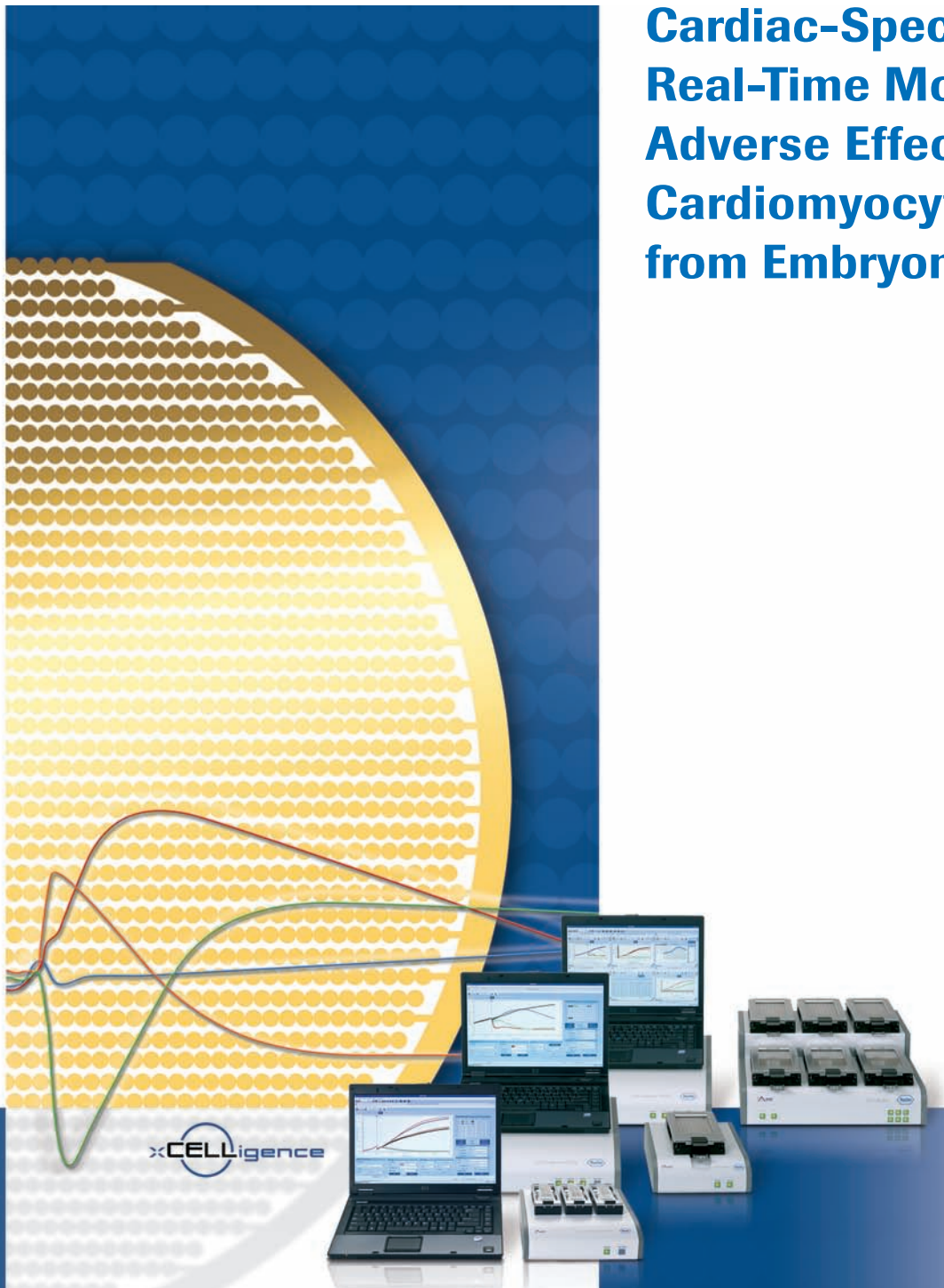
Cardiac-Specific Toxicity – Real-Time Monitoring of Adverse Effects on Cardiomyocytes Derived from Embryonic Stem Cells



ACEA Biosciences, Inc.

axioGENESIS

Cardiac-Specific Toxicity – Real-Time Monitoring of Adverse Effects on Cardiomyocytes Derived from Embryonic Stem Cells

Introduction

Organ specific apoptosis, cytotoxicity, unwanted hERG effects, QT-effects, repeated dose, chronic and acute effects, are all factors contributing to the phenomenon called cardiotoxicity[1]. Up to now the pre-clinical analysis of all these parameters is hampered by the lack of both (1) a standardized, pure cardiac cell and tissue model to monitor cardiac-specific toxicity and (2) a suitable technology-platform for continuous, label-free analysis of cell function and integrity. These impediments are now addressed by the xCELLigence Real-Time Cell Analyzer (RTCA) System, in combination with pure cardiomyocytes generated from mouse embryonic stem cells[2].

