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ORIGINAL ARTICLE

Doxorubicin-induced cardiomyocyte toxicity - protective effects of endothelial cells in a tri-culture model system

Eliesmaziah Alias,[1†] Vijay Parikh,[1†] Araida Hidalgo-Bastida,[1] Malcolm Wilkinson,[2] Kelly S. Davidge,[2] Tim Gibson,[3] Duncan Sharp,[3] Rameen Shakur[4] & May Azzawi[1*]

[1] *Cardiovascular Research Group, School of Healthcare Science, Manchester Metropolitan University, Manchester M1 5GD, UK*

[2] *Kirkstall Ltd. Rotherham, S60 1FB, UK*

[3] *Elisha Systems Ltd, Wakefield, West Yorkshire WF3 4AA, UK*

[4] *Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, University of Cambridge, Cambridge CB10 1SA, UK*

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Correspondence to
May Azzawi, Cardiovascular Research Group, School of Healthcare Science, Manchester Metropolitan University, Manchester M1 5GD, UK.
Tel: +44 161 247 3332;
E-mail: m.azzawi@mmu.ac.uk

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Abstract

Doxorubicin-induced cardiomyopathy is a clinically prevalent pathology, occurring as a sequelae following chemotherapy for cancer patients. In particular, the "first dose" effect has been particularly challenging, given the heterogeneous and multifactorial nature of this pathophysiology. Here, we describe the development of a physiologically relevant in vitro model for cardiotoxicity testing, using human cells. Primary cardiomyocytes, endothelial, and smooth muscle cells were tri-cultured in 2D, or within nano-fibrous scaffolds in a 3D environment, under dynamic nutrient flow, using the Quasi Vivo® system. State-of-the-art sensor chips were used to detect troponin I levels, 2 h after acute exposure to doxorubicin. We demonstrate a significant improvement in cardiomyocyte viability when grown in a 3D tri-culture environment over a 5-day period and a 10-fold reduction in doxorubicin-induced toxicity. Our tri-culture model can be used as a valuable tool for physiologically relevant assessment of drug-induced cardiotoxicity in vitro.

Introduction

Doxorubicin is a widely used chemotherapeutic agent but has long been associated with cardiac dysfunction in cancer patients (Bartlett et al., 2017). Doxorubicin acts directly on cardiomyocytes (CMCs), generating reactive oxygen species and reactive nitrogen species (Cappetta et al., 2017). These moieties can result in the upregulation of inflammatory markers, the induction of apoptosis, and dysregulation of lysosomal autophagy, eventually leading to cardiomyopathy and heart failure

[†]Authors contributed equally.

(Bartlett et al., 2017; Cappetta et al., 2017; Zhao & Zhang, 2017). Understanding the "first dose" acute effects of doxorubicin is critical to monitoring later events associated with its cardiotoxicity, because even very low doses have been associated with subclinical toxicity in childhood cancer (Leger et al., 2015).

There have been a number of approaches to assess cardiotoxicity; however, existing in vitro toxicity assays that normally involve the culture of isolated CMCs alone do not mimic the complexity of the physiologically dynamic environment (Kirkpatrick et al., 2007; Chaudhari et al., 2018). Furthermore, determining safe dosages has relied heavily on experimental animal models, where precise mechanisms are difficult to ascertain due to interacting molecular pathways in vivo. In addition, findings from animal studies are often not representative of the outcomes in human subjects and are associated with higher costs, technical demands, and ethical issues (U.K. Government, 22 Oct. 2015; Novakovic et al., 2014).

Within cardiac tissue, CMCs exist along with other cells, including endothelial cells (ECs), smooth muscle cells (SMCs), fibroblasts, and macrophages (Azzawi et al., 1997), responding to cues from the extracellular matrix and the surrounding cells (Bartlett et al., 2017; Zhang & Shah, 2014). CMCs form the bulk of the myocardial tissue, supplied by a rich network of blood vessels and capillaries, such that ECs, SMCs, and CMCs lie in very close proximity to each other. Despite their challenges, recent studies have suggested that culturing CMCs with other cell types in a 3D environment improves their survival, biochemical, and physiological responses, including the ability to retain their contractile properties (Chan et al., 2015; Narmoneva et al., 2004) and their spatial organisation in a 3D configuration (Narmoneva et al., 2004).

In order to reproduce a 3D environment, investigators have used a range of strategies to host the cells, from matrigel discs, simple spheroids, to scaffold biomaterial structures (Pononchuk et al., 2017; Roth & Singer, 2014). The scaffolds can be synthesised using synthetic (such as polyesters and poly (vinyl alcohol) (PVA)), natural (such as collagen and gelatine), or a blend of both types of material (reviewed in Novakovic et al., 2014). Electrospinning technology enables the fabrication of nanostructured scaffolds with complex porous architecture resembling the extracellular matrix. With the use of appropriate polymers, these constructs can replicate the biomechanical and structural properties of native tissue, providing support and high surface area for cell attachment, proliferation, and differentiation (Sill & von Recum, 2008). The maintenance of cell seeded scaffolds under flow conditions also enables the replication of mechanical forces (shear stress) imparted on cells by flowing interstitial fluid (Nithianantha et al., 2017).

In the present study, we report the fabrication of a 3D PVA-gelatin nano-fibrous scaffold, which is used to seed CMCs, in tri-culture with ECs and SMCs, under dynamic nutrient flow, using the Quasi Vivo® system (Pagliari et al., 2014). The construct is used to analyze the effects of the common cardiotoxic drug doxorubicin, integrating biophysical variables to mimic the physiological state and provide efficient and accurate drug toxicity screening. Such a system has a strong translational potential and can be used for screening applications in preclinical drug development. Furthermore, we used a novel biosensor-based, highly sensitive assay to assess the levels of the biomarker cardiac Troponin I (cTnI) for doxorubicin toxicity (Atas et al., 2015; Thygesen et al., 2012).

