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TITLE:

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

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Cardiac surgery, Myocardial protection, Hypothermia, Apoptosis, Mild Hypothermia, Moderate Hypothermia, Deep Hypothermia

SUMMARY:

The distinct effects of different degrees of hypothermia on myocardial protection have not been thoroughly evaluated. The goal of the present study was to quantify the levels of cell death following different hypothermia treatments in a human cardiomyocyte-based model, laying the foundation for future in-depth molecular research.

ABSTRACT:

Ischemia/reperfusion-derived myocardial dysfunction is a common clinical scenario in patients after cardiac surgery. In particular, the sensitivity of cardiomyocytes to ischemic injury is higher than that of other cell populations. At present, hypothermia affords considerable protection against an expected ischemic insult. However, investigations into complex hypothermia-induced molecular changes remain limited. Therefore, it is essential to identify a culture condition similar to in vivo conditions that can induce damage similar to that observed in the clinical condition in a reproducible manner. To mimic ischemia-like conditions in vitro, the cells in these models were treated by oxygen/glucose deprivation (OGD). In addition, we applied a standard time-temperature protocol used during cardiac surgery. Furthermore, we propose an

approach to use a simple but comprehensive method for the quantitative analysis of myocardial injury. Apoptosis and expression levels of apoptosis-associated proteins were assessed by flow cytometry and using an ELISA kit. In this model, we tested a hypothesis regarding the effects of different temperature conditions on cardiomyocyte apoptosis in vitro. The reliability of this model depends on strict temperature control, controllable experimental procedures, and stable experimental results. Additionally, 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.

INTRODUCTION:

Ischemia/reperfusion-derived myocardial dysfunction is a common clinical scenario in patients after cardiac surgery[1,2]. During nonpulsatile low flow perfusion and periods of total circulatory arrest, damage involving all types of heart cells still occurs. In particular, the sensitivity of cardiomyocytes to ischemic injury is higher than that of other cell populations. At present, therapeutic hypothermia (TH) affords substantial protection against an expected ischemic insult in patients undergoing cardiac surgery[3,4]. TH is defined as a core body temperature of 14-34 °C, although no consensus exists regarding a definition of cooling during cardiac surgery[5-7]. In 2013, an international panel of experts proposed a standardized reporting system to classify various temperature ranges of systemic hypothermic circulatory arrest[8]. Based on electroencephalography and metabolism studies of the brain, they divided hypothermia into four levels: profound hypothermia (≤ 14 °C), deep hypothermia (14.1-20 °C), moderate hypothermia (20.1-28 °C), and mild hypothermia (28.1-34 °C). The expert consensus provided a clear and uniform classification, allowing studies to be more comparable and provide more clinically relevant outcomes. This protection afforded by TH is based on its capacity to reduce the metabolic activity of cells, further limiting their rate of high-energy phosphates consumption[9,10]. However, the role of TH in myocardial protection is controversial and may have multiple effects depending on the degree of hypothermia.

Myocardial I/R is well known to be accompanied by increased cell apoptisis[11]. Recent reports have observed that programmed cardiomyocyte death increases during open-heart surgery, and may coincide with necrosis, thereby increasing the number of dead myocardial cells[12]. Therefore, reducing cardiomyocyte apoptosis is a useful therapeutic approach in clinical practice. In the mouse atrial HL-1 cardiomyocyte model, therapeutic hypothermia was shown to reduce the mitochondrial release of cytochrome c and apoptosis-inducing factor (AIF) during reperfusion[13]. However, the effect of temperature in regulating apoptosis is controversial and appears to depend on the degree of hypothermia. Cooper and colleagues observed that compared to a normothermic cardiopulmonary bypass control group, the apoptosis rate of myocardial tissue from pigs with the deep hypothermic circulatory arrest was increased[14]. In addition, the results of some studies have suggested that deep hypothermia may activate the apoptosis pathway, while less aggressive hypothermia appears to inhibit the pathway[12,15,16]. The reason for this result may be due to confounding effects associated with ischemic injury and a lack of understanding of the mechanisms by which temperature affects myocardial tissue. Therefore, the temperature limits at which apoptosis is enhanced or attenuated should be accurately defined.

