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Slicing and Culturing Pig Hearts under Physiological Conditions

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Corresponding Author:	Tamer M A Mohamed, PhD University of Louisville Louisville, KY UNITED STATES
Corresponding Author's Institution:	University of Louisville
Corresponding Author E-Mail:	TAMER.mohamed@louisville.edu
Order of Authors:	Qinghui Ou
	Riham R. E. Abouleisa
	Xian-Liang Tang
	Hamzah R. Juhardeen
	Moustafa H. Meki
	Jessica M Miller
	Guruprasad Giridharan
	Ayman El-Baz
	Roberto Bolli
	Tamer M A Mohamed, PhD
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UNIVERSITY OF LOUISVILLE

Tamer M A Mohamed, Ph.D.
Assisstant Professor of Medicine
Institute of Molecular Cardiology
Email: Tamer.Mohamed@louisville.edu
Tel: 502-852-8428

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Editorial Office
JoVE

Dear Dr. Cao,

In response to your invitation for resubmission, please find enclosed our revised manuscript entitled "**Protocol for Slicing and Culturing Pig Heart under Physiological Conditions**" for consideration as a JoVE methods paper. This manuscript is detailing the protocol for a reliable and easily reproducible culture system that maintains the viability and functionality of pig heart slices for at least 6 days under physiological conditions which was recently published in Circulation Research (Ou et al., *Circ Res* **125**, 628-642). In this revised manuscript we addressed all the comments raised by the editor as detailed in the response to the reviewers.

Thank you for your consideration.

Sincerely,

Tamer Mohamed, PhD
Assistant Professor of Medicine
Institute of Molecular Cardiology
University of Louisville

TITLE:

Slicing and Culturing Pig Hearts under Physiological Conditions

AUTHORS AND AFFILIATIONS:

Qinghui Ou[1,*], Riham R. E. Abouleisa[1,*], Xian-Liang Tang[1], Hamzah R. Juhardeen[1], Moustafa H. Meki[2], Jessica M. Miller[2], Guruprasad Giridharan[2], Ayman El-Baz[2], Roberto Bolli[1], Tamer M. A. Mohamed[1,3,4,5,6]

[1]Institute of Molecular Cardiology, Department of Medicine, University of Louisville, KY, USA
[2]Department of Bioengineering, University of Louisville, KY, USA
[3]Diabetes and Obesity Center, Department of Medicine, University of Louisville, KY, USA
[4]Department of Pharmacology and Toxicology, University of Louisville, KY, USA
[5]Institute of Cardiovascular Sciences, University of Manchester, UK
[6]Faculty of Pharmacy, Zagazig University, Zagazig, Egypt

*These authors contributed equally.

Corresponding Author:

Tamer M. A. Mohamed (tamer.mohamed@louisville.edu)

Email Addresses of Co-Authors:

Qinghui Ou (qinghui.ou@louisville.edu)
Riham R. E. Abouleisa (riham.abouleisa@louisville.edu)
Xian-Liang Tang (xian-liang.tang@louisville.edu)
Hamzah R. Juhardeen (hjuhardeen@alfaisal.edu)
Moustafa H. Meki (moustafa.meki@louisville.edu)
Jessica M. Miller (jessica.miller.3@louisville.edu)
Guruprasad Giridharan (guruprasad.giridharan@louisville.edu)
Ayman El-Baz (ayman.elbaz@louisville.edu)
Roberto Bolli (roberto.bolli@louisville.edu)

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gene therapy, cardiotoxicity, heart, electric stimulation, calcium, electrophysiology, biomimetic culture, pharmacology

SUMMARY:

This protocol describes how to slice and culture heart tissue under physiological conditions for 6 days. This culture system could be used as a platform for testing the efficacy of novel heart failure therapeutics as well as reliable testing of acute cardiotoxicity in a 3D heart model.

ABSTRACT:

Many novel drugs fail in clinical studies due to cardiotoxic side effects as the currently available in vitro assays and in vivo animal models poorly predict human cardiac liabilities, posing a multi-billion-dollar burden on the pharmaceutical industry. Hence, there is a worldwide unmet medical

need for better approaches to identify drug cardiotoxicity before undertaking costly and time consuming 'first in man' trials. Currently, only immature cardiac cells (human induced pluripotent stem cell-derived cardiomyocytes [hiPSC-CMs]) are used to test therapeutic efficiency and drug toxicity as they are the only human cardiac cells that can be cultured for prolonged periods required to test drug efficacy and toxicity. However, a single cell type cannot replicate the phenotype of the complex 3D heart tissue which is formed of multiple cell types. Importantly, the effect of drugs needs to be tested on adult cardiomyocytes, which have different characteristics and toxicity responses compared to immature hiPSC-CMs. Culturing human heart slices is a promising model of intact human myocardium. This technology provides access to a complete multicellular system that mimics the human heart tissue and reflects the physiological or pathological conditions of the human myocardium. Recently, through optimization of the culture media components and the culture conditions to include continuous electrical stimulation at 1.2 Hz and intermittent oxygenation of the culture medium, we developed a new culture system setup that preserves viability and functionality of human and pig heart slices for 6 days in culture. In the current protocol, we are detailing the method for slicing and culturing pig heart as an example. The same protocol is used to culture slices from human, dog, sheep, or cat hearts. This culture system has the potential to become a powerful predictive human in situ model for acute cardiotoxicity testing that closes the gap between preclinical and clinical testing results.