Methods

Materials

Human primary cells were purchased from suppliers as follows: Primary human CMCs (PromoCell, GmbH), human coronary artery ECs (PromoCell, GmbH), and human coronary artery SMCs (Cellworks, UK). For each cell type, their respective media was purchased from the same supplier. They are all supplemented with foetal calf serum (0.05 mL/mL) and growth factors, including basic fibroblast growth factor (10 ng/mL) and epidermal growth factor (5 ng/mL). The EC culture media is additionally supplemented with vascular endothelial growth factor (0.5 ng/mL). Full details are provided by the respective suppliers. Other reagents and materials were supplied as indicated in each section below.

Flow chamber set-up

A bespoke perfusion bioreactor chamber (Quasi Vivo®, Kirkstall Ltd., UK) was used for the experiments (Pagliari et al., 2014). The chambers were interconnected in series, using tubing, linked to a reservoir bottle. Perfusion of the circulating media was supplied by a peristaltic pneumatic micro-pump (Parker PF22x0103) to provide constant recirculation under flow conditions (Vinci et al., 2011), (Fig. 1).

Scaffold synthesis and characterisation

Scaffolds were synthesised using the electrospinning process, at room temperature and normal atmospheric

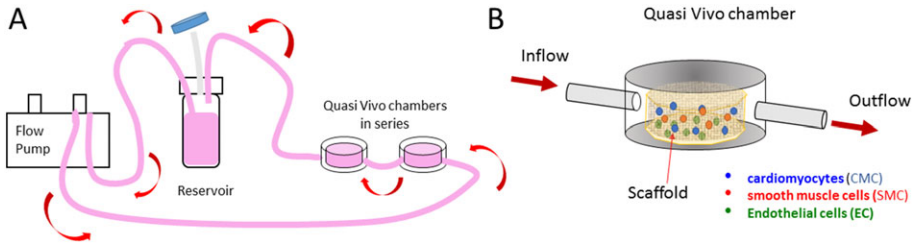

Figure 1. Schematic sketch of the Quasi Vivo flow perfusion chamber set-up (A), with illustration of the chamber including cells seeded within the scaffold (B).

humidity. PVA (99% hydrolyzed, M.Wt. 89,000-98,000 gm/mol; Sigma Aldrich, UK) solution 12.5% *w/v* in deionised water was mixed with Gelatine Type A (Ge; approx. 300 Bloom; Aldrich Chemical Co., UK), 12.5% *w/v* in Acetic Acid:water, at a ratio 2:8 (PVA: Ge). The electrospinning process was carried out at 16 kV and a flow rate of 1 mL/h. The needle gauge was 18G, and tip to collector distance was 15 cm. The synthesised scaffolds were allowed to dry overnight to ensure evaporation of residual solvents, then cross-linked with methanol and stored in a sterile container. Fibre diameter and orientation was determined using scanning electron microscopy. Samples were washed in PSS, then fixed overnight in 2.5% glutaraldehyde, followed by dehydration in serial alcohol and drying using a vacuum desiccator. Samples were then sputter coated with gold palladium and observed using the Zeiss scanning electron microscope (Zeiss Supra™ 40VP).

Cell culture

Cells were cultured in a humidified atmosphere of 5% CO_2, 95% air, at 37°C, in their respective media, as recommended by the supplier. The cells were maintained at low passage (<8), and the media changed every 2 to 3 days. When cells were ~80% confluent, they were enzymatically passaged using TrypLE express reagent (Thermo fisher scientific, UK) and frequently characterised to confirm their phenotype. CMCs, ECs, and SMCs were tagged using CellTracker™ blue, green, and red (Invitrogen, UK), respectively. For the tri-culture cells were grown (1:1:1) at the same seeding density (3.33×10^3 cells/mL) on either a 2D TCPS tissue-culture polystyrene coverslip (11 mm^2 diameter; Nunc, UK, incubated in human fibronectin (Sigma Aldrich) at 50 μg/mL prior to use) or on a 3D scaffold. Electrospun 3D scaffolds were cut into discs of 2 mm thickness and sterilised using a UV steriliser. Cell seeded scaffolds or cells-coverslip constructs were placed either in the perfusion chamber or in a 24-well plate and held down using CellCrown™ 24 (Scaffdex, Finland). The media was used at 1:1:1 proportion of the cell's respective medium. The biochemical composition of the individual and mixed media was initially assessed using the Stat Profile Prime (Nova Biomedical, UK) media profiler. While there was a small increase in glucose levels over the 5-day period of the mixed media, there was no overall difference in composition and all the values were within the physiological range (Fig. 2). Cells were maintained either in either static conditions or under flow (at 50 μl/min) conditions. Once the cells reached 80% confluency, they were maintained at 37°C (4% CO_2; 95% humidity) over a 5-day period.

Cells were identified using cell trackers and quantified using live-dead reagent. Briefly, the samples were washed for 5 min in phosphate buffered solution (PBS) and incubated in a solution of 2-μM calcein-AM (live) and 4-μM ethidium homodimer-1 (dead) solution in PBS for 30 min. After rinsing, the samples were imaged with an inverted fluorescence microscope (Leica DMI6000B; Leica Microsystems, Germany) using a conventional fluorescein long pass filter. The quantification of the cells was done with Image J software (US National Institutes of Health). At each time interval analysed, images were taken from five random fields per slide, for at least three slides. Cells numbers were expressed per field of view (FOV = 1 mm^2). Cells were fixed in 10% neutral buffered formalin, rinsed in PBS, and observed using the fluorescent microscope (Leica microsystem, UK). Cells were stained using anti-smooth muscle actin and DAPI (nucleus).