To predict the pharmacological and toxicological effects of a drug, scientists use either recombinant cell systems such as cell lines expressing specific ion channels[3,4] or primary cardiomyocytes[5] prepared from *e.g.* neonatal rats[6,7]. The disadvantages of these systems are that recombinant cell lines lack the physiological ion channel environment and functional humoral regulation. Freshly isolated primary cardiomyocytes, although showing *in vivo* physiological properties, are costly and time consuming to produce and difficult to standardize[5]. Furthermore, the possibility of contamination with other cell types increases the variability of data and reduces the reliability of test results.

To address these problems, the biotechnology company, Axiogenesis (Cologne, Germany) has developed Cor.At® ready-to-use cardiomyocytes to predict *in vivo* physiological responses[2]. The cells are 99.9% pure, can be produced in large quantities and are stored frozen in liquid nitrogen. After thawing, Cor.At® cardiomyocytes can be cultured for up to three weeks, maintaining 99.9% purity throughout the culture period. It was demonstrated that they retain their functional and morphological integrity through in both immunostaining and functional analysis[8] (Fig. 3, Fig. 4).

The new xCELLigence Real-Time Cell Analyzer (RTCA) System was developed by Roche Applied Science and ACEA Biosciences[9,10]. RTCA Instruments produce an electronic readout after measuring impedance using microelectrodes found at the bottom of each culture plate well, enabling the detection and quantification of changes in density, growth and morphology, in real-time, without using exogenous labels. Cells are seeded in special 96-well culture plates, called E-plates 96. Cultured cells contact the gold microelectrode network covering 80% of the bottom of each well, to measure changes in cell impedance that not only indicates cell viability, but also correlates with cell number and changes in cell morphology.

This impedance measuring technology is an innovative way to overcome the limitations in currently available assay systems. Impedance measurements are both continuous and non-invasive, so that cells remain in a normal physiological state during the entire assay of cell proliferation, cell behavior and cytotoxicity. The RTCA System does not require labeling the cells with interfering reagents.

To harness these advantages, Roche Applied Science and Axiogenesis AG® have defined a complete system using Cor.At® cardiomyocyte technology to produce physiologically relevant data for candidate substances in cardiac research.

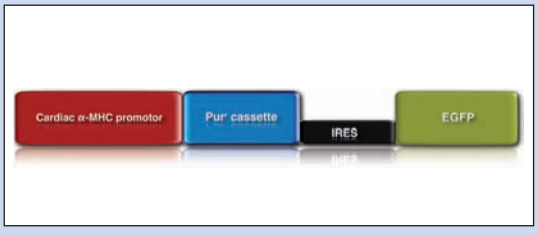
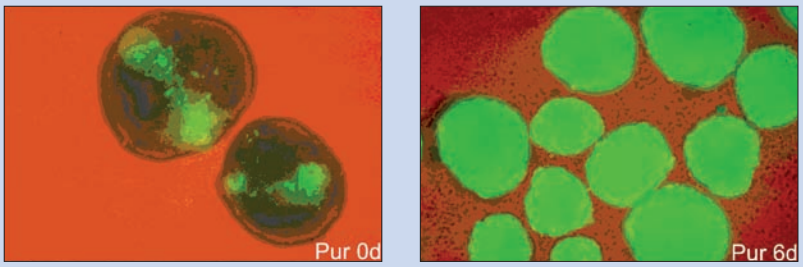


Figure 1: Vector Construct



(A) **(B)**

Figure 2: Expression of GFP in EBs pre (A) and post puromycin treatment (B)