To gain a better understanding of the mechanisms associated with the efficacy of hypothermia and provide a rational basis for its implementation in humans, it is essential to identify a culture condition similar to in vivo conditions that can produce damage similar to that observed for the clinical condition in a reproducible manner. An essential step towards achieving this goal is to establish the optimal conditions for inducing cardiomyocyte apoptosis. Accordingly, in the present study, we explored the methodological details regarding oxygen-glucose deprivation experiments with cultured cells, a facile in vitro model of ischemia-reperfusion. Furthermore, we evaluated the effect of different hypoxic-ischemic times on cardiomyocyte apoptosis, and verified our hypothesis regarding the effect of different temperature conditions on cell apoptosis in vitro.

PROTOCOL:

Information regarding commercial reagents and instruments are listed in the **Table of Materials**. The AC16 human cardiomyocyte cell line was derived from the fusion of primary cells from adult ventricular heart tissue with SV40-transformed human fibroblasts[17], which were purchased from BLUEFBIO (Shanghai, China). The cell line develops many biochemical and morphological features characteristic of cardiomyocytes. In addition, the cell line is widely used to evaluate myocardial damage and myocardial function in vitro[18,19].

1. Cell culture

NOTE: The basal culture medium consists of serum-free Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 1% cardiac myocyte growth supplement, and 1% penicillin/streptomycin solution. Store the medium at 4 °C and prewarm to 37 °C before use.

1.1. Remove the cryopreserved human cardiomyocyte (HCM) cells from liquid nitrogen and thaw them in a water bath at 37 °C.

1.2. Gently shake the vial (<1 minute) in a 37 °C water bath until only a small piece of ice remains in the vial.

1.3. Transfer the vial into a sterile laminar flow hood. Wipe the outside of the vial with a cotton ball dipped in 70% alcohol.

1.4. Transfer 4 mL of prewarmed complete growth medium dropwise into the centrifuge tube containing the thawed cells.

1.5. Centrifuge the cell suspension at 200 × *g* for 10 minutes. After centrifugation, discard the supernatant and resuspend the pellet in 5 mL of complete medium.

1.6. Maintain the cells at 37 °C in a humidified incubator under an atmosphere with 95% air and 5% CO_2.

NOTE: Before harvesting the cells for experiments, the cells are allowed to grow until reaching approximately 60-70% confluency.

2. Establishment of an oxygen-glucose deprivation (OGD) model

NOTE: Two hours before the study period, replace the growth medium with serum-free medium, and the cells were reincubated in a humidified incubator for 2 h at 37 °C under an atmosphere with 5% CO_2.

2.1. Aspirate the medium from a 6-well plate and gently wash the cells three times with phosphate buffered saline (PBS).

2.2. Add 2 mL of fresh sugar-free DMEM per well.

2.3. Culture the cells at a constant temperature in a three-gas incubator under an atmosphere with a mixture of 95% N_2, 5% CO_2, and 0.1% O_2 at 37 °C for 1, 2, 4, 8, 12, or 16 h.

2.4. After the hypoxia treatment, aspirate the medium from the 6-well plate and wash the cells with PBS (pH 7.4) three times.

2.5. Add 2 mL of complete DMEM per well.

2.6. Maintain the cells at 37 °C in a humidified incubator under an atmosphere with 95% air and 5% CO_2.

3. Time-temperature protocol

NOTE: A standard time-temperature protocol is used during cardiac surgery, as described previously by others[20,21]. Treat HCMs according to the following protocol (**Figure 1**): timepoint 1 (T1) indicates the end of induction, timepoint 2 (T2) indicates the end of maintenance and timepoint 3 (T3) indicates the end of rewarming. Analyze control cells maintained under continuous normothermic conditions (37 °C). The temperature conditions are created using a tri-gas incubator, which allows precise temperature regulation.