INTRODUCTION:

Drug induced cardiotoxicity is a major cause of market withdrawal[1]. In the last decade of the 20th century, eight non-cardiovascular drugs were withdrawn from the market as they resulted in sudden death due to ventricular arrhythmias[2]. In addition, several anti-cancer therapies (while in many cases effective) can lead to several cardiotoxic effects including cardiomyopathy and arrhythmias. For example, both traditional (e.g., anthracyclines and radiation) and targeted (e.g., trastuzumab) breast cancer therapies can result in cardiovascular complications in a subset of patients[3]. A close collaboration between cardiologists and oncologists (via the emerging field of "cardio-oncology) has helped make these complications manageable ensuring that patients can be treated effectively[2]. Less clear are the cardiovascular effects of newer agents, including Her2 and PI3K inhibitors, especially when therapies are used in combination. Therefore, there is a growing need for reliable preclinical screening strategies for cardiovascular toxicities associated with emerging anti-cancer therapies prior to human clinical trials. The lack of availability of culture systems for human heart tissue that is functionally and structurally viable for more than 24 h is a limiting factor for reliable cardiotoxicity testing. Therefore, there is an urgent need to develop a reliable system for culturing human heart tissue under physiologic conditions for testing drug toxicity.

The recent move towards the use of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) in cardiotoxicity testing has provided a partial solution to address this issue; however, the immature nature of the hiPSC-CMs and the loss of tissue integrity compared to multicellular nature of the heart tissue are major limitations of this technology[4]. A recent study has partially overcome this limitation through fabrication of cardiac tissues from hiPSC-CMs on hydrogels and subjecting them to gradual increase in electrical stimulation over

time[5]. However, their electromechanical properties did not achieve the maturity seen in the adult human myocardium. Moreover, the heart tissue is structurally more complicated, being composed of various cell types including endothelial cells, neurons and various types of stromal fibroblasts linked together with a very specific mixture of extracellular matrix proteins[6]. This heterogeneity of the non-cardiomyocyte cell population[7-9] in the adult mammalian heart is a major obstacle in modeling heart tissue using individual cell types. These major limitations highlight the importance of developing methods to enable culture of intact cardiac tissue for optimal studies involving physiological and pathological conditions of the heart[5].

Culturing human heart slices is a promising model of intact human myocardium. This technology provides access to a complete 3D multicellular system that is similar to the human heart tissue which could reliably reflect the physiological or pathological conditions of the human myocardium. However, its use has been severely limited by the short period of viability in culture, which does not extend beyond 24 h using the most robust protocols reported until 2018[10-12]. This limitation was due to multiple factors including the use of air-liquid interface to culture the slices, and the use of a simple culture medium that does not support the high energetic demands of the cardiac tissue. We have recently developed a submerged culture system which is able to provide continuous electrical stimulation and optimized the culture media components to keep cardiac tissue slices viable for up to 6 days[13]. This culture system has the potential to become a powerful predictive human in situ model for acute cardiotoxicity testing to close the gap between preclinical and clinical testing results. In the current article, we are detailing the protocol for slicing and culturing the heart slices using a pig heart as an example. The same process is applied to human, dog, sheep, or cat hearts. With this protocol, we are hoping to spread the technology to other laboratories in the scientific community.

PROTOCOL:

All animal procedures were in accordance with the institutional guidelines of the University of Louisville and approved by the Institutional Animal Care and Use Committee.

1. Preparation for slicing (one day before slicing)

1.1. Preparation of the vibrating microtome

1.1.1. Place the ceramic blade into its holder by following these steps: after carefully unwrapping the blade, place the sharp edge first into the slot of the blade tool. Then, fit the blade into the holder by loosening the two screws on the arms of the holder and slide the blade under each washer and push it firmly back against the rear stops. Tighten the screws to secure the blade.

NOTE: The screws should not be overtightened.

1.1.2. Calibrate the blade using the calibration protocol and tools according to the manufacturer's protocol.

NOTE: Briefly, in order to facilitate alignment of the blade with the axis of travel and to minimize the Z axis deflections, the vibrating microtome uses a demountable calibration device. When the calibration device is plugged into the instrument its presence will automatically be detected, and the vibrating microtome will take control of adjusting the amplitude and frequency settings of the blade to a magnitude which best allows the adjustment of the blade alignment error.

1.1.2.1. Press the **Slice** button to start the alignment process which will automatically move the blade so that the cutting edge is in optimal position relative to the calibration device for best alignment evaluation. Follow the on-screen instructions to make adjustments to the blade using the provided screwdriver to ensure that the Z-alignment is within 1 µm.

1.1.3. Autoclave the inner bath and all the metal parts of the vibrating microtome.

1.2. Autoclave the large metal trays (for collecting the slices and soaking the electrical stimulation plate covers), any glass containers, regular rectangle blades, forceps, scissors, printer timing belt (cut them into 6 mm wide pieces), metal washers and 5 L of double distilled water (ddH_2O).

1.3. Sterilize one 1 L jar, one 500 mL jar and one plastic tray for the heart in situ and in vitro perfusion with the cardioplegic solution.

1.4. Prepare 2 L of the slicing Tyrode's solution in a 2 L beaker.

1.4.1. For 1 L of the slicing Tyrode's solution, mix 3 g/L 2,3-butanedione monoxime (BDM), 140 mM NaCl (8.18 g), 6 mM KCl (0.447 g), 10 mM D-glucose (1.86 g), 10 mM HEPES (2.38 g), 1 mM $MgCl_2$ (1 mL of 1 M solution), 1.8 mM $CaCl_2$ (1.8 mL of 1 M solution), up to 1 L of ddH_2O. Adjust the pH to 7.40 using NaOH. Then, filter the solution using a 1000 mL, 0.22 µm, vacuum filter/storage system and store at 4 °C overnight.

1.5. Prepare 4 L of the cardioplegic solution.

1.5.1. For 1 L of the cardioplegic solution, mix 110 mM NaCl (6.43 g), 1.2 mM $CaCl_2$ (1.2 mL of 1 M solution), 16 mM KCl (1.19 g), 16 mM $MgCl_2$ (3.25 g), 10 mM $NaHCO_3$ (0.84 g), 1 U/mL heparin, up to 1 L of ddH_2O. Adjust the pH to 7.40 using NaOH. Then, filter the solution using a 1000 mL, 0.22 µm, vacuum filter/storage system and store at 4 °C overnight.

NOTE: Add 1,000 U/L heparin in cardioplegia solution the day of slicing (see step 2.1).