The Cor.At® cells

The advantage of the murine ES cells is their susceptibility for genetic manipulation[11,12] the unlimited availability of undifferentiated manipulated cells, and the close to 100% physiological behavior after cardiomyocate cell differentiation is complete. In order to purify and identify ES cell–derived cardiomyocytes, bicistronic vectors were used, in which the cardiac-specific promoter αMHC drives the expression of both the puromycin resistance gene and an EGFP cassette (see Figure 1). When differentiating in embryoid bodies (EBs)[13], the first clusters of EGFP+ cells are detected in the EBs on days 7 to 8 of development (see Figure 2A), with spontaneous cell beating at approximately 12 to 24 hours later. Due to the tissue specific expression of puromycin resistance, addition of puromycin on days 9 to 10 (see Figure 2B) results in a dramatic increase in EGFP fluorescence and contractile activity of the EGFP+ clusters after 24 to 72 hours, indicating high level cardiomyocyte enrichment. After day 6 of puromycin treatment, most of the cells in the EBs consist of beating EGFP+ clusters of cardiac cells. Both cell differentiation and cell selection has been scaled up using a mass-culture protocol for producing typical yields of several billion cells per lot. The cell purity of 99.9% has been shown by transplantation experiments in which subsequent to transplantation of such cells in syngeneic mice, no teratocarcinomas could be detected[8]. Moreover, it was shown that Cor.At® cardiomyocytes can be frozen and thawed, and still retain their functional and morphological integrity, as judged by both immunohistochemical (see Figure 3) and functional analysis.

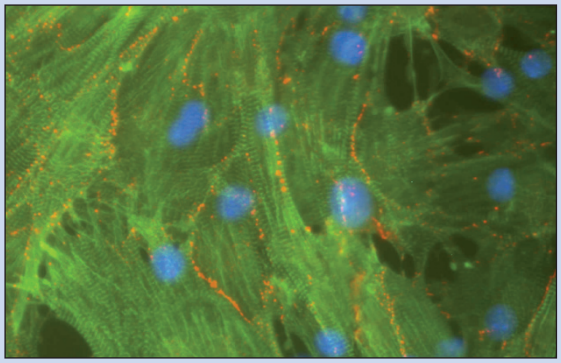


Figure 3: Immunohistology of Cor.At® cells.
After 12 daysthe cells were stained for expression of Cx4314 (red dots).Actinin filaments display striations; Hoechst stain to detect nuclei.

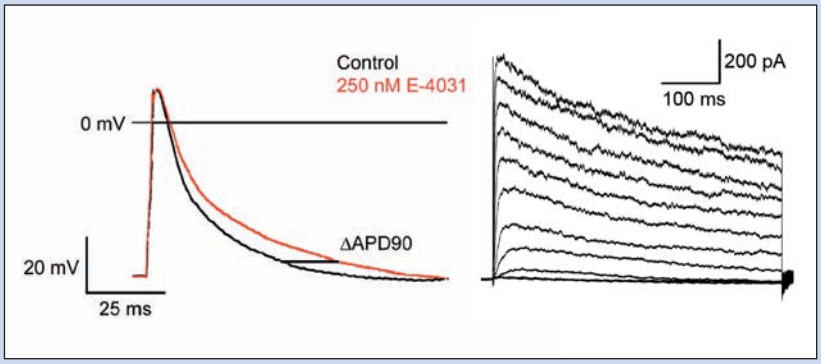


Figure 4A: Patch-clamp experiment with Cor.At® cells. Cells wereanalyzed using manual patch clamp technology to demonstrate the effects of the hERG blocker E-4031 on the action potential (left) and the presence of the outward potassium current (right).

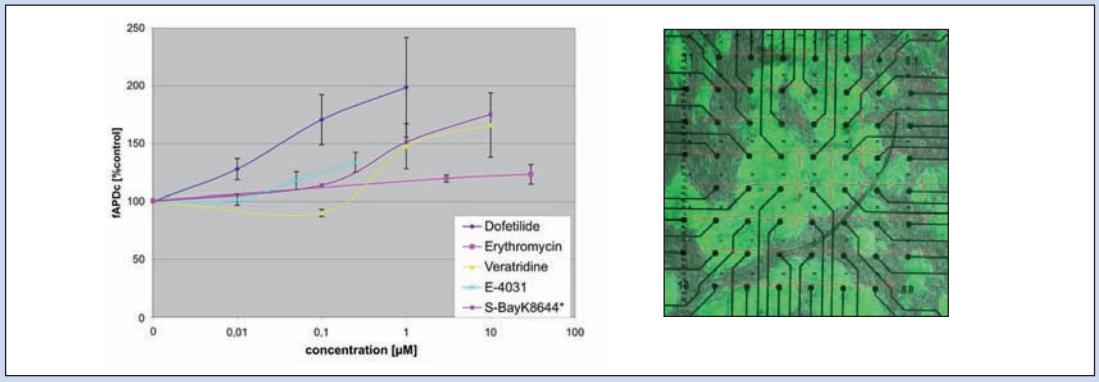


Figure 4B: In this experiments drugs inducing fAPDc prolongation in Cor.At® cells on a multielectrode array identify drug effects in intact cardiac tissue using the calcium channel activator S-BayK 8644 and sodium channel activator Veratridine, and hERG blockers E-4031, Dofetilide, and Erythromycin.