3.1. Two hours before the experiments, aspirate the culture medium from a 6-well plate and add 2 mL of fresh serum-free DMEM per well.

3.2. Re-incubate the cells in a humidified incubator for 2 h at 37 °C under an atmosphere with 5% CO_2.

3.3. After 2 h, aspirate the medium from the 6-well plate and wash the cells with PBS (pH 7.4) three times.

3.4. Add 2 mL of fresh serum-free DMEM per well.

3.5. Reincubate the cells in the tri-gas incubator.

NOTE: Replace the medium to remove unattached cells and debris.

3.6. Immediately change the temperature by placing the culture dishes a tri-gas incubator.

3.7. After 1 h of cooling, quickly aspirate the medium from the 6-well plate and add 2 mL of fresh sugar-free DMEM per well.

3.8. Culture the cells in a tri-gas incubator under an atmosphere comprising 95% N_2, 5% CO_2, and 0.1% O_2 at 37 °C for 12 h to establish hypoxia.

3.9. Set the temperature as described below.

NOTE: The protocol starts with 10 h of a low-temperature treatment, followed by a rewarming phase for 2 h up to 37 °C, and 24 h of normothermia (37 °C). At every time point, three Petri dishes are removed for analysis.

3.10. After low-temperature treatment, aspirate the medium from the 6-well plate and wash with PBS (pH 7.4) three times.

3.11. Add 2 mL of complete DMEM per well.

3.12. Maintain the cells at 37 °C in a humidified incubator under an atmosphere with 95% air and 5% CO_2.

4. CCK-8 viability assay

4.1. Warm the 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution and PBS to 37 °C prior to use.

4.2. Aspirate the medium, rinse the cells with 1 mL of PBS and then add 0.5 mL of 0.25% trypsin along the wall of the well. Incubate at 37 °C until almost all HCMs are detached (approximately 1 min).

4.3. Add 1 mL of DMEM complete medium to the wells to neutralize the trypsin.

4.4. Transfer the cell suspension to a 15 mL centrifuge tube and pellet the HCMs by centrifugation at 500 × g for 3 min. Aspirate the supernatant without disturbing the pellet.

4.5. Dispense 100 µL aliquots of the cell suspension (5000 cells/well) into a 96-well plate. Preincubate the plate for 24 h in a humidified incubator (at 37 °C.)

4.6. Use the cultured cells in the 96-well plates to generate different treatment groups.

4.7. Incubate the plate for an appropriate length of time (16 h) in an incubator.

4.8. Add 10 µL of CCK-8 solution to each well of the plate.

4.9. Incubate the plate for 1 hour in the incubator.

4.10. Measure the absorbance at 450 nm using a microplate reader.

CAUTION: Be careful not to introduce bubbles to the wells, as they interfere with OD reading measurements.

5. Flow cytometry for apoptosis analysis

5.1. Perform trypsinization and centrifugation steps by following steps 4.2-4.4.

NOTE: Cells are harvested with trypsin without EDTA. To assess apoptosis, both floating and adherent cells are collected.

5.2. Centrifuge the cells for 5 min at 1000 × g. Discard the supernatant and resuspend the pellet in 1 mL of PBS.

5.3. Count the cells using a hemocytometer. Using a pipette, transfer 100 µL of Trypan Blue-treated cell suspension to a hemocytometer. Using a hand tally counter, count the live, unstained cells in one set of 16 squares and then use PBS to generate a cell suspension at 1×10^7 cells/ml.

5.4. Obtain 200 µL of the cell suspension (5×10^5 -1×10^6 cells).

5.5. Centrifuge for 5 min at 1000 × g and resuspend the pellet in Annexin V-FITC binding solution.

5.6. Add 5 µL of Annexin V-FITC to dye the cells.

5.7. Add 10 µL of propidium iodide into the cell suspension.

5.8. Gently mix the cells and incubate them for 20 minutes at room temperature in the dark.

NOTE: The cells are resuspended 2-3 times during the incubation to improve staining.