1.6. Freshly prepare 500 mL of culture medium in the original bottle in a biosafety cabinet: mix medium 199, 1x insulin-transferrin-selenium (ITS) supplement, 10% fetal bovine serum (FBS), 5 ng/mL vascular endothelial growth factor (VEGF), 10 ng/mL FGF-basic, and 2x antibiotic-antimycotic. Filter sterilize through the 0.22 µm, vacuum filter/storage system and store at 4 °C overnight.

NOTE: Do not adjust pH, add VEGF and FGF freshly before use.

1.7. Prepare 4% agar gel plates.

1.7.1. Add 200 mL of sterilized ddH_2O in a 500 mL sterilized flask. Weigh 8 g of agarose, and gently pour into the flask. Boil for 2 min in a microwave, then cool for 5 min at room temperature.

1.7.2. Pour 25 mL in each 100 mm Petri dish in a biosafety cabinet (prepare 6 dishes) and wait for 1 h to solidify. Then, seal the plates with paraffin film, wrap with clean wrap, and store at 4 °C.

1.8. Prepare 4 L of 70% ethanol by diluting the 200-proof absolute ethanol in autoclaved ddH_2O.

1.9. Prepare 2x antibiotic-antimycotic in sterilized ddH_2O: mix 980 mL of sterilized ddH_2O with 20 mL of antibiotic-antimycotic under the biosafety cabinet.

2. Pig heart perfusion

2.1. Add 1 mL of 1,000 U/mL heparin to each 1 L of cardioplegic solution. Keep the solution on ice.

NOTE: At least 3 L are needed for the perfusion and preservation of the heart during the transfer to the tissue culture room.

2.2. Take the following items to the pig surgery room: one large ice bucket full of ice, one sterilized metal tray (keep on ice bucket for heart perfusion), one sterilized 500 mL jar, one 1 L jar (to transfer the heart in cardioplegia solution back to the tissue culture room, on ice), and one sterilized plastic tray full of ice (to keep cardioplegic solution on ice).

2.3. Prepare an in-situ heart perfusion system containing a 3-way stopcock, a 60 mL syringe, an 18 G butterfly needle catheter, and extension tubing to the cardioplegic solution reservoir.

2.4. Anesthetize the Yorkshire male pig (2−3-month-old, 20−25 kg) first with an intramuscular injection of a ketamine (20 mg/kg)/xylazine (2 mg/kg) cocktail. Confirm proper anesthesia by the loss of jaw tension. Then, transfer the pig to the surgery room, intubate and mechanically ventilate the lungs as previously described[14].

2.5. Maintain anesthesia with (1.5−2%) isoflurane.

2.6. Place the animal on the right lateral position and shave the fur from the chest area. Clean the skin with alcohol scrub, then sterilize the surgical site with 10% (w/v) povidone-iodine solution.

2.7. Open the chest by left thoracotomy incision using a scalpel at the 4^{th} intercostal space, and use a chest retractor between ribs to hold the chest open and expose the heart.

NOTE: Use all sterilized instruments for this procedure.

2.8. Insert the butterfly needle catheter into the left atrium and fix the needle with a purse string closure. After intravenous injection of a bolus dose of heparin (100 U/kg), start to perfuse the heart in situ with 500 mL of ice-cold cardioplegic solution (to slow down the heart rate) via the left atrial catheter.

2.9. Deeply anesthetize the pig with 5% isoflurane. Quickly excise the heart out of the chest with surgical scissors. Cannulate the aorta with an aortic cannula and perfuse the heart in vitro with another 1 L of ice-cold cardioplegic solution (100 mL/min) to flush out vasculature blood.

2.10. Following heart perfusion, keep the heart in a 1 L jar filled with ice-cold cardioplegia solution and keep on ice during the transfer to the tissue culture room.

3. Pig heart tissue slicing

3.1. Set up the tissue bath on the vibratome, add ice to the tissue bath cooling jacket, then add Tyrode's solution into the tissue bath. Set up 1 L plastic jar to collect the disposal of melted ice from the tissue bath cooling jacket.

3.2. Under the biosafety cabinet, transfer the pig heart to a tray containing 1 L of fresh cold cardioplegic solution.

3.3. Dissect the heart to isolate the left ventricle using retractable sterile scalpels. Then, cut the left ventricle into blocks of 1–2 cm^3 each using razor rectangle blades. Use one piece for slicing and keep the remaining pieces in a 50 mL tube in cold Tyrode's solution on ice for cutting later, if needed.

NOTE: Massage the tissue before slicing with hands, especially if this is the second block. Massaging helps to restore the correct muscle fiber configuration and prevents any stiffness within the heart block.

3.4. Add 1–2 drops of tissue glue (**Table of Materials**) to the metal sample holder and stick a piece of the 4% agar block with a surface area of approximately 1 cm^2.

3.5. Add 1–2 drops of tissue glue on the agar.

3.6. Stick the heart block to the agar with the cardiac epicardium side facing down on the tissue glue and make sure it is as flat as possible. Then, transfer the tissue holder with the heart block to its position in the slicing bath of the vibratome.

3.7. Attach the oxygen tube to the slicing bath and the metal tray filled with Tyrode's solution for collecting the slices (oxygenated Tyrode's bath). Then, add 40 µm cell strainers in the metal tray to collect the slices after cutting.

3.8. Adjusting the slicing position

3.8.1. Using the vibratome operating software and the dashboard, adjust the height of the blade/sample. Select the **height** button and follow the on-screen instructions for adjusting the height. Ideally, have the blade close to the top of the tissue but below the papillary muscles and make sure there is liquid covering the tissue and the blade before slicing.

3.8.2. Adjust where to begin slicing the tissue by selecting the **advance** button. Press the **slice** button and increase the speed using the knob to move the blade towards the edge of the tissue. Then, press **slice** again to stop, and press the **advance** button to inactivate the process.

3.8.3. Adjust the cutting parameters: advance speed = 0.03 mm/s, vibration frequency = 80 Hz, and horizontal vibration amplitude = 2 mm. Then, start slicing by selecting the **slice** button.