Doxorubicin challenge

Cells were cultured for 5 days, then challenged with doxorubicin (Sigma Aldrich; 0.01 nM to 100 μM) over a 2-h acute exposure period. Cell viability was assessed using Alamar blue (AB) reagent according to the manufacturer's instructions (Thermo fisher, UK). AB is a sensitive metabolic assay based on the reduction of

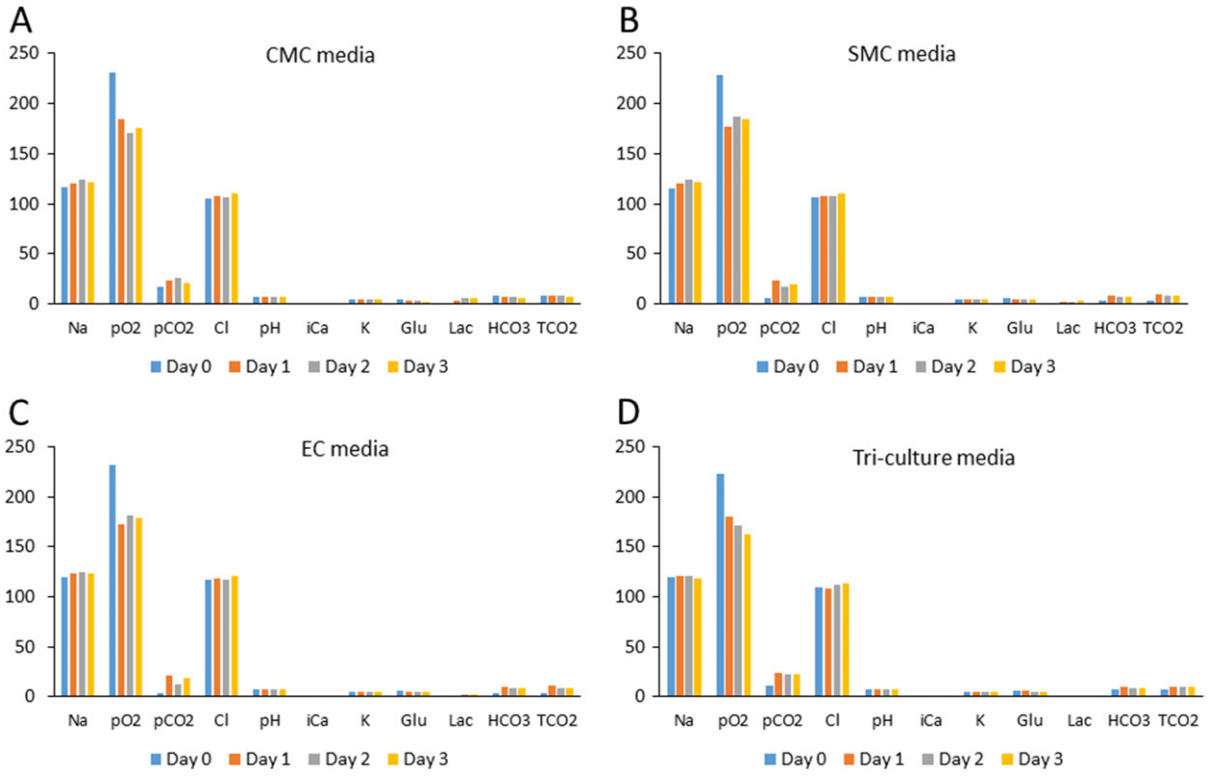

Figure 2. Biochemical composition of the culture media using the media profiler. The composition of the individual media for the respective cell types (Cardiomyocytes (CMCs, A), smooth muscle cells (SMCs, B), endothelial cells (ECs, C)), as well as the mixed media for their tri-culture (D), was determined over a 3-day period. All biochemical indices were within the physiological ranges for Sodium (Na, 100-180 mmol/L); partial pressure of oxygen (pO_2, 5-800 mmHg); partial pressure of carbon dioxide (pCO_2, 5-130 mmHg); chloride (Cl, 65-140 mmol/L); pH (6.5-8.2); ionised calcium (iCa, 0.25-2.5 mmol/L); potassium (K, 2.0-9.0 mmol/L); glucose (Glu, 1.1-38.9 mmol/L); lactate (Lac, 0.3-20.0 mmol/L); bicarbonate (HCO_3, 1.0-85.0 mmol/L); total CO_2 or bicarbonate (TCO_2, 5.0-50.0 mmol/L). CMC, cardiomyocyte; EC, endothelial cell; SMC, smooth muscle cell.

resazurin (blue) to the highly fluorescent resorufin (red) inside metabolically active cells only. Hence, it is used as a measure of cellular health and viability and has been widely used to assess cell cytotoxicity in a range of biological applications (Rampersad, 2012). Briefly, cells were rinsed in PBS and 100 μl of diluted AB reagent (1:10 in medium) added to each well and incubated over 4 h in the incubator at 37°C (protected from light). AB fluorescence was quantified in a microplate reader (excitation wavelength 530 nm; emission wavelength 590 nm) (Molecular Devices, Menlo Park, CA, USA). The background fluorescence of media alone (plus AB reagent) was subtracted in the analysis. Cells treated with 2% Triton X-100 was included in each assay as a control. Displayed results are from three independent experiments carried out in triplicates.

Measurement of nitric oxide levels

To measure the nitric oxide (NO) levels, cultured cells were exposed to the agonist acetylcholine (ACh, 0.01 μM) for 0-30 min and NO was quantified using the Griess reaction. Briefly, equal volumes of sample and Griess reagent (1% sulfanilamide in 2.5% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) were incubated at room temperature for 10 min. The absorbance was measured at 570 nm using the microplate reader. The content of nitrite was calculated based on a standard curve constructed with $NaNO_2$ (3.12 to 400 μM). The assay sensitivity is approximately 125 pMoL (Gómez et al., 2013).