5.9. Start the flow cytometer and make sure the software is working appropriately.

5.10. Open two dot plot windows in the flow cytometry software.

5.11. Select forward scattered light (FSC) on the X axis and side scattered light (SSC) on the Y axis to exclude cell debris and/or clumps in terms of their size and granularity, respectively.

5.12. Select the PE (590 mm) detection channel and FITC (530 mm), which is used to measure the fluorescence intensity of the cells.

5.13. Place the blank (HCMs that have not been dyed) sample tube on the flow cytometer.

5.14. Click **Record** to collect particles from the suspension in the blank sample tube and then gate the cell population for further analysis in the first dot plot.

5.15. Place the single-stained samples on the tube support arm. Click **Record** to collect particles from the suspension and then gate the cell population for further analysis in the first dot plot.

5.16. Collect the HCMs in other sample tubes and optimize the measurement by adjusting the voltages of the fluorescence channels.

NOTE: Unstained and single-stained samples are used as compensation controls during the experiment.

5.17. Display the statistics of each sample tube and calculate the rate of apoptosis of each sample.

6. Mitochondrial depolarization assessment

6.1. Perform trypsinization and centrifugation by following steps 4.2-4.4.

6.2. Resuspend the cells in 500 µL of complete medium, and then adjust the cell density adjusted to 1×10^5 - 6×10^6 cells

6.3. Add 0.5 mL of JC-1 working solution to each tube.

6.4. Incubate the cells in a cell incubator at 37 °C for 20 minutes.

NOTE: During the incubation period, add 1 mL of 5× JC-1 staining buffer to 4 mL of distilled water to prepare the JC-1 staining buffer.

6.5. After incubation, centrifuge the cells at 600 × g for 3 min at 4 °C.

6.6. Discard the supernatant and resuspend the pellet in 1 mL of JC-1 staining buffer.

6.7. Repeat steps 6.5 to 6.6 three times.

6.8. Resuspend the HCMs in 1 mL of ice-cold staining buffer in a 1.5 mL centrifuge tube and use the cells for analysis within 30 min.

6.9. Select the PE (590 nm) detection channel and FITC (530 nm) to measure the fluorescence intensity of JC-1 dye in the cells.

NOTE: Set up the flow cytometer by following steps 5.10-5.17.

7. Reactive oxygen species assay

7.1. Perform trypsinization and centrifugation by following steps 4.2-4.4.

7.2. Stain the cells in culture medium with 10 µM DCFDA and adjust the cell density to 1×10^6 - 1×10^7 cells

7.3. Incubate for 30 minutes at 37 °C.

7.4. Gently pipette the cells up/down every 3-5 min.

7.5. After the incubation, wash the cells three times with serum-free cell culture medium.

7.6. Analyze the cells on a flow cytometer by following steps 5.10-5.17.

NOTE: DCF is excited at 488 nm and the emission intensity measured at 530 nm.

8. Measurement of Caspase 3/ Caspase 8 Activity

8.1. Perform trypsinization by following steps 4.2-4.4.

8.2. Collect the cells by centrifugation at 600 × g at 4 °C for 5 minutes.

8.3. Add 100 µL of lysate buffer per 2×10^6 cells.

8.4. Lyse the cells for 15 minutes on ice.

8.5. After incubating for 15 min in an ice bath, centrifuge the sample at 1.6×10^4 x g at 4 °C for 15 minutes.

8.6. Transfer the supernatant to an ice-cold centrifuge tube for use.

NOTE: The protein concentration in the sample should be at least 1-3 mg/mL.

8.7. Remove Ac-DEVD-pNA (2 mM) and place it on an ice bath for use.

8.8. Accurately add 40 µL of buffer solution to the enzyme-labeled plate, add 80 µL of the sample, and finally add 10 µL of Ac-DEVD-pNA (2 mM).