3.8.4. Let the vibratome slice until it reaches the end of the tissue but before it hits the back end of the specimen holder. At this point, press **slice** again to stop. Hit the **Return** button to go back to the start position.

3.8.5. Select **auto repeat** (clicking it twice) which will allow the vibrating microtome to auto repeat the slicing process for up to 99 times.

3.9. Collecting slices

3.9.1. Wait until slices are full length and appear good (after getting past the papillary muscle layers), then start collecting the slices.

3.9.2. Collect the slices using a plastic Pasteur pipette filled with cold Tyrode's solution to gently grab the tissue from the bath. If necessary, use forceps and spring scissors to dissociate the slice from the heart block if the tissue is still attached.

3.9.3. Transfer the slice to one cell strainer in the oxygenated Tyrode's bath (see step 3.7) and use the liquid in the plastic Pasteur pipette to get the tissue to lie flat on one of the cell strainers, then add a metal washer on the top to hold the tissue down.

NOTE: The slices need to be incubated at least for 1 h in the Tyrode's solution before processing (this is for the BDM to relax the tissue).

4. Culturing heart slices

4.1. Preparing the slices for culture

4.1.1. Trim the slice from the edges to avoid any uneven edges. Then, glue it from both ends to sterilized polyurethane 6 mm wide printer timing belt with metal wires embedded using tissue

glue.

4.1.2. Transfer the supported heart slices to 6 well plates that contain 6 mL of culture medium in each well.

4.1.3. Place the stimulation plate cover (**Table of Materials**) on the top of the 6 well plate and connect the cover to the cell culture electrical stimulator (**Table of Materials**). Adjust the stimulator to electrically stimulate the heart slices at 10 V, 1.2 Hz continuously all the time.

NOTE: After plugging in the stimulation, the heart slices will start to beat, and this movement will be visually obvious.

4.1.4. Transfer the plates to an incubator and maintain at 37 °C with humidified air and 5% CO_2.

4.2. Change culture medium 3x per day and add 6 mL of culture medium per well during each media change.

NOTE: Culture medium is oxygenated for 5 min prior to each media change. Medium must be changed at least 1x per day; this is okay on weekends if necessary. Two days of only 1 media change per day is the maximum that is tested.

4.3. Change the stimulation plate cover every day to prevent release of toxic graphite particles in the culture medium (usually during the mid-day media change).

4.3.1. Remove the stimulation plate cover from the culture dish and insert the white foam plug where the cover connects to the cable for the cell culture electrical stimulator, to prevent water damage to the electric circuit.

4.3.2. Place the plate cover in a bath of autoclaved water with 2x antibiotic-antimycotic. Keep it in this bath (i.e., rinsing bath) overnight.

4.3.3. The next day, move the plate cover into a 70% ethanol bath for 5−15 min to decontaminate. Shake off as much liquid from the device before switching baths.

4.3.4. Then, transfer the plate cover to the third and final bath (i.e., clean bath), which consists of autoclaved water and 2x antibacterial-antimycotic, to rinse any remaining ethanol traces.

4.3.5. After rinsing the plate cover in the clean bath, use a clean lint-free wipe (sprayed down with ethanol) to dry off any residual water on the plastic parts, then remove the white foam piece and carefully place the plate cover back on the culture plate.

NOTE: Do not touch the black graphite electrodes that go into the well, as the device will no longer be sterile.

4.3.6. Using sterile forceps, make sure the tissue is in the center of the well so the plate cover does not touch the tissue. Ensure that the curved side of the plate cover matches up with the angled side of the plate and that the square corners line up.

NOTE: To minimize contamination of the stimulation plate covers, keep them in clean and empty 6 well plates after finishing the experiment.

4.4. Perform an MTT assay, calcium measurements and contractile function assessments on fresh pig heart slices (day 0), and after 6 days in culture according to Ou et al.[13].

REPRESENTATIVE RESULTS:

Using a commercially available cell culture electrical stimulator that can accommodate eight 6 well plates at once, we emulated the adult cardiac milieu by inducing electrical stimulation at the physiological frequency (1.2 Hz), and screened for the fundamental medium components to prolong the duration of functional pig heart slices in culture[13]. Since pig and human hearts are similar in size and anatomy[15], we developed a biomimetic heart slice culture system using pig hearts and subsequently validated it in human heart slices. Here, we detailed the protocol for pig heart slices which is the same procedure for human heart slicing and culturing. The new biomimetic culture setup described here, maintained the viability of the pig heart slices for 6 days as assessed by MTT assay **(Figure 1A)**. We confirmed the functional viability of these slices over 6 days by assessing their calcium homeostasis and contractile force. In the first 6 days, there is no spontaneous calcium transients in cardiomyocytes, and the cardiomyocytes responded to external electrical stimulation as well as β-adrenergic stimulation similar to fresh heart slices **(Figure 1B)**. In addition, the contractile force and responses to isoproterenol are maintained in cultured heart slices for up to 6 days, similar to that of fresh heart slices **(Figure 1C,D)**.

FIGURE AND TABLE LEGENDS:

Figure 1: Validation of the viability and functionality of pig heart slice culture. (**A**) Quantification of heart slice viability over time using the MTT assay in either stimulated or unstimulated culture system in optimized medium (n = 5 pig hearts each in triplicate, SEM is represented as error bars; two-way ANOVA test was conducted to compare between groups, *$p < 0.05$). (**B**) Representative calcium signal trace from a fresh heart slice (day 0) and after 6 days in culture (day 6) with and without 1 µM isoproterenol (Iso) stimulation. Transients were recorded after loading the heart slices with Fluo-4 calcium dye and using 1 Hz/20 V electrical stimulation at the time of recording. Quantification of calcium signal amplitude with and without stimulation with isoproterenol shows a similar pattern of calcium transients at day 0 and day 6 in culture (n = 4 pig hearts, 10–15 cells analyzed in each, SEM is represented as error bars; two-way ANOVA test was conducted to compare between groups, *$p < 0.05$ comparing baseline to Iso stimulation at the same time point, p-value $_{D0\ vs\ D6} = 0.95$, and p-value $_{D0\ Iso\ vs\ D6\ Iso} = 0.93$). (**C**) Contractile force assessment setup (left panel); the heart slice (HS) is hung between a force transducer (FT) and a stable post (P) between two electric electrodes (EE) for electrical stimulation. Upon stimulation, the slice contracts and the contractions are recorded using the designated software (right panel). (**D**) Bar graph shows the quantification of the contractile force in the presence or absence of isoproterenol (Iso) generated by fresh pig heart slices (day 0) and after 6 days in biomimetic

culture (n = 4 pig hearts each in triplicate, SEM is represented as error bars; two-way ANOVA test was conducted to compare between groups, *p < 0.05 comparing baseline to Iso stimulation at the same time point, twitch force: p-value $_{D0\ vs\ D6}$ = 0.96, p-value $_{D0\ Iso\ vs\ D6\ Iso}$ = 0.81; time to 50% relaxation: p-value $_{D0\ vs\ D6}$ = 0.47, p-value $_{D0\ Iso\ vs\ D6\ Iso}$ = 0.43). This figure has been modified from Ou et al.[13].

Table 1: Comparison between the slicing and culturing conditions used in Fischer et al.[16] and Ou et al.[13].

DISCUSSION:

Here we describe the detailed video protocol for our recently published method for simplified medium throughput (processes up to 48 slices/device) method that enables culture of pig heart slices for a period sufficiently long to test acute cardiotoxicity[13]. The proposed conditions mimic the environment of the heart, including frequency of electrical stimulation, nutrient availability, and intermittent oxygenation. We attribute the prolonged viability of heart slices in our biomimetic stimulated culture to our focus on recreating the physiological conditions experienced by the intact heart. This concept is supported by our data showing that electrical stimulation alone, without providing essential nutrients, is not sufficient to maintain heart slice viability[13]. Therefore, the new components of the culture medium are the major driver for prolonging the heart slice viability and functionality in culture. We found that it is essential to include FBS in the medium to maintain the viability, which is likely due to the requirement for a variety of fatty acids, trace elements, enzymes, proteins, macromolecules, chemical components, and hormones, which are usually present in the serum and delivered to heart tissue in vivo[17]. Furthermore, we found that the addition of FGF and VEGF to the culture medium are needed to enhance the tissue viability. FGF and VEGF are known angiogenic factors which are essential for maintenance of cultured endothelial cells[18]. Therefore, it is likely that FGF and VEGF are needed to maintain the endothelial cells and connective tissues in culture to support tissue viability. Our work is the first to modify the simplified culture medium previously reported for culturing heart slices, and it opens the possibility for further optimization of the media components in subsequent studies.

In a timely manner with our new method, there were three other studies that have demonstrated the importance of continuous electromechanical stimulation in maintaining slices in culture in bioengineered systems[16,19,20]. However, these studies used the basal M199/ITS medium, which is not sufficient to maintain the metabolic needs of heart slices. This led to several compromises in the slice contractility and calcium homeostasis early during culture[16,19,20]. Qiao et al.[19] developed a highly sophisticated bioengineered chips, which can maintain the electrophysiological properties of the heart slices for 4 days, but no assessment of the contractile function was provided. Watson et al.[20] have bioengineered a low throughput culture system (processes up to 4 slices/device) to demonstrate the importance of diastolic sarcomere length for the maintenance of cardiac muscle properties for 24 h. Finally, Fischer et al.[16] have shown that failing human heart slices can be maintained in vitro for up to 4 months when cultured under 0.2 Hz stimulation, auxotonic loading and media agitation in a bioengineering device. However, these conditions induced a variety of changes in the heart slices, as reflected in their RNAseq

data, which showed over 10 fold downregulation of cardiac gene expression as early as the first time point of assessment (day 8)[16]. However, in our optimized culture system, only 2 and 5 transcripts were significantly differentially expressed after 2 and 6 days in culture compared to fresh heart tissue, respectively. However, after 10 days in culture, there were significant changes in the expression of over 500 transcripts. The downregulated genes after 10 days in culture were mostly related to cardiac muscle, while the upregulated genes are related to fibroblasts, extracellular matrix, and inflammation[13]. **Table 1** includes a full comparison of slicing and culture protocols between Fischer et al.[16] and Ou et al.[13]. Therefore, our biomimetic stimulated culture system emulates the controllable conditions experienced by the heart in situ and, therefore, should provide a reliable readout of the functional and the structural outcomes of drug treatment with regard to acute cardiotoxicity or efficacy compared to compromised culture systems.

One of the major limitations of this culture system is that although our culture system can emulate several physiological conditions, it cannot mimic changes in mechanical load and shear stress during the cardiac contractile cycle. Further optimization of physiological and metabolic factors could help prolong heart slice viability and function. Additionally, we noticed an early adaptation which leads to diminishment of oxidative phosphorylation which might instigate deterioration of the heart slice viability[13]. Unfortunately, so far, we were not able to identify an optimal method to maintain oxidative phosphorylation. It is likely that enhancing fatty acid metabolism in heart slices is not as simple as adding fatty acids to the growth medium, and will require further optimization, which could be addressed in future studies. Furthermore, a general weakness of this approach is the missing measurement of action potentials on single cardiomyocytes within the heart slice, which is essential to demonstrate disruption in electrocardiogram and ventricular arrhythmia. Therefore, there is a need to optimize protocols to isolate single cardiomyocytes from the heart slices after treatment with cardiotoxins that induce disruption in electrocardiogram and assess their effect on the action potential on the isolated cardiomyocytes.

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DISCLOSURES:

TMAM holds equity in Tenaya Therapeutics. The other authors report no conflicts.