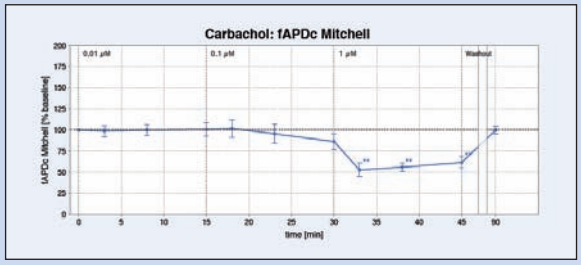


Figure 4C: Carbachol-induced changes on frequency corrected field action potential duration (fAPDc). Frequency correction was established according to ref. 15. All data points are displayed as % of baseline (0 min). Error bars are standard error of the mean. Differences between the carbachol group (n = 6) and the DMSO-vehicle group (n = 4) were tested for statistical significance with the Student's t-test for unpaired experiments.

	ES	Cor.At juvenile phenotype	Primary Heart
Voltage-dependent calcium channels			
L-type α1c subunit			
T-type α1h subunit			
Voltage-gated sodium channel, type V, alpha			
Voltage-gated potassium channels			
Kcnd3 (Kv4.3)			
Kcnh2 (merg1a)			
Sodium calcium exchanger			
Slc8a1 (NCX1)			
Sodium potassium ATPase catalytic alpha subunit			
ATP1A2			
Calcium ATPase; catalytic alpha subunit			
ATPase (SERCA)			

Table1: Gene expression analysis

Figures 4, panels A, B and C illustrate the electrophysiological behavior of the Cor.At® cells. Both patch clamp analysis and multi-electrode array measurement data clearly indicate functional behavior of the most relevant ion channels. Furthermore humoral regulation of Cor.At® cardiomyocytes is demonstrated (see Figure 4C).

Cor.At® cardiomyocytes permit both cell stockpiling and the easy distribution of the cells for purposes of studying functional drug development, cardiotoxicity analysis, cardiac electrophysiology studying (manual and automated patch clamp analysis), tissue engineering and in vivo transplantation experiments.

Cor.At® cardiomyocyte gene expression pattern was also analyzed in detail. cDNA was profiled using a whole genome chip (Affymetrix), as shown in Table I. There is a high correlation between Cor.At® and primary heart cells.

Materials and Methods

Standard end point experiments

NRU assay

Cor.At® ready-to-use cardiomyocytes were cultured for 72 hours after thawing, and incubated with test compounds for 48 hours. Effect of compounds was determined by neutral red uptake (NRU) test. Mouse embryonic fibroblasts (MEF) were used as control cells to distinguish cardiac specific toxicity from general cytotoxicity. The compound class of anthracyclines is known to induce physiologically relevant cardiotoxic reactions[16]. The cells were cultured in E-Plates 96 to compare the NRU data to the label free data.

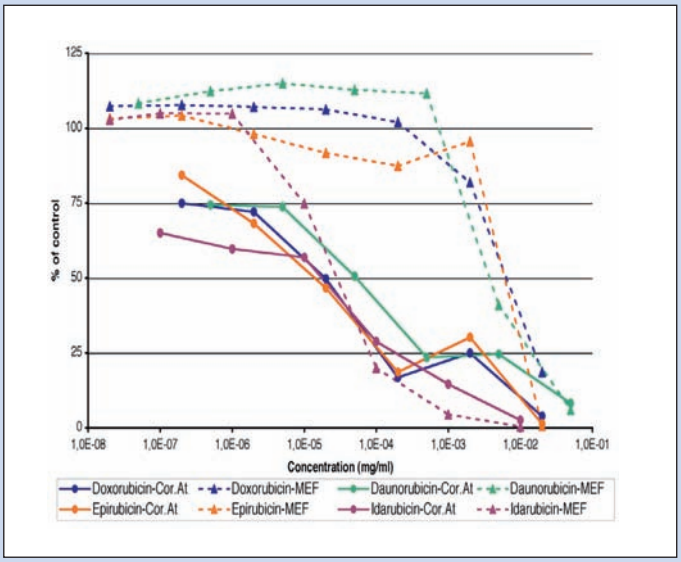


Figure 5: NRU (neutral red uptake) endpoint analysis with Cor.At® cells and embryonic fibroblasts. Both cultures were incubated with decreasing concentrations of **doxorubicin, daunorubicin,** epirubicin or idarubicin.

Note the clear difference in treatment sensitivity between fribroblasts (MEF) and Cor.At® cells.