8.9. Incubate the sample at 37 °C for 120 minutes.

8.10. Measured the A405 value on a microplate reader according to the manufacturer's instructions.

NOTE: The absorbance produced by the pNA generated by caspase-3/caspase-8 is calculated by subtracting the A405 value of the blank control from that of the sample.

REPRESENTATIVE RESULTS:

The effect of OGD exposure on the viability of HCMs was determined by CCK-8 assay. Compared with that observed in the control group, cell viability was significantly decreased in a time-dependent manner (**Figure 2A**). The apoptosis rates of HCMs at different times after reperfusion showed a specific trend, where from 0 to 16 h, the apoptosis rates gradually increased and reached the maximum rate at the 16 h time point (**Figure 2B**). As OGD for 12 h reduced cell activity by ~50%, 12 h OGD was used to induce cell damage in subsequent experiments.

We subsequently examined the effect of temperature on the process of apoptosis. Compared with that observed in the OGD group, the percentage of viable cells was higher in the three groups treated with hypothermia (**Figure 3B**). In addition, cells in the deep hypothermia group had the highest viability (>92%), 2.1-fold higher than that observed in the OGD group. In addition, the flow cytometry results showed that hypothermia prevented the apoptosis of HCMs under OGD conditions (**Figure 3A&3C**). Because mitochondrial dysfunction is associated with apoptosis, we obtained additional data to assess mitochondrial disorders. The intracellular ROS levels were determined using a DCFH-DA assay. Compared with that observed in the OGD group, the hypothermia treatment decreased the intracellular ROS levels in HCMs (**Figure 4A**). In addition, mitochondrial membrane potential was detected with JC-1 staining. Following OGD treatment, the red fluorescence of JC-1 was significantly reduced, and the green fluorescence was significantly increased. In contrast, Hypothermia treatment significantly inhibited the OGD-induced effect and increased the red to the green ratio by a large margin (**Figure 4B**). Moreover, the decrease of caspase 3/caspase-8 was also observed in the cells that were treated with hypothermia treatments (**Figure 4C,4D**)

FIGURES AND TABLES:

FIGURE AND TABLE LEGENDS:

Figure 1: Flow chart of the experimental procedure. HCMs were treated according to the following protocol: time point 1 (T1) indicates the end of induction (cooling for 2h); time point 2 (T2) indicates the end of maintenance (hypothermia for 10h at the desired temperature); and

time point 3 (T3) indicates the end of rewarming (rewarming for two h up to 37 °C). The temperature was maintained at the desired temperature: 34 °C for mild hypothermia, 31°C for moderate hypothermia, and 17 °C for severe hypothermia. Control cells maintained under continuous normothermic conditions (37 °C) were analyzed. The temperature conditions were created using a tri-gas incubator, which allows for precise temperature regulation.

Figure 2: Evaluation of cell viability and apoptosis by the CCK-8 and Annexin V/PI assays.
(A) Cell viability was measured by using a cell viability assay.
(B) Apoptosis was analyzed by flow cytometry. *$p < 0.05$,***$p < 0.001$ versus Normal group.

Figure 3: Evaluation of cell viability and apoptosis following hypothermia treatments.
(A) Cell apoptosis was detected by flow cytometry.
(B) Quantitative analysis of apoptosis.
(C) Cell viability was measured by using a cell viability assay. **$p < 0.01$,***$p < 0.001$ versus Normal group.

Figure 4: Analysis of mitochondrial function and caspase-3/ caspase-8 activity.
(A) Intracellular ROS levels.
(B) Quantification of mitochondrial membrane potential.
(C&D)The caspase-8/caspase-3 activity was estimated using an ELISA kit. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus OGD group.