Troponin I levels

Cardiac troponin I (cTnI) levels in culture medium was detected and analysed by Elisha Systems Ltd (Wakefield, UK), using novel biosensors based on commercially available dual electrodes and an immobilised polyclonal antibody against Troponin-I. Polyclonal rabbit anti-Troponin-I (Raybiotech, Rabbit anti-Troponin) was diluted in phosphate buffer pH 7.0 and immobilised using a proprietary method to allow antibody proximity

to the surface of working electrode 2 of a dual gold electrode (DropSens Dual customised gold electrode Ref. CX2220AT). A polyclonal sheep anti-digoxin (prepared in-house) was used as the non-specific internal control immobilised onto working electrode 1. Solutions of Troponin-I in culture medium were used to calibrate the biosensors. Samples were analysed by exposing the upper surfaces of the biosensors to 30 μl of sample, incubating for 5 min, then rinsing with 10× PBS. Biosensors were then connected to a SP200 BioLogic potentiostat with an integral AC impedance monitor. Impedance of the surfaces were measured over a frequency range of 1–1000 Hz in a 10 mM Ferricyanide/Ferrocyanide PBS (10×) solution. Total impedance values at 1 Hz were used to generate the graphical data, correcting for non-specific binding by

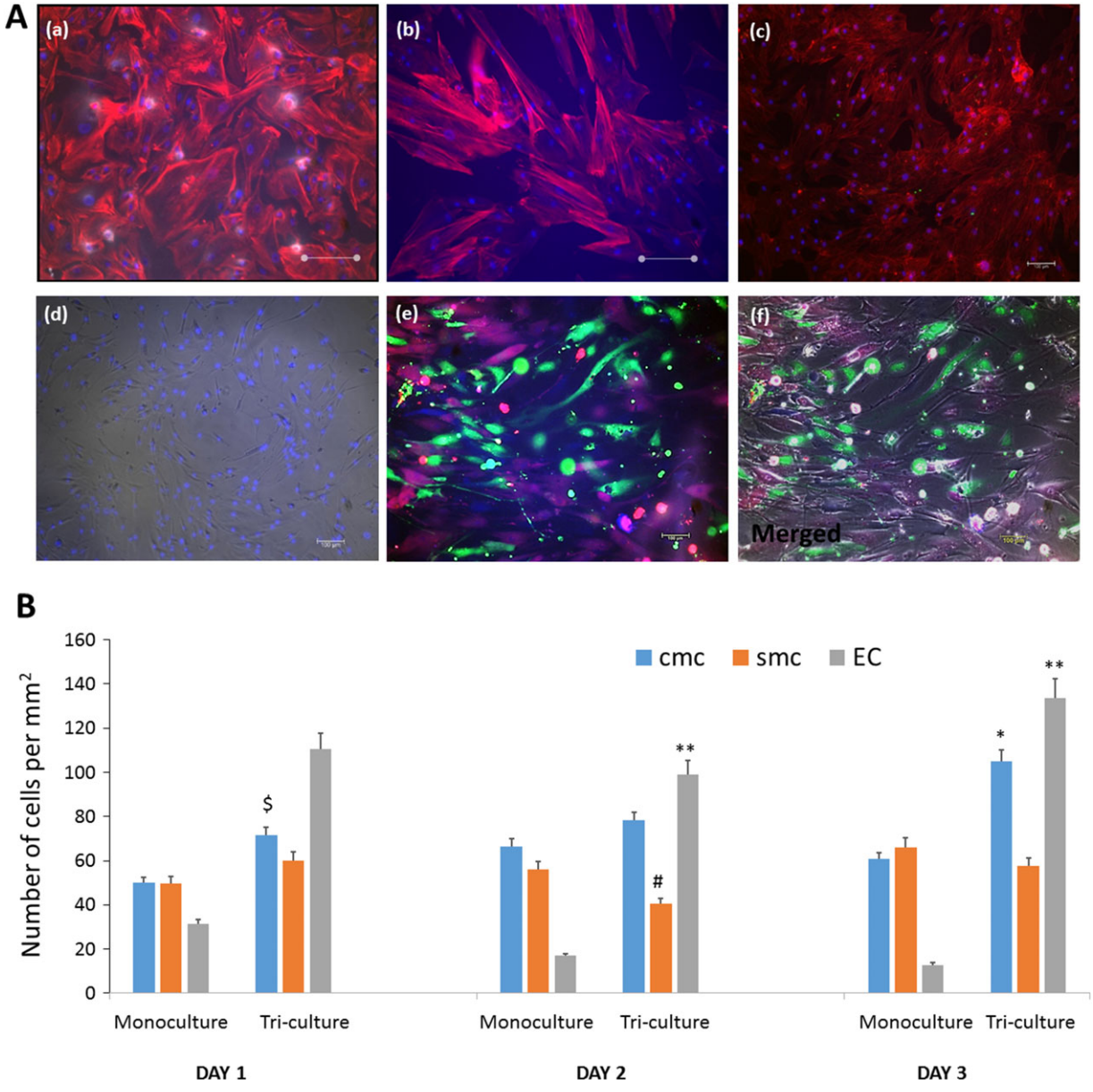

Figure 3. Establishing a tri-culture of cells in static conditions. (A) Light micrograph images of isolated cells after 5 days in 2D culture in static conditions. Cardiomyocytes (CMCs) (a), smooth muscle cells (SMCs) (b), and Endothelial cells (ECs) (c), in monoculture were stained blue (DAPI, nuclear stain); and red (F-actin Phalloidin, cytoskeletal stain). A bright field image of all three cell types in tri-culture (d), and the image of the fluorescently tagged cells (e) is merged in figure (f) (CMCs blue; SMCs red; ECs green). Scale bar 100 μm. (B) Cell survival in monoculture and tri-culture conditions. The total number of CMCs, SMCs, and ECs grown after 1, 3, and 5 days, in isolation (monoculture) or in combination (tri-culture), under 2D static conditions. $^{\$}P < 0.05$; $\#P < 0.01$; $^{*}P < 0.001$; $^{**}P < 0.0001$.

subtracting the impedance values of working electrode 1 from those of working electrode 2 and converting to percentage impedance change to normalise the data. Results from several biosensors were pooled to give an overall average response. Internal software algorithms were used to calculate standard deviation, standard errors, and percentage variability of the coefficient of variation (CV%). Coefficient of variation values acceptable for the assay were ±15%.