Label free continuous cell monitoring with the xCELLigence System

Cells

The Cor.At® cells used in this study were taken from different lots that were cryopreserved for 1 to 20 weeks. Lots were standardized and quality-controlled for uniform results in subsequent assays.

Thawing and preparation of Cor.At® cells

Cor.At® vials was removed from liquid nitrogen and thawed for 2 minutes in a +37° C water bath. Cells were transferred to a 50 ml tube pre-filled with 18 ml Cor.Atx medium A, and washed by centrifugation (1000 rpm, swing-out bucket) for 5 minutes. After aspiration of the supernatant, 5 ml fresh Cor.Atx medium B was added. Cells were counted in a Neubauer chamber using Trypan blue exclusion to identify viable cells. Cell concentration was adjusted to 1.5 Mio cells per ml and transferred to E-Plates 96, pre-coated with Cor.Atx coating solution, 50 µl per well, for 2 hours.

Seeded E-Plates 96 were cultured for 24 to 48 hours, with the RTCA SP Instrument placed in a standard incubator at +37°C, 5% CO_2, 95% humidity, prior to addition of test compounds. During this period the impedance was monitored in 1 minute sweeps. After 5 days cell culture, media exchange (130µl Cor.Atx medium B/well) was performed every day using the following method:

1. **Pre-warmed medium was always used**
2. **At least 15 µl medium in each individual well was not removed to prevent artifacts by disrupting cell attachment**
3. **Each well was filled by gentle pipetting the media so as not to disturb the cell monolayer**
4. **The total volume per well never exceeded 180 µl.**

Result

Is it possible to demonstrate "organ-specific" toxicity?

The optimal cell concentration

To identify optimal parameters, cardiomyocytes were titrated in the E-Plates. As shown in Figure 6, typical cell responses were detected using 15,000 to 30,000 cells per well. However, to maintain cardiomyocytes for more than 2 days with an exchange of 50 to 80% of the media daily, all further experiments were performed with 15,000 cells per well. Culture media for all experiments was Cor.At culture medium containing an optimized cardiomyocytes serum. Data depicted in Figure 6B, underscore the uniform behavior and the degree of standardization achieved using Cor.At® cells and Cor.At® culture medium.

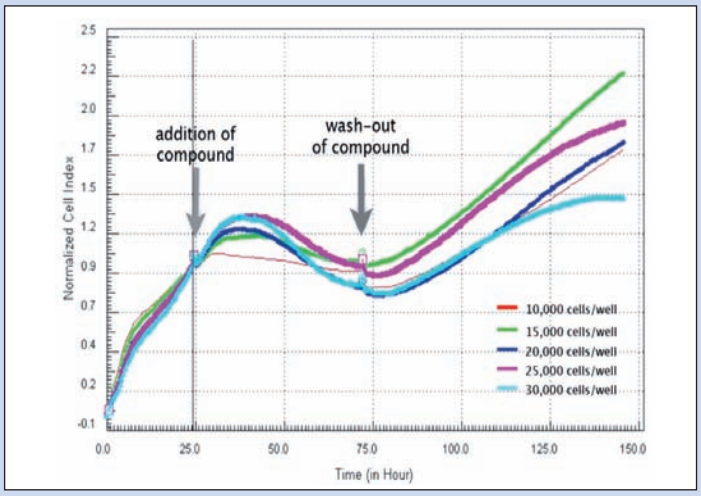


Figure 6A: xCELLigence analysis of Cor.At® cells. Cell amounts were titrated as indicated, and 0.1 µM doxorubicin was added after 24 hours.

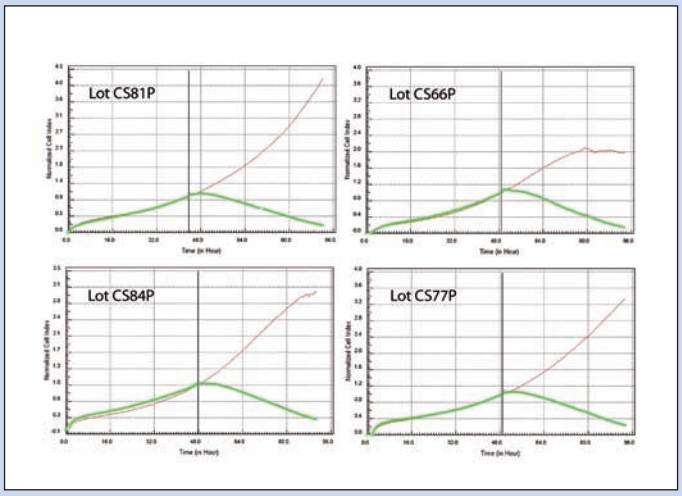


Figure 6B: xCELLigence analysis of different lots of Cor.At® cells. Cor.At® cells were treated with 1 µM doxorubicin (green line) or media only (control- purple line) after 48 hours.