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Figure 1

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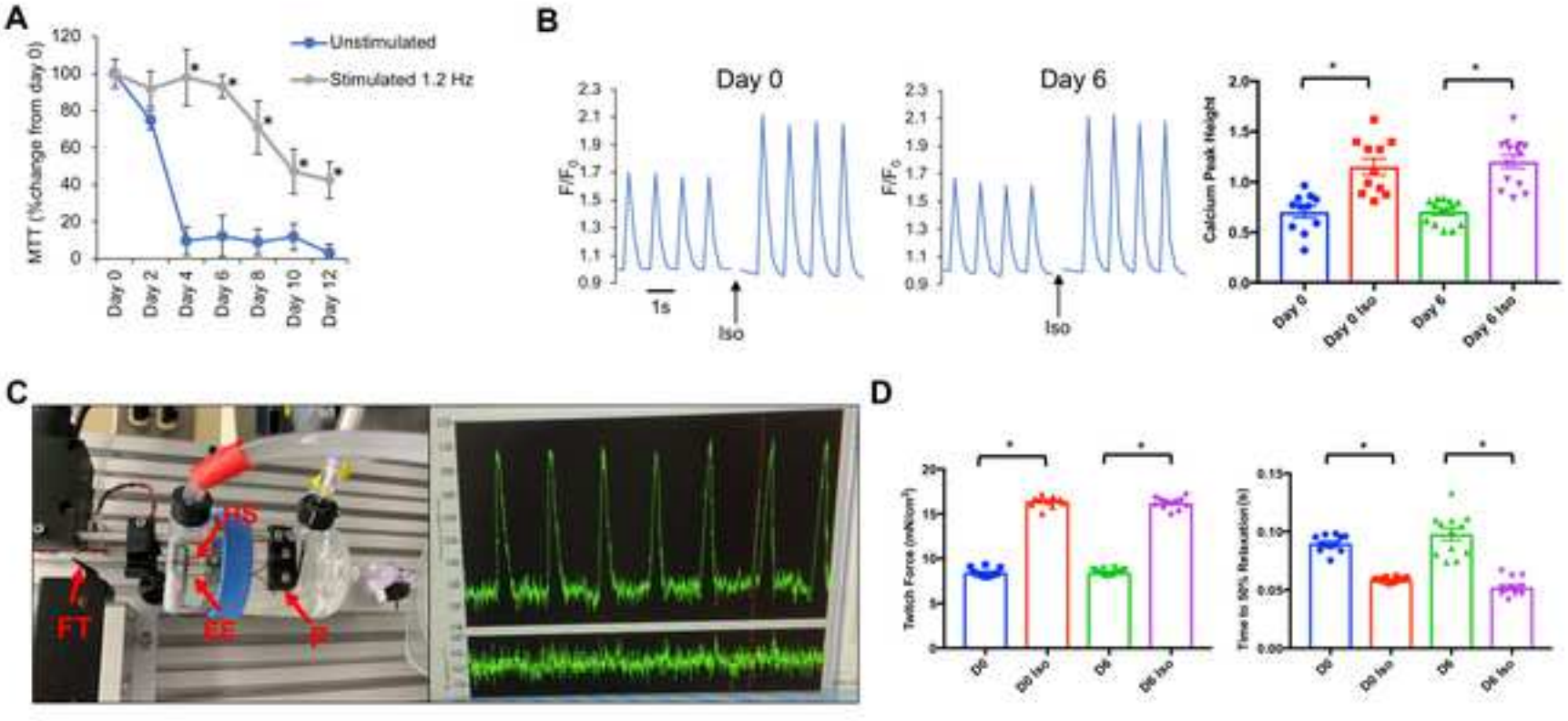

Condition
Species
Disease
Preservation
Slice thickness
Medium Oxygenation
Culture Medium
Agitation
Pacing rate
Duration of the culture
Cuture Temperature
Mechanical loading
Tested time points with no change in gene expression compared to fresh heart slice
First tested time point to show change in gene expression

Fischer et al.[16]
Human
Failing hearts
Preserved tissue
300 µm
No
M199/ITS
Yes
0.2 Hz
4 months with compromise
37
auxotonic loading

N/A

Day 8

Ou et al.[13]
Pig and human
Normal healthy hearts
Fresh tissue
300 μm
Intermittant oxygenation
M199/ITS/FBS/VEGF/FGF
No
1.2 Hz
6 days with no compromise
37
no loading

2 and 6 days

Day 10

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1000ml, 0.22µm, Vacuum Filter/Storage Systems	VWR	28199-812	
2,3-Butanedione monoxime (BDM)	Fisher	AC150375000	
500ml, 0.22µm, Vacuum Filter/Storage Systems	VWR	28199-788	
6-well C-Dish Cover (electrical-stimulation-plate-cover)	Ion Optix	CLD6WFC	
6-well plates	Fisher	08-772-1B	
Agarose	Bioline USA	BIO-41025	
Antibiotic-Antimycotic	Thermo	15-240-062	
C-Pace EM (cell-culture-electrical-stimulator)	Ion Optix	CEP100	
Calcium Chloride ($CaCl_2$)	Fisher	C79-500	
Ceramic Blades for Vibrating Microtome	Campden Instruments	7550-1-C	
Cooley Chest Retractor	Millennium Surgical	63-G5623	
D-Glucose	Fisher	D16-1	
Disposable Scalpel # 20	Biologyproducts.com	DS20X	
Falcon Cell Strainers, Sterile, Corning	VWR	21008-952	
Fetal Bovine Serum	Thermo	A3160502	
Graefe Forceps	Fisher	NC9475675	
Heparin sodium salt	Sigma-Aldrich	H3149-50KU	
HEPES	Fisher	BP310-1	