Data analysis

Repeated measures analysis of variance and student's *t* test were used to assess statistical significance, using IBM SPSS. Values of $P < 0.05$ were considered significant. Data are presented as mean ± standard error of mean. The half maximal inhibition concentration (IC50; 50% of growth inhibition) was calculated using *Origin Lab* software.

Results

Establishing a dynamic tri-culture of cardiac cells

Tri-culture of cells in a 2D environment

In a 2D culture on coverslips, all cell types grew to confluency. At early time points, cells grew in the middle where they were initially seeded and later spread to the periphery over time. We demonstrate cell survival in monoculture and tri-culture for all three cell types (Fig. 3A), as can be seen by the formation of an intense meshwork of cellular connections in the tri-culture system. We then quantified the cell numbers over the 5-day incubation period and found that while the SMC numbers were maintained at a relatively constant level, the CMC and EC numbers increased significantly in the tri-culture system when compared with the monoculture system (significantly higher numbers for ECs on day 3; twofold and 10-fold increase in CMC and EC numbers in tri-culture, respectively; $P < 0.0001$) (Fig. 3B).

Physiological responsiveness of endothelial cells under static and flow conditions

Subsequently, the physiological responsiveness of ECs was assessed. The cells were grown over 5 days and then challenged with Ach, at a concentration of 0.01 μM (established as the maximal concentration to provide vasodilator responses ex vivo over a 30-min period) (Mohamed et al., 2017). A small non-significant increase in NO levels after 20 min of exposure to the agonist for both mono and tri-culture models was observed (Fig. 4). In a separate set of experiments, cells were cultured under flow conditions at 50 μl/min. Under flow conditions, cell numbers for each cell type were maintained and the morphology of the cells was elongated, when compared with a spherical morphology for cells cultured under static conditions. Furthermore, we observed a significant increase in CMC cell number after 5 days of culture, both under static conditions and flow conditions. SMC and EC numbers were significantly increased after 5 days under flow conditions (Fig. 5A, B).

Establishment of the 3D cell culture system

For the 3D cell culture, PVA-gelatine nanostructured 3D scaffolds were initially fabricated and characterised. The fibre diameter ranged from 160–300 nm, and the scanning electron microscopy images of the scaffold indicated overlapping fibres without any specific orientation (Fig. 6). The fabricated 3D scaffolds promoted CMC cell growth and attachment (Fig. 6, inset).

Cellular responses to acute doxorubicin exposure

A tri-culture model of cardiomyocytes protects against doxorubicin-induced cardiomyocyte toxicity

At the start of each experiment, in the absence of doxorubicin exposure, viability of all cell types was >99%. As expected, acute exposure to doxorubicin resulted in a dose-dependent reduction in CMC cell viability when these cells were cultured in isolation, in both 2D and 3D environments, under static conditions (Fig. 7A). However, when CMCs were grown in tri-

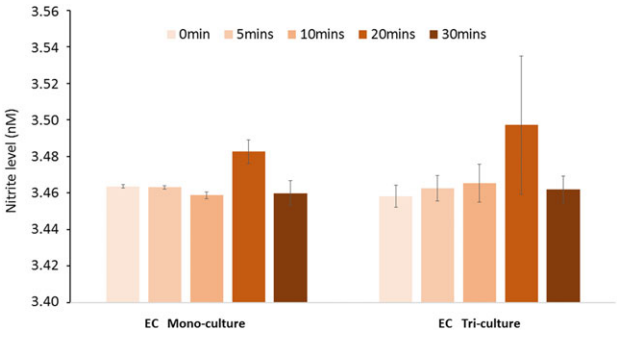

Figure 4. Physiological responsiveness of ECs in cell culture. Cells were cultured for a period of 5 days and then challenged with Ach (0.01 μM) in monoculture and tri-culture, in a 2D culture system under static conditions. EC, endothelial cell.