Comparing cardiomyocytes with fibroblasts

In Figure 7, typical experiments are shown demonstrating the differences between mouse embryonic fibroblasts (*i.e.*, nonspecialized reference cells; see Figure 7A) versus Cor.At® cardiomyocytes (see Figure 7B). The comparision of the two cell types clearly demonstrates a cell type–specific, *i.e.* cardiomyocyte specific, effect of doxorubicin in this system. Interestingly, the *in vitro* effects of anthracyclins appear to mirror the well known clinical delayed side effects, dependent on the accumulation of anthracyclins.

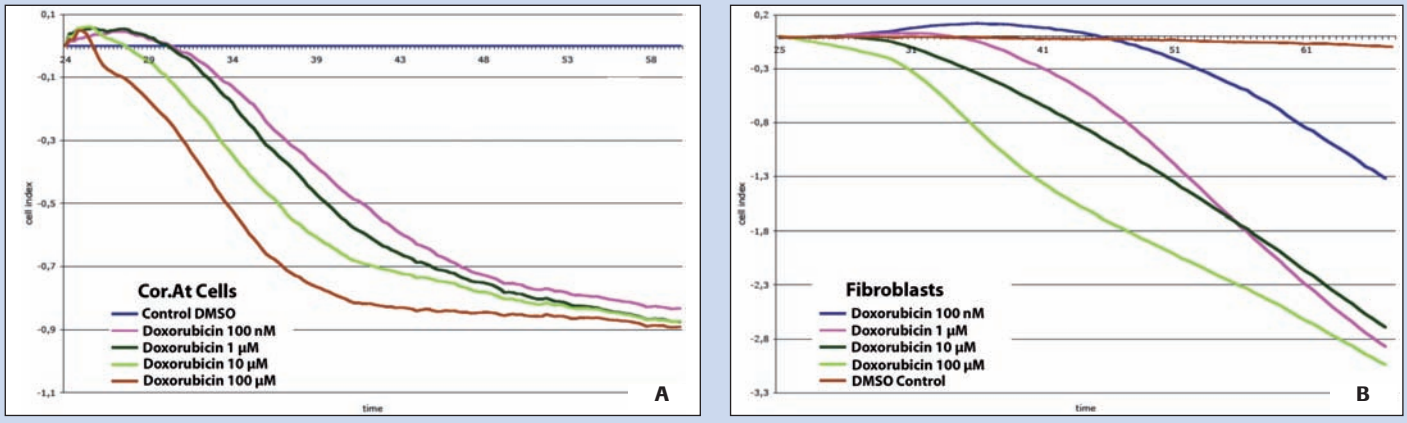


Figure 7: xCELLigence analysis of Cor.At® cells. Cor.At® cells (A) and mouse embryonic fibroblasts (B) were treated with the indicated concentrations of doxorubicin.

There is a difference between the developmental stages of the Cor.At® cells

Comparison of juvenile (day 1 to 5) and adult (day 12 to 15) Cor.At® cell phenotypes

Multiple functional characteristics of cardiomyocytes are age dependent[17]. Figure 8 demonstrates the effects of a single doxorubicin treatment, clearly demonstrating the difference between the two "age-groups" of juvenile and adult *Cor.At® cell* phenotypes.