SUPPLEMENTARY FILES:

DISCUSSION:

The complexities of intact animals, including the interactions between different types of cells, often prevent detailed studies of specific components of I/R injury. Therefore, it is necessary to establish an in vitro cell model that can accurately reflect the molecular changes after ischemia in vivo. Research on OGD models has been previously reported[13,22], and many sophisticated methods have been established[23,24]. The preparation process of OGD models includes two key steps: oxygen deprivation and glucose deprivation. In the present study, glucose deprivation was performed by culturing cell in glucose-free medium, and oxygen deprivation was achieved by substitution with nitrogen, which is currently a well-established method to prepare OGD models[25,26]. However, depending on cell type, two factors must be considered, including:1) cell seeding density and 2) duration of OGD exposure. The greater the number of attached cells, the stronger the resistance to OGD stress, such that the duration of seeding prior to OGD is crucial. HCMs (2×10^5 cells/wells) were seeded in 6-well plate for 30-34h before OGD, at which time cells were approximately 65% confluent. Higher cell density will reduce the impact of OGD on cells. In addition, it is crucial to minimize the potential of loss of the cells during the washing steps. The effect of the duration of OGD exposure is another important factor in evaluating the efficacy of the OGD model. For example, to study the protective effects of drugs, it is appropriate to choose a duration that causes 40–50% cell death without treatment. If cell death is too extreme, e.g., 80%, then it will be challenging to quantify the protective effect of the reagent being analyzed. For HCMs, exposure to OGD for 12 hours resulted in 42% cell death.

Therefore, in the subsequent experiments, a 12-hour OGD treatment was used to induce cell damage.

Due to difference in hypothermic kinetics between in vivo and in vitro environments, the optimal approach and mechanisms of hypothermia induction for cardiomyocyte model remains unclear. In the past few decades, several in vitro models have been developed to study cardiomyocytes at low temperatures. For example, Jana Krech et al. established a moderate hypothermia cell model to study the effect of temperature on myocardial apoptosis after ischemia-reperfusion[13]. Although many studies have focused on the physiological effects of cooling, there are also a large number of harmful side effects that occur during rewarming[27]. The results of previous studies have shown that rewarming can induce contractile dysfunction in the isolated cardiomyocytes[27,28]. Therefore, the temperature and speed of rewarming is particularly essential. To strictly control the effect of temperature, we applied the standard time-temperature protocol used during cardiac surgery, as previously mentioned[20,21]. In this model, the temperature is accurately controlled within the required temperature range, including three stages: 1) the cooling period (1 h); 2) the temperature maintaining period (10 h); and 3) the temperature rewarming period (2 h). In addition, the temperatures used in this study are typical of mild (34° C), moderate (31 ° C), and severe (17 ° C) low temperatures, comparable to those used in previous publications[29-31]. Finally, we also tested our hypothesis regarding the effects of different temperature conditions on cardiomyocyte apoptosis in vitro. As expected, the results showed that temperature significantly reduced ROS levels, restored MMP, and decreased caspase-8 / caspase-3 activity.

We are aware that this study was carried out using both a non-contractile cell line and an in vitro model, which was not affected by any body fluids. Despite these limitations, the significant improvement in cell apoptosis resulting from hypothermia treatment emphasizes the importance of performing further investigation, including in vivo studies. Therefore, 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.

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DISCLOSURES:

The authors have nothing to disclose.