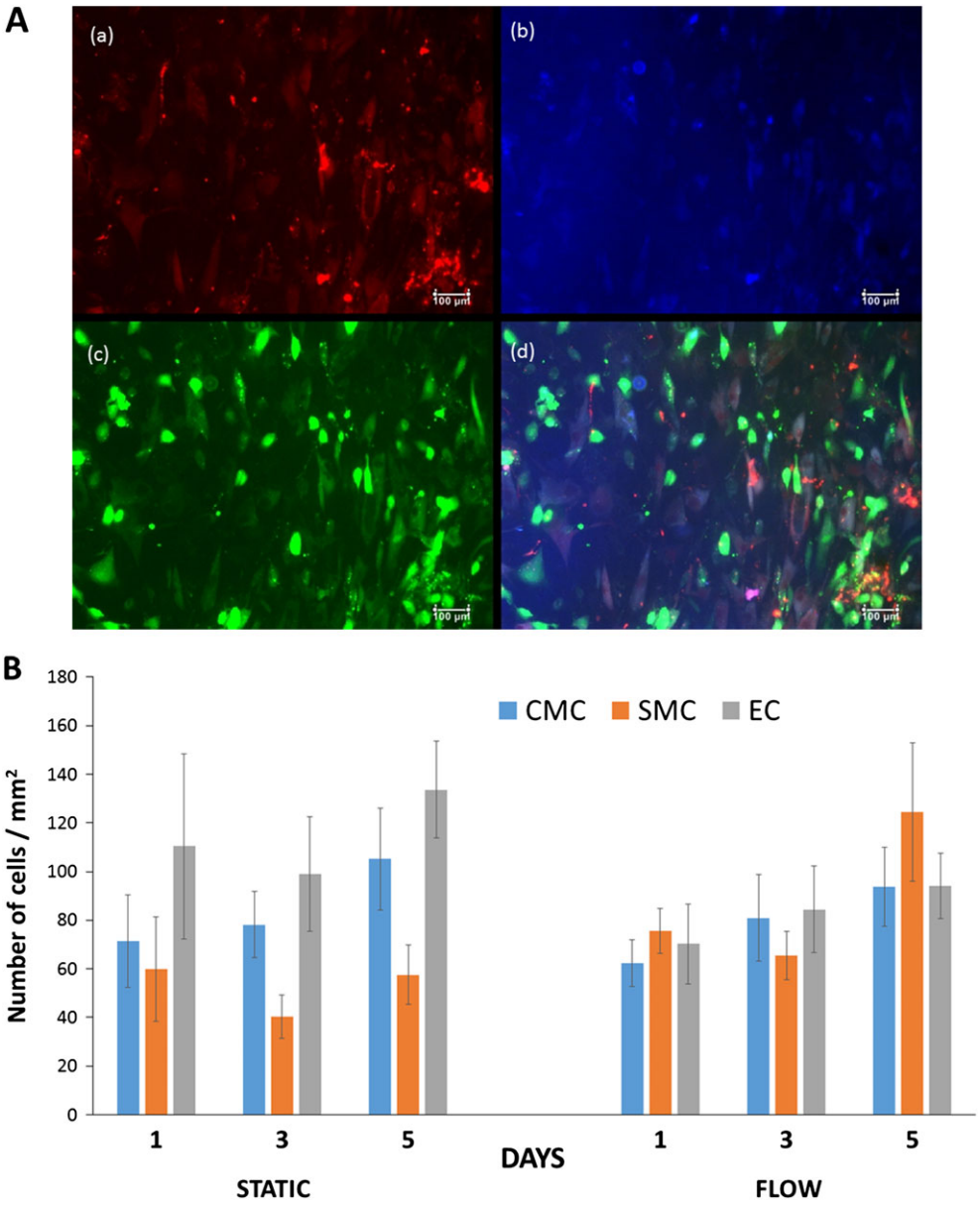

Figure 5. Establishing a dynamic tri-culture of cells. (A) Light microscopy images of cells isolated after 5 days in 2D culture under flow conditions. CMCs (a), SMCs (b), and ECs (c), in tri-culture (d, merged) are fluorescently tagged (CMCs blue; SMCs red; ECs green). (B) Cell survival under flow conditions. The total number of ECs, CMCs, and SMCs grown after 1, 3, and 5 days, under 2D static and flow conditions. CMC, cardiomyocyte; EC, endothelial cell; SMC, smooth muscle cell.

culture, along with SMCs and ECs under static conditions, the toxicity of doxorubicin, hence the dose-dependent reduction in viability, was markedly reduced by approximately a factor of 10. Hence, there was a 10-fold reduction in IC50 values (IC50 in 2D: 0.65 and 0.085 nM for in monoculture and tri-culture, respectively; IC50 in 3D: 5.43 and 0.32 nM in monoculture and tri-culture, respectively (Fig. 7A)).

We next assessed the effect of doxorubicin toxicity on CMC viability for cells cultured under flow conditions. While the doxorubicin-induced toxicity was dose-dependent, as under static conditions, cells cultured under flow conditions showed reduced toxicity to doxorubicin in comparison with the static culture model, for both the 2D culture system and the 3D culture system. The IC50 values showed a marked reduction (by approximately 10-fold) from 7.46 to 0.92 nM; and from 41 nM down to 2.7 nM, in 3D and 2D culture, respectively. Doxorubicin toxicity was minimal for the tri-culture 3D system under flow conditions (Fig. 7B).

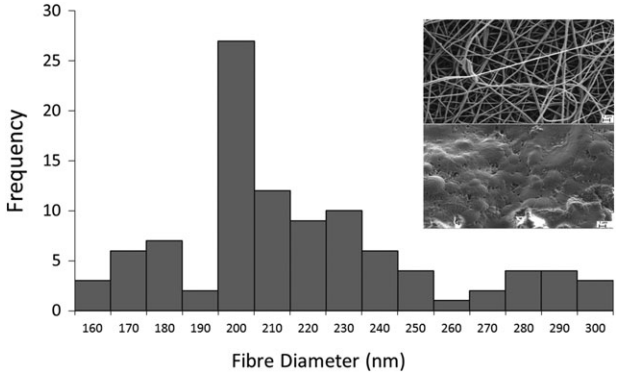

Figure 6. Fabrication of 3D scaffolds using electrospinning. Fibre diameter frequency and scanning electron microscopy image in the absence and presence of seeded cells (upper and lower inset), after a 5-day culture.

Endothelial cells are protective against doxorubicin-induced cardiomyocyte toxicity

All cell types were exposed to doxorubicin, in isolation, and in tri-culture, in 2D and 3D (Fig. 7C and D, respectively). In order to assess the protective effect of ECs on CMCs, CMCs were grown in co-culture with ECs, and the response to doxorubicin-induced toxicity was assessed in 2D (Fig. 7C) and 3D (Fig. 7D). Initially, in the absence of doxorubicin exposure, viability for all cell types was >99%. Doxorubicin toxicity to CMCs was reduced when they were co-cultured in the presence of ECs (IC50 values were 0.92 nM vs. 8.4 nM and 2.7 nM vs. 39.8 nM for CMCs alone vs. CMCs co-cultured with ECs, respectively). Analysis of samples (from both CMC monoculture and tri-culture) for cTnI levels using the biosensors demonstrated higher levels of cTnI in the monoculture than in the tri-culture (averaged at 7.60 and 3.89 pg/mL after doxorubicin challenge, vs. 0.32 and 0.00 pg/mL in the absence of doxorubicin, in monoculture and tri-culture, respectively; calculated from normalised data) (Fig. 8A, B).