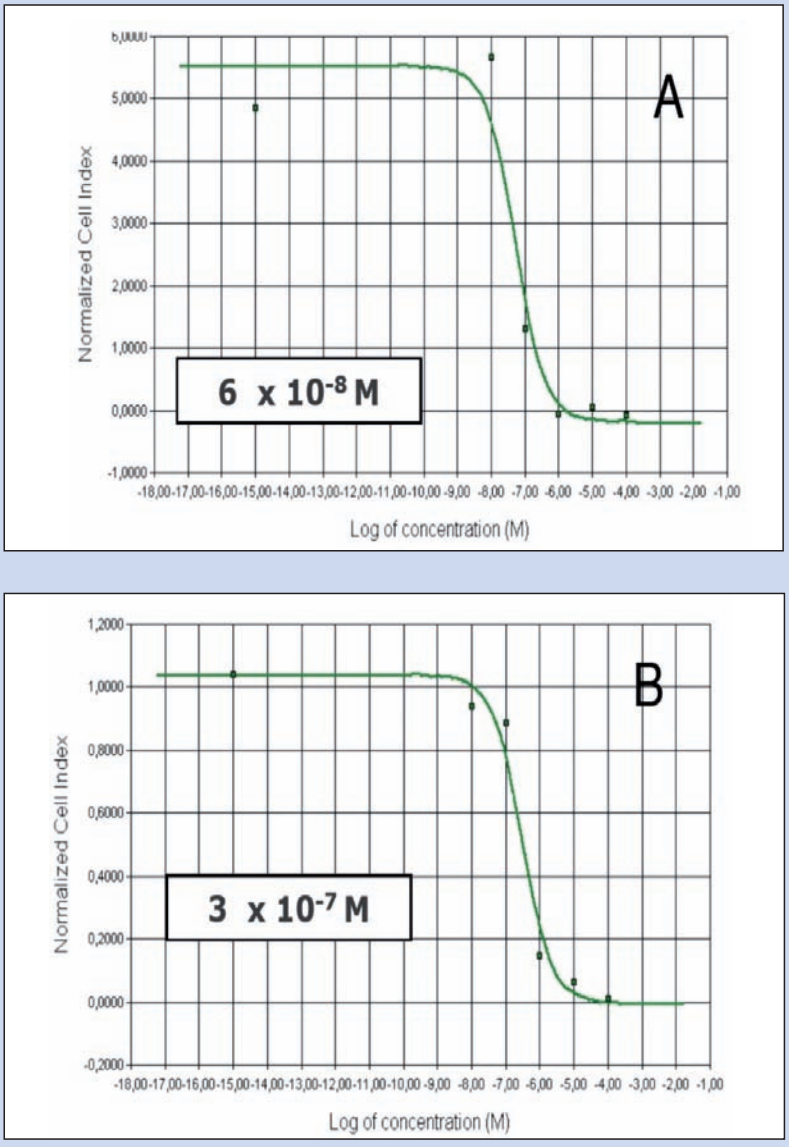


Figure 8: xCELLigence Analysis of Cor.At® cells. IC50 calculations were performed 3 days after addition of doxorubicin. A. Calculations done on day 5 (juvenile) Cor.At® cells B. Calculations done on day 18 (adult phenotype) Cor.At® cells.

Result continued

It is possible to measure the effects of repeated Treatments

Analysis of adult Cor.At® cardiomyocytes (day 10 to 15) under single or repeated doxorubicin additions

Figure 9 demonstrates the effects of a single addition (see Figure 9A) versus multiple treatments with doxorubicin (see Figure 9B) . The Cor.At® cells were pre-cultured for 15 days (360 hours) in order to complete the maturation process to the adult cardiac cell phenotype. It is importanct to note that repeated treatments with anthracyclines can lead to toxic events even at 100 nM concentrations, a dose showing no effect after just a single treatment.

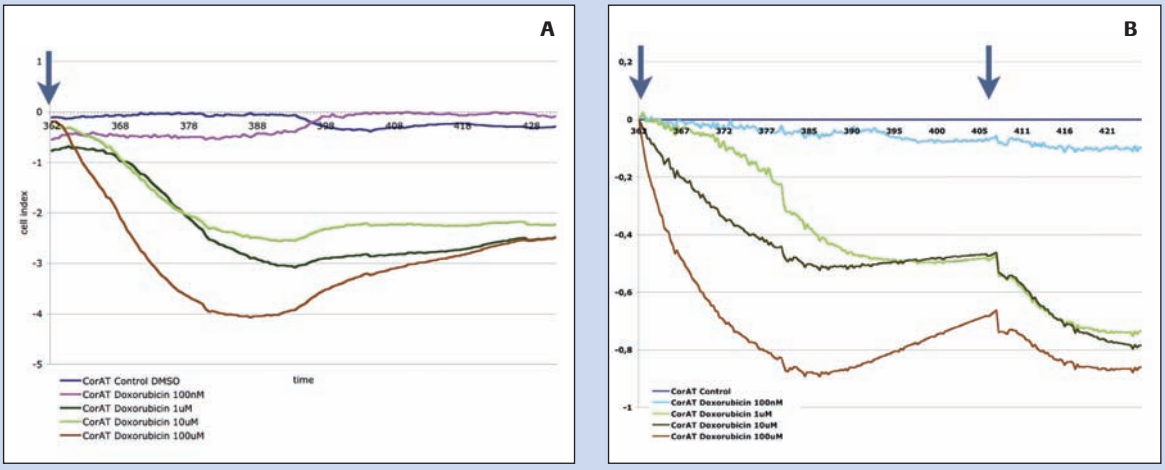


Figure 9: xCELLigence Analysis of Cor.At® cells. Change of relative cell densities after single (A) and double (B) treatments of doxorubicin at different concentrations (The curve in graph B is normalized to the media only control).