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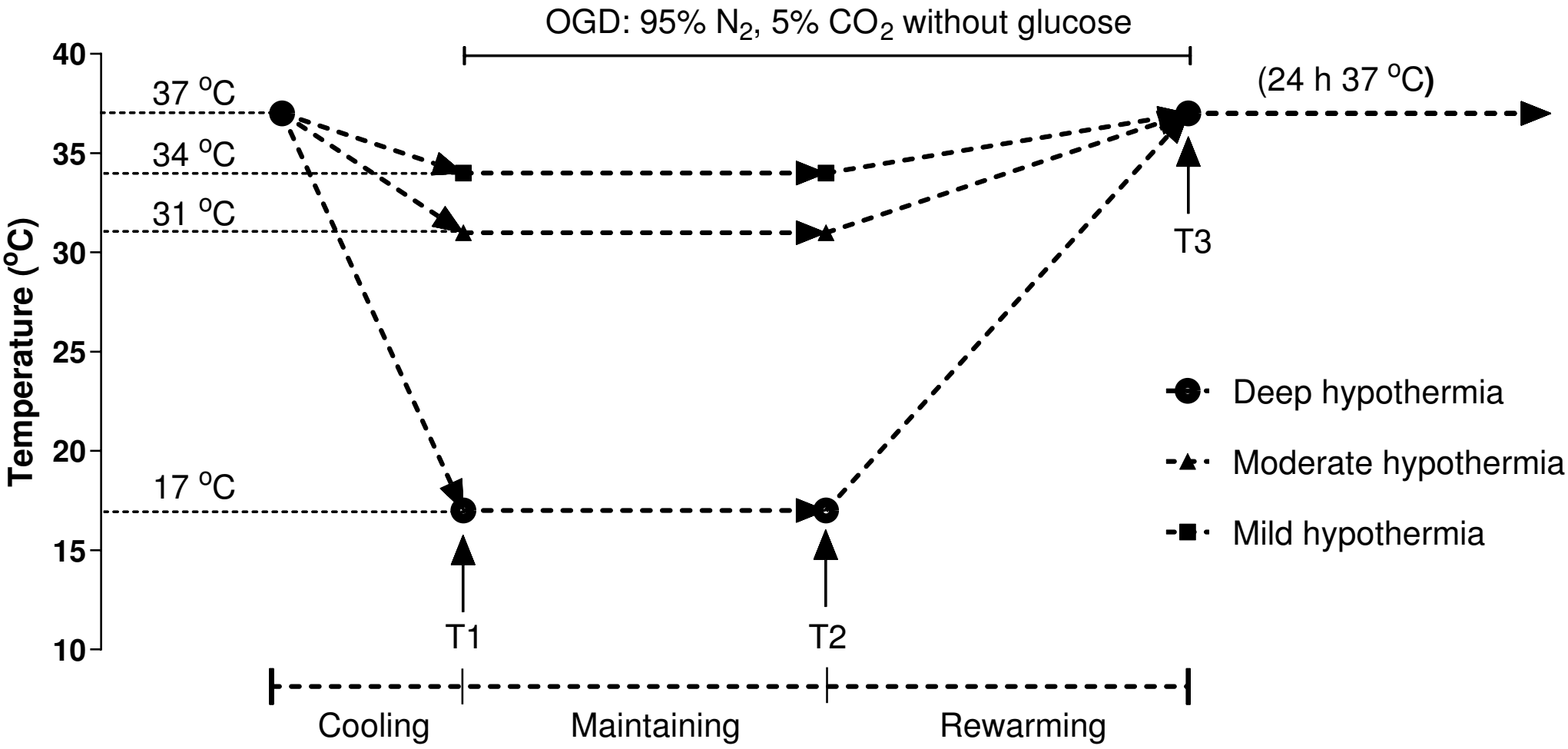

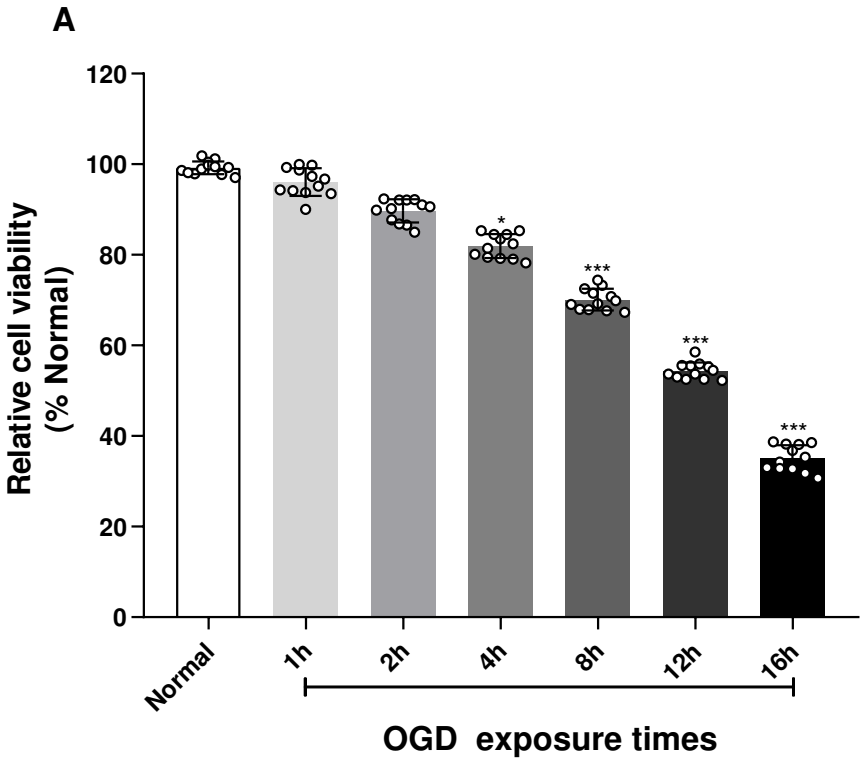

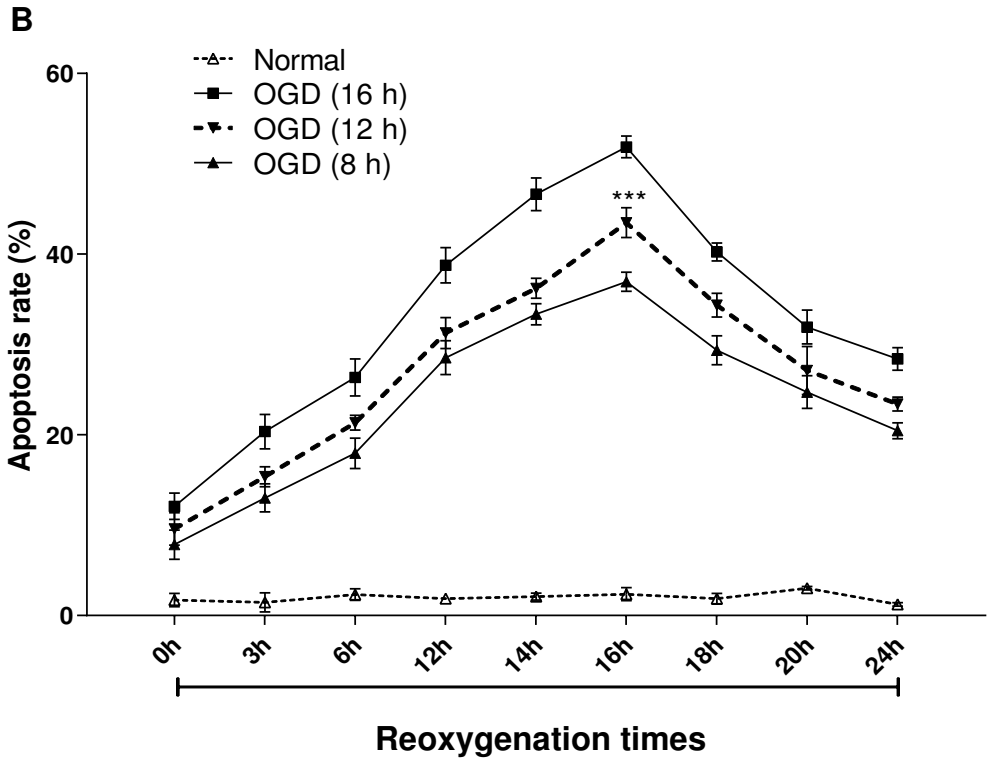