Discussion

It is important that in vitro models recapitulate in vivo physiological conditions when studying the effects of drugs on the cell environment and cell functionality, in order to be used in translational medicine. In the present study, we describe the development and characterisation of a 3D cell culture system for CMCs, tri-cultured in the presence of other cell types such as the ECs and the SMCs that have the ability to form a

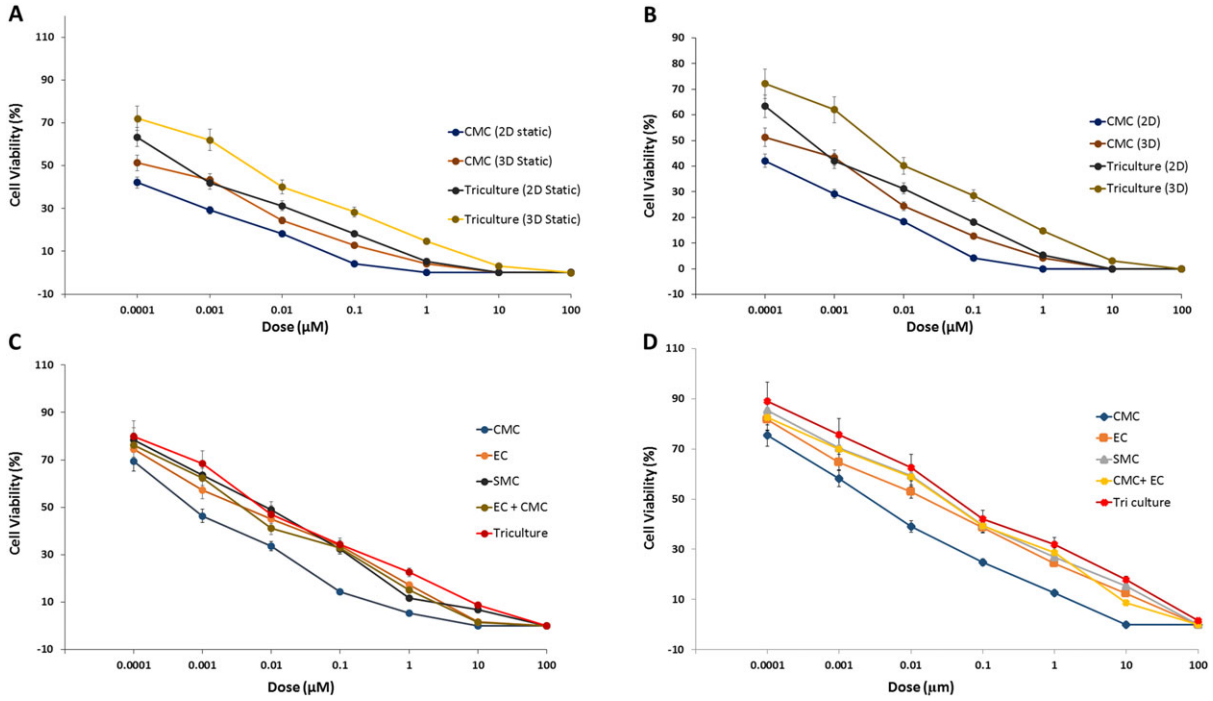

Figure 7. Cellular responses to acute doxorubicin exposure. CMC viability in monoculture and tri-culture, under static (A) and flow (B) conditions. Least toxicity was achieved for tri-cultured cells, under flow conditions, in the 3D environment. Cell viability for all cardiac cells in monoculture and tri-culture under flow conditions is compared in 2D (C) and 3D (D) environments. CMC, cardiomyocyte; EC, endothelial cell; SMC, smooth muscle cell.

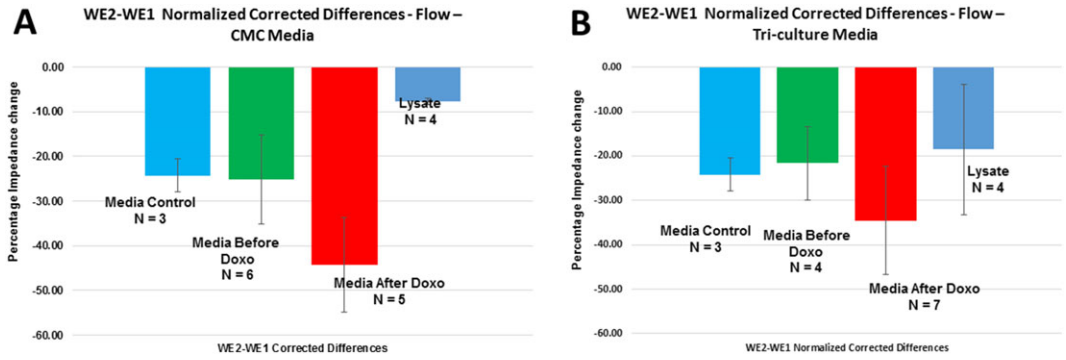

Figure 8. Detection of cTnI levels using biosensors. cTnI levels demonstrate higher levels of cTnI in monoculture (A) than in tri-culture (B), using novel biosensor technology.

3D matrix that closely resembles the in vivo environment. In this tri-culture model, we demonstrate that CMCs grown with ECs and SMCs show reduced doxorubicin-induced cardiotoxicity, in comparison with their growth in monoculture. Furthermore, under flow conditions that model the shear stress in vivo, we show a further reduction in doxorubicin-induced toxicity against CMCs.