Dynamic monitoring of signaling events

In addition to the patch-clamp and MEA analysis, adrenergic and muscarinergic agonist stimulation were examined. Both showed a clear cellular response compared to DMSO controls. It became evident that both physiological effects can be discriminated using the RTCA SP Instrument with Cor.At® cells (see Figure 10). These proof of concept experiments are currently being expanded to produce a complete picture comprising results from cellular integrity analyses and the differentiation of ion channels and associated humoral events underlying cardiac cell differentiation.

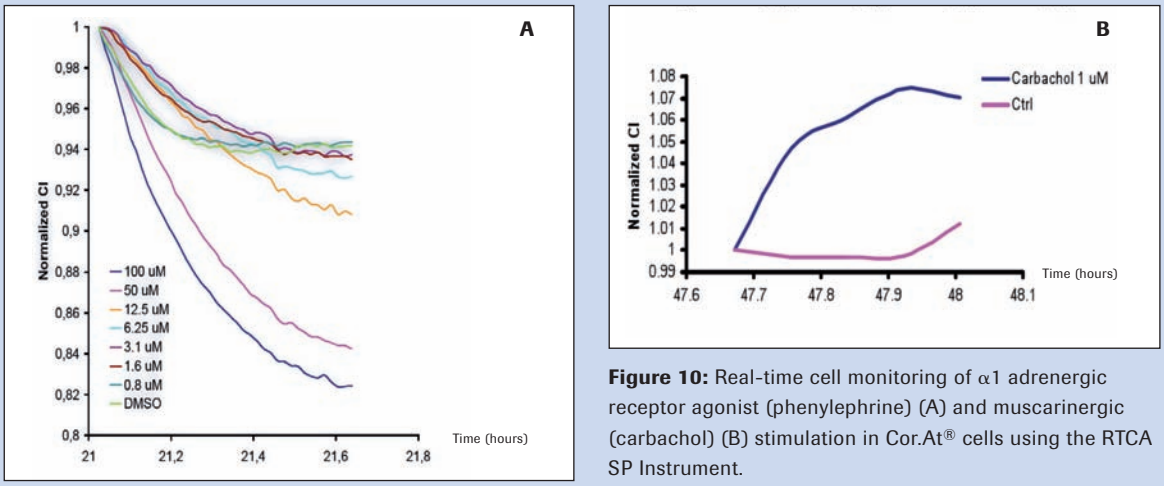


Figure 10: Real-time cell monitoring of α1 adrenergic receptor agonist (phenylephrine) (A) and muscarinergic (carbachol) (B) stimulation in Cor.At® cells using the RTCA SP Instrument.

Conclusion

A very high correlation exists between primary cardiomyocytes and Cor.At® cells derived from ES cells. Both cell types appear to express the same typical surface markers and functionally intact ion channels. The signal pathways in Cor.At® cells appear to be virtually identical to their primary cell counterparts.

A number of important advantages were identified when combining Cor.At® cells with the xCELLigence Real-Time Cell Analyzer (RTCA) System.

*Cor.At® cells, stored in liquid nitrogen, are produced in uniform lot sizes (4 -7*10^9 cells). Phenotypic and functional standardization of individual lots of Cor. At® cells is performed using xCELLigence Instruments.*

Cor.At® cells and associated consumables are standardized and optimized for use with the xCELLigence System (the availablity of Cor.At® cells is guaranteed for at least 5 years).

The above described studies underscore the feasibility of performing repeated drug treatments in combination with constant cell monitoring using the xCELLigence System with Cor.At® cells to investigate cardiotoxicity.

Analyzing the effects of drugs used in clinical oncology on the responses of Cor.At® cells showed high correlation to effects found in human cardiomyocytes. The combined use of Cor.At® cells and the technology of the xCELLigence System thus opens a new opportunity to obtain data with high physiological relevance to clinical findings.
In contrast to other label-free technologies, the xCELLigence Real-Time Cell Analyzers permit not only short-term, but also long-term experiments. This feature facilitates the establishment of cell differentiation age-related strategies for testing pharmaceutical compounds, as well as the anaysis of repeated dose effects and chronic effects of substances. In addition, functional drug effects on Cor.At® cells, such as GPCR mediated responses can be addressed in combination with the RTCA Instruments.

The benefits of using ES cell derived "cardiac primary tissue" in the xCELLigence System can now be explored horizontally to other clinically relevant organ tissues of mouse origin, and vertically to other model systems like human induced pluripotent stem (iPS) cell based research.

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