Histoacryl BLUE Tissue glue	Amazon		https://www.amazon.com/HISTOACRYL-FLEXIBLE-1051260P-Aesculap-Adhesive/dp/B074WB5185/ref=sr_1_2?ie=UTF8&qid=1522101102&sr=8-2&keywords=histoacryl
Iris Spring scissors	Fisher	NC9019530	
Iris Straight Scissors	Fisher	731210	
Isoflurane, USP	Piramal	NDC 66794-017-25	
ITS Liquid Media Supplement	Sigma-Aldrich	I3146-5ML	
Ketamine HCl (500 mg/10 mL)	West-Ward	NDC 0143-9508	
Magnesium Chloride ($MgCl_2$)	Fisher	M33-500	
Mayo SuperCut Surgical Scissors	AROSurgical Instruments Corporation	AROSuperCut™ 07.164.17	
Medium 199, Earle's Salts	Thermo	11-150-059	
Oxygen regulator	Praxair		
Oxygen tanks -	Praxair		
Plastic Pasteur pipettes	Fisher	13-711-48	
Potassium Chloride (KCl)	Fisher	AC193780010	
Printer Timing Belt	Amazon		https://www.amazon.com/Uxcell-a14081200ux0042-PRINTER-Precision-Timing/dp/B00R1J3KDC
Razor rectangle blades	Fisher	12-640	
Recombinant Human FGF basic	R&D Systems	233-FB-025/CF	
Recombinant Human VEGF	R&D Systems	293-VE-010/CF	
Retractable scalpels	Fisher	22-079-716	
Sodium Bicarbonate ($NaHCO_3$)	Fisher	AC217125000	
Sodium Chloride (NaCl)	Fisher	AC327300010	
Vibrating Microtome	Campden Instruments	7000 SMZ-2	

Xylazine HCl (100 mg/mL)	Heartland Veterinary Supply	NADA 139-236	

We would like to thank the editor for his constructive comments regarding our manuscript which highly improved the manuscript. In this revised version, we addressed all editorial comments. We detailed our responses below:

Editorial comments:

1. Please thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response:

We run the text by 2 native English speakers to ensure there is no spelling or grammar issues.

2. Please note that the editor has formatted the manuscript to match the journal's style and made minor changes to the protocol (merging some relevant steps, combining shorter steps, etc.). Please review the protocol for accuracy. The updated manuscript is attached and please use this version to incorporate the changes that are requested.

Response:

We thank the editor for his careful review of our manuscript and we fully agree with all the changes. There were some edits elsewhere which are tracked.

3. Please address specific comments marked in the attached manuscript. Please turn on Track Changes to keep track of the changes you make to the manuscript.

Response:

We responded to all the specific comments marked in the formatted manuscript.

4. Once formatted please ensure that the highlighted content is no more than 3 pages long including heading and spacings. Please do not highlight any steps describing anesthetization.

Response:

Due to the size limitation to 3 pages, we had to exclude section 2 “Pig heart perfusion” from the highlighted video protocol.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please replace "C-dish" and "C-pace" with generic terms throughout the manuscript.

Response:

We changed "C-dish" to “electrical-stimulation-plate-cover” and "C-pace" to “cell-culture-electrical-stimulator”. In addition, we updated the table of materials accordingly.

6. As a co-author is affiliated with UK institutions, please print and sign the attached Author License Agreement - UK. Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account. Please also check whether open access is required by your funding agencies.

Response:

We filled and signed the Author License Agreement – UK. Open access is not required as we don’t receive funding from the UK.

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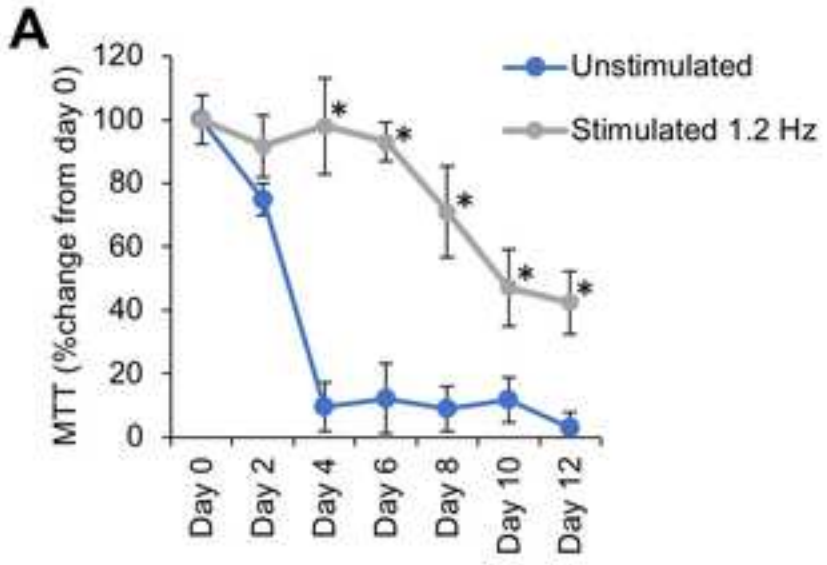

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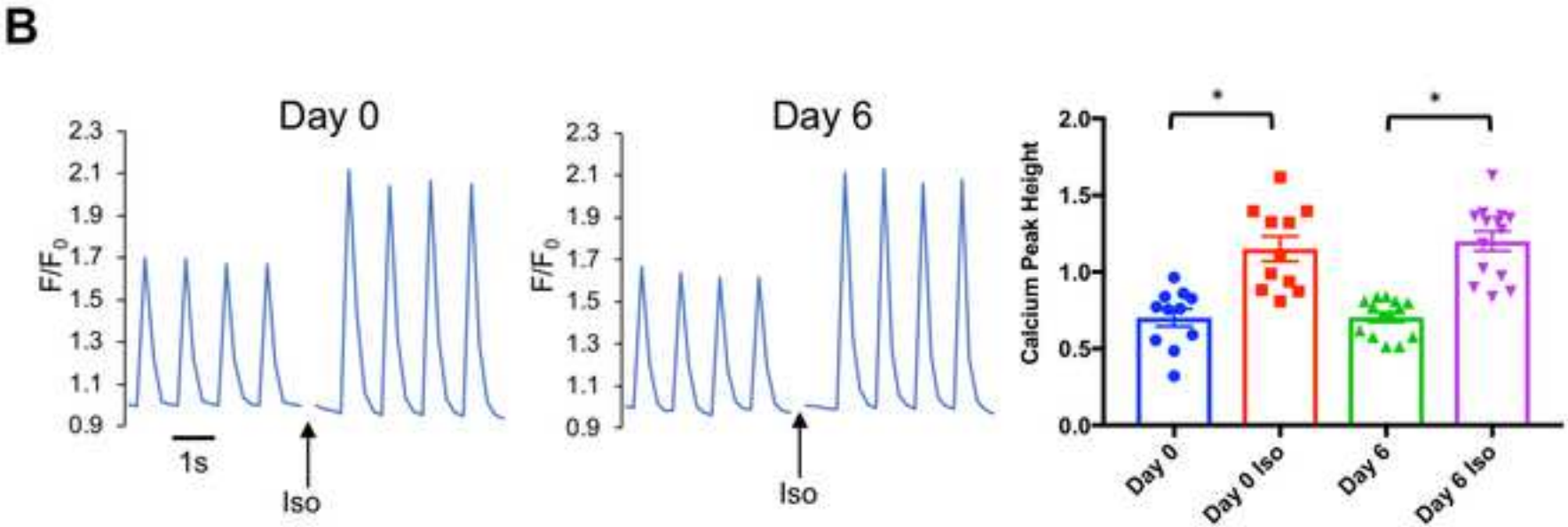

Figure 1C

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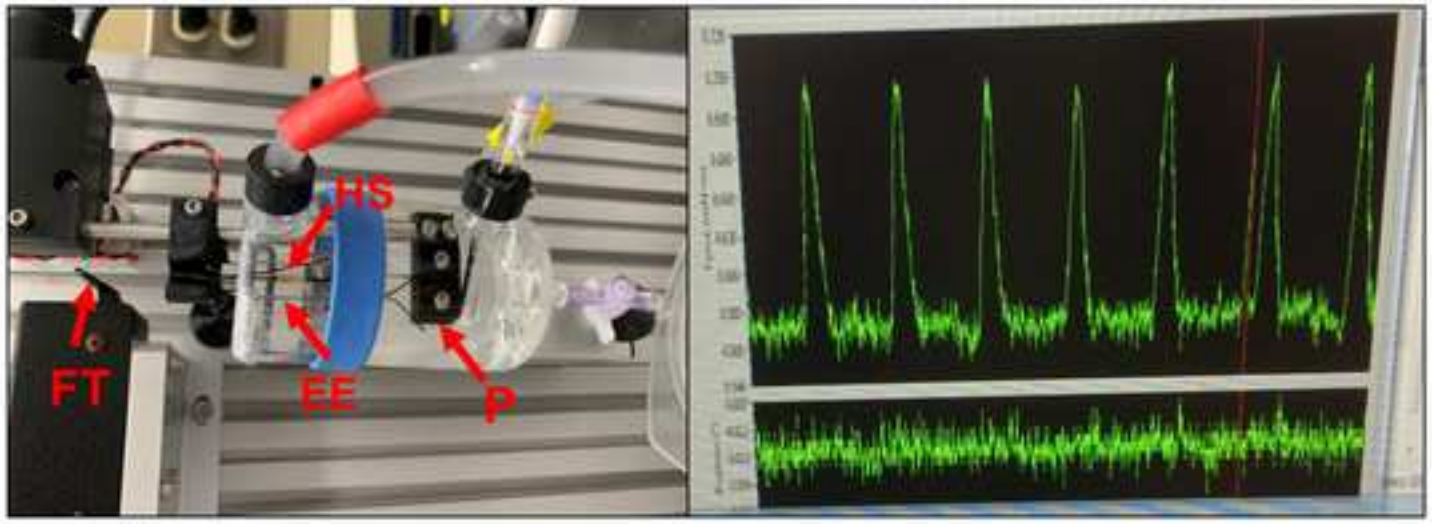
C



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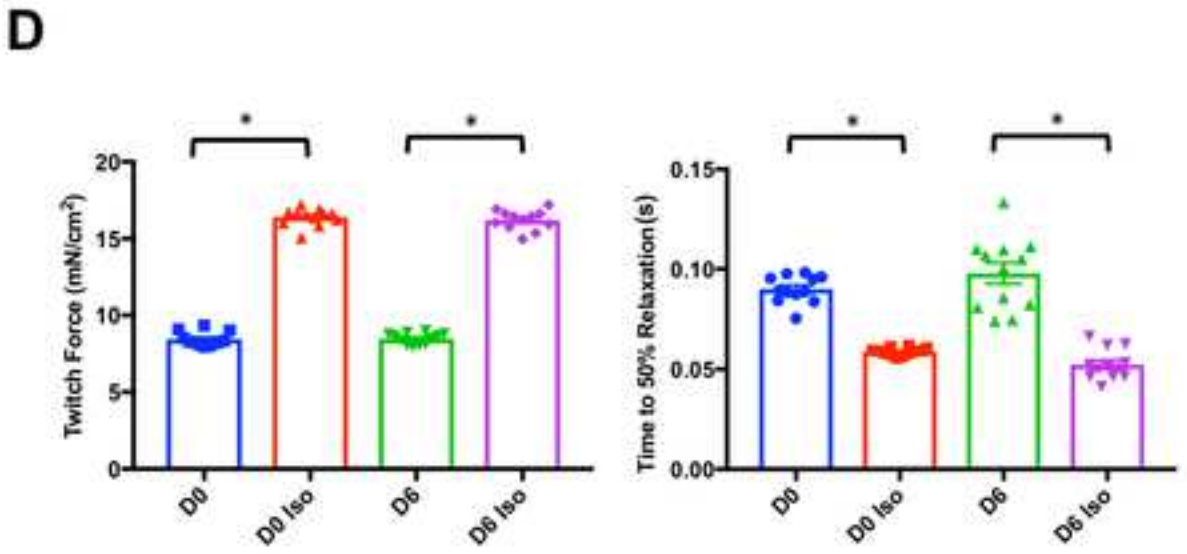

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