We demonstrate that the maintenance of CMCs in tri-culture supports their viability over a period of 5 days. This is also observed for ECs, which maintain their physiological responsiveness to agonist stimulation in tri-culture, as shown by maximal NO levels, 20 min after ACh challenge. Nitrite levels were within the nM range for all time points, which is well within the assay sensitivity used in the present study (Gómez et al., 2013). Although ECs are the classic cell type associated with NO release, a key cardiovascular signalling molecule, eNOS is also associated with caveoli structures within the plasma membrane of SMCs and CMCs; hence, their NO contribution cannot be excluded (Zhang & Shah, 2014; Bernhardt et al., 1991). Using a milli-fluidic system, cells were subjected to physiological rates of interstitial fluid flow (5.59×10^{-4} dyn/cm^2) (Sill & von Recum, 2008; Mazzei et al., 2010; Kang et al., 2017; Rashidi et al., 2016) and grown within a nanostructured scaffold that did not adversly affect cell attachment and viability.

We further demonstrate the dose-dependent reduction in doxorubicin-induced CMC toxicity when tri-cultured with ECs and SMCs, in particular when grown under flow conditions, in a 3D environment. While the dose-dependent cumulative toxicity of doxorubicin is well documented for CMCs, we also demonstrate a significant dose-dependent reduction in EC and SMC numbers (Wojcik et al., 2015; Bielak-Zmijewska et al., 2014). Doxorubicin-induced EC apoptosis and disturbed crosstalk between ECs and CMCs has recently been demonstrated using VEGF-B gene therapy in a murine model (Rasanen et al., 2016). NO from ECs plays a protective role in CMCs from doxorubicin, probably via eNOS uncoupling. The exposure to doxorubicin has been shown to increase the expression and protein activity of eNOS (Kalivendi et al., 2001). Others have also shown the protective effects of ECs as a result of factors such as Neuregulin-1 when adjacent to CMCs (Lemmens et al., 2006). Furthermore, ECs line the coronary vasculature and play an important role in diameter regulation and homeostasis through the release of mediators, including NO. Importantly, they form a selective barrier between the myocardium and the circulating blood, regulating the exchange of oxygen and nutrients. Wilkinson et al. (2016) have demonstrated that doxorubicin can inhibit EC–cell tight junction formation resulting in increased drug permeability. Hence, it is plausible that the protective effect of ECs in our study is mediated by the barrier function of ECs by minimising the exposure of CMCs to doxorubicin. This, however, needs to be confirmed by analysing the organisational structure of CMCs and ECs in a co-culture system. Other cell types within the myocardium may also influence doxorubicin toxicity. For example, in a cardiac spheroid model, Pononchuk et al. (2017) have highlighted important crosstalk between fibroblasts, CMCs, and ECs. While NO released from ECs protects CMCs against doxorubicin-induced apoptosis, doxorubicin induces reactive oxygen species production and eNOS over-expression in cardiac fibroblasts, leading to peroxynitrous acid formation, which can override the protective effects of co-cultured ECs (Pononchuk et al., 2017). Within our tri-culture system, the specific role of the SMCs in influencing

doxorubicin-induced CMC-toxicity requires further investigation, by also assessing apoptotic cell death, as an important parameter in doxorubicin toxicity, for all cell types. Furthermore, possible variation in exposure conditions and uptake kinetics of drugs and reagents between 2D and 3D culture cannot be excluded as a contributing factor to the observed reduced doxorubicin-induced toxicity in our 3D environment (Casey et al., 2016).

The concentration ranges of doxorubicin used in the present study are within the ranges that the cells are exposed to in vivo (Gunven et al., 1986; Barpe et al., 2010). For example, in normal weight patients, infusion of 60 mg/m^2 of doxorubicin led to peak plasma concentrations of 630.4 ± 22.1 ng/mL within 0.66 h, reducing to 39.8 ± 15.3 ng/mL after 24.66 h after infusion (Barpe et al., 2010). This equates to 315 and 20 nM, respectively, which is within the wide range used herein (0.01 nM to 100 μM). Furthermore, the documented intracellular concentration of doxorubicin within tumour cells range from 0.007 to 0.013 nmol/mg protein and 0.027 to 0.086 nmol/mg protein, before and after concomitant therapy with verapamil and cyclosporine (Tidefelt et al., 1994).

We detected cTnI levels as an indicator of cell toxicity within the cell seeded PVA-gelatin scaffolds. cTnI is a predictive "gold standard" biomarker for cardiac injury (Atas et al., 2015) and myocardial infarction (Thygesen et al., 2012) with high myocardial tissue specificity and clinical sensitivity. cTnI has previously been assayed using commercial ELISA kits with a lower limit of detection of 1 ng/mL (Atas et al., 2015). We used a state-of-the-art assay for sensitive and specific cTnI detection using robust biosensors with a detection levels in pico molar range. CMCs grown in monoculture had elevated levels of cTnI, which were reduced as the cells were cultured in a tri-culture system, indicating that the presence of ECs and SMCs has a protective effect on CMCs. We also detected negligible levels of cTnI in the absence of doxorubicin exposure, and we postulate that this low concentration of cTnI is released by CMCs as an intact protein as a result of stretch-related mechanism mediated by integrin molecules (Hessel et al., 2008).

In conclusion, we utilised a novel tri-culture dynamic system to demonstrate a protective role for ECs against doxorubicin-induced CMC toxicity. Our model is an attractive non-animal alternative approach to drug testing and assessing new cardio protective strategies against doxorubicin toxicity.

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Conflict of Interest

Kirkstall Ltd. is the Owner of the Quasi Vivo trade mark and manufactures and sells the Quasi Vivo cell culture equipment. ELISHA Systems Ltd has a proprietary manufacturing method for the Troponin biosensors.

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