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Title: In vitro assessment of myocardial protection following hypothermia-preconditioning in a human cardiac myocytes model

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interview Statements are read by JoVE's voiceover talent.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 21

Number of Shots: 44

Introduction

1. Introductory Interview Statements

V*ideographer: All interview statements will be read by VO, please skip to protocol.*

1.1. The goal of the present study was to quantify the levels of cell death following different hypothermia treatments in a human cardiomyocyte-based model.

1.1.1. *2.6.1.*

1.2. This model can be used to study the molecular mechanism of hypothermic cardioprotection, which may have important implications for the development of complementary therapies for use with hypothermia.

1.2.1. *5.3.1.*

Protocol

2. Establishment of an oxygen-glucose deprivation model and time -temperature protocol.

2.1. To culture the HCM cells, thaw the cryopreserved cells from liquid nitrogen in a 37-degree Celsius water bath **[1-TXT]** and maintain them in a humidifier incubator under an atmosphere with 95% air and 5% carbon dioxide **[2]**.

2.1.1. Talent thawing the HCM cells on water bath. **TEXT: HCM-Human cardiomyocyte**

2.1.2. Talent putting the cells in the humidifier incubator

2.2. Once the cells reach 60 to 70% confluence, aspirate the medium from the 6-well plate **[1]** and gently wash the cells three times with PBS **[2]**.

2.2.1. Talent aspirating the cell medium.

2.2.2. Talent washing the cells with PBS.

2.3. Add 2 milliliters of fresh sugar-free medium into each well **[1]** and culture the cells at a constant temperature in a three-gas incubator under a mixture of 95% nitrogen, 5% carbon dioxide, and 0.1% oxygen at 37 degrees Celsius **[2]**.

2.3.1. Talent sugar-free medium to the wells.

2.3.2. Talent putting the cells in the incubator.

2.4. For the time-temperature protocol, aspirate the medium from the 6-well plate **[1]** and add 2 milliliters of fresh sugar-free medium **[2]**. Culture the cells in a tri-gas incubator for 12 hours to establish hypoxia **[3-TXT]**. *Videographer: This step is important!*

2.4.1. Talent aspirating the old medium.

2.4.2. Talent adding the fresh medium.

2.4.3. Talent culturing the cells in the incubator. **TEXT: 95% N_2, 5% CO_2, and 0.1% O_2 at 37 °C.** NOTE: The oxygen concentration in the video is for demonstration only.

2.5. Set the temperature program starting with 10 hours of low-temperature treatment, followed by a rewarming phase for 2 hours, and 24 hours of normothermia **[1]**. Remove three Petri dishes at every time point for analysis **[2]**. *Videographer: This step is important!*

2.5.1. Talent setting the temperature program of the incubator. *Videographer: Obtain multiple usable takes of this shot, it will be reused in the conclusion.*

2.5.2. Talent removing the Petri dishes.

2.6. After the low-temperature treatment, aspirate the medium from the 6-well plate **[1]** and wash three times with PBS **[2]**.

2.6.1. Talent aspirating the medium from the plate.

2.6.2. Talent washing the plate with PBS.

2.7. Add 2 milliliters of fresh medium to each well **[1]**, then maintain the cells at 37 degrees Celsius in a humidified incubator under an atmosphere with 95% air and 5% carbon dioxide **[2]**.

2.7.1. Talent adding fresh medium in the well.

2.7.2. Talent keeping the cells in the incubator.

3. CCK-8 viability assay and analysis of flow cytometry for apoptosis

3.1. Add 8 microliters of CCK-8 solution to each well of the plate **[1-TXT]** and leave the plate for 1 hour in the incubator **[2]**.

3.1.1. Talent adding CCK-8 solution in the well. **TEXT: CCK-8 – Cell counting kit 8**

3.1.2. Talent keeping the plate in the incubator.

3.2. After the incubation, measure the absorbance at 450 nanometers using a microplate reader **[1]**.

3.2.1. Talent taking the reading of the plate on the microplate reader machine.

3.3. For apoptosis analysis, add 5 microliters of Annexin V-FITC to dye the cells **[1]**. Then, add 10 microliters of propidium iodide to the cell suspension **[2]**. Gently mix the cells **[3]** and incubate them for 20 minutes at room temperature in the dark **[4]**.
Videographer: This step is difficult!

3.3.1. Talent adding Annexin V-FITC to the cells.

3.3.2. Talent adding propidium iodide in the cell suspension.

3.3.3. Talent mixing the cells in the plate.

3.3.4. Talent incubating the plates in dark.

3.4. In the flow cytometry software, open two dot plot windows and select **forward scattered light** on the X-axis and **side scattered light** on the Y-axis **[1]**.

3.4.1. SCREEN: 5.10-5.12.mkv

3.5. Select the **PE** detection channel and **FITC**. Click **Record** to collect particles from the suspension in the blank sample tube, then gate the cell population for further analysis in the first dot plot **[1]**.

3.5.1. SCREEN: 5.14-5.15.mkv

3.6. Next, place the single-stained samples on the tube support arm **[1]**. Click **Record** to collect particles form the suspension and gate the cell population for further analysis **[2]**.

3.6.1. Talent placing the samples on the tube support arm

3.6.2. SCREEN: 6.9.mkv

4. Mitochondrial depolarization assessment and reactive oxygen species assay

4.1. For mitochondrial assessment, add 0.5 milliliters of JC-1 working solution to each tube containing the trypsinized cells **[1]** and incubate them in a 37-degree Celsius incubator for 20 minutes **[2]**.

4.1.1. Talent adding JC-1 solution in the tube.

4.1.2. Talent incubating the tube in the incubator.

4.2. After incubation, centrifuge the cells at 600 times *g* for 3 minutes at 4 degrees Celsius **[1]** and resuspend the HCMs in 1 milliliter of ice-cold staining buffer in a 1.5-milliliter centrifuge tube **[2-TXT]**. Measure the fluorescence intensity of JC-1 by selecting the PE and FITC detection channels **[3]**.

4.2.1. Talent centrifuging the cells.

4.2.2. Talent adding the obtained HCMs in staining buffer. **TEXT: Use cells within 30 minutes**

4.2.3. Talent measuring the fluorescence intensity.

4.3. For the reactive oxygen species assay, stain the cells in culture medium with 10 micromolar DCFDA and adjust the cell density to around 1 to 10 million cells **[1]**.

4.3.1. Talent adding DCFDA stain to the cells.

~~4.3.2. Talent adjusting the cell density~~

4.4. Incubate these cells for 30 minutes at 37 degrees Celsius **[1]**, then analyze them on a flow cytometer **[2]**.

4.4.1. Talent incubating the cells in incubator.

4.4.2. Talent analyzing the cells in flow cytometer machine.

5. Measurement of Caspase 3/ Caspase 8 activity

5.1. Add 40 microliters of buffer solution to the enzyme-labelled plate **[1]**, then add 80 microliters of the sample **[2]**.

5.1.1. Talent adding buffer solution to enzyme plate

5.1.2. Talent adding the cell sample.

5.2. Then, add 10 microliters of 2 millimolar Ac-DEVD-pNA *(pronounce acetyl-D-E-V-D-p-nitroanilide)* **[1]** and incubate the plate at 37-degrees Celsius for 120 minutes **[2]**.

5.2.1. Talent adding Ac-DEVD-pNA

5.2.2. Talent incubating the plate in incubator.

5.3. Measure the A405 value on a microplate reader machine using the manufacturer's instructions **[1]**.

5.3.1. Talent using the plate reader.

Results

6. Evaluation of cell viability, apoptosis, mitochondrial function, and caspase activity

6.1. The temperature conditions in the time-temperature protocol were created and maintained using a tri-gas incubator **[1]**, which allows for precise temperature regulation at three time points **[2]**.

6.1.1. LAB MEDIA: Figure 1.

6.1.2. LAB MEDIA: Figure 1. *Video Editor: Emphasize T1, T2, and T3 on the plot.*

6.2. The effect of oxygen and glucose deprivation, or OGD, on the viability of HCMs was determined using the CCK-8 assay **[1]**. Compared with the control group, cell viability was significantly decreased in a time-dependent manner **[2]**.

6.2.1. LAB MEDIA: Figure 2A.

6.2.2. LAB MEDIA: Figure 2A. *Video Editor: Emphasize the OGD bars.*

6.3. The apoptosis rates of HCMs gradually increased between 0 and 16 hours after reperfusion **[1]**, then reached the maximum rate at the 16-hour time point **[2]**.

6.3.1. LAB MEDIA: Figure 2B. *Video Editor: Emphasize the 0 – 16h points data.*

6.3.2. LAB MEIDA: Figure 2B. *Video Editor: Emphasize the 16h data points.*

6.4. Compared with the OGD group, the percentage of viable cells was higher in the three groups treated with hypothermia **[1]**. In addition, cells in the deep hypothermia group had the highest viability, 2.1-fold higher than that observed in the OGD group **[2]**.

6.4.1. LAB MEDIA: Figure 3B

6.4.2. LAB MEDIA: Figure 3B. *Video Editor: Emphasize the deep hypothermia bar.*

6.5. Flow cytometry results confirmed that hypothermia prevented the apoptosis of HCMs under OGD conditions **[1]**.

6.5.1. LAB MEDIA: Figure 3C

6.6. Hypothermia treatment also decreased the intracellular ROS levels in HCMs **[1]**. Mitochondrial membrane potential was detected with JC-1 staining **[2]**. After OGD treatment, the red to green fluorescence ratio was decreased **[3]**. Hypothermia significantly inhibited this OGD-induced effect **[4]**.

6.6.1. LAB MEDIA: Figure 4A.

6.6.2. LAB MEDIA: Figure 4B.

6.6.3. LAB MEDIA: Figure 4B. *Video Editor: Emphasize the OGD bar.*

6.6.4. LAB MEDIA: Figure 4B. *Video Editor: Emphasize the hypothermia bars.*

6.7. Moreover, a decrease of caspase-8 and caspase-3 was observed in the cells that were treated with hypothermia **[1]**.

6.7.1. LAB MEDIA: Figure 4C and D.

Conclusion

7. Conclusion Interview Statements

7.1. The reliability of this model depends on strict temperature control, controllable experimental procedures, and stable experimental results.

7.1.1. *2.5.1.*

7.2. This method can be used to further explore the specific mechanism of hypothermic treatment on cell apoptosis and to study the molecular mechanism of hypothermic cardioprotection. It is of great significance for the development of hypothermic adjuvant therapy **[1]**.

7.2.1. *4.4.2 or LAB MEDIA: Figure 3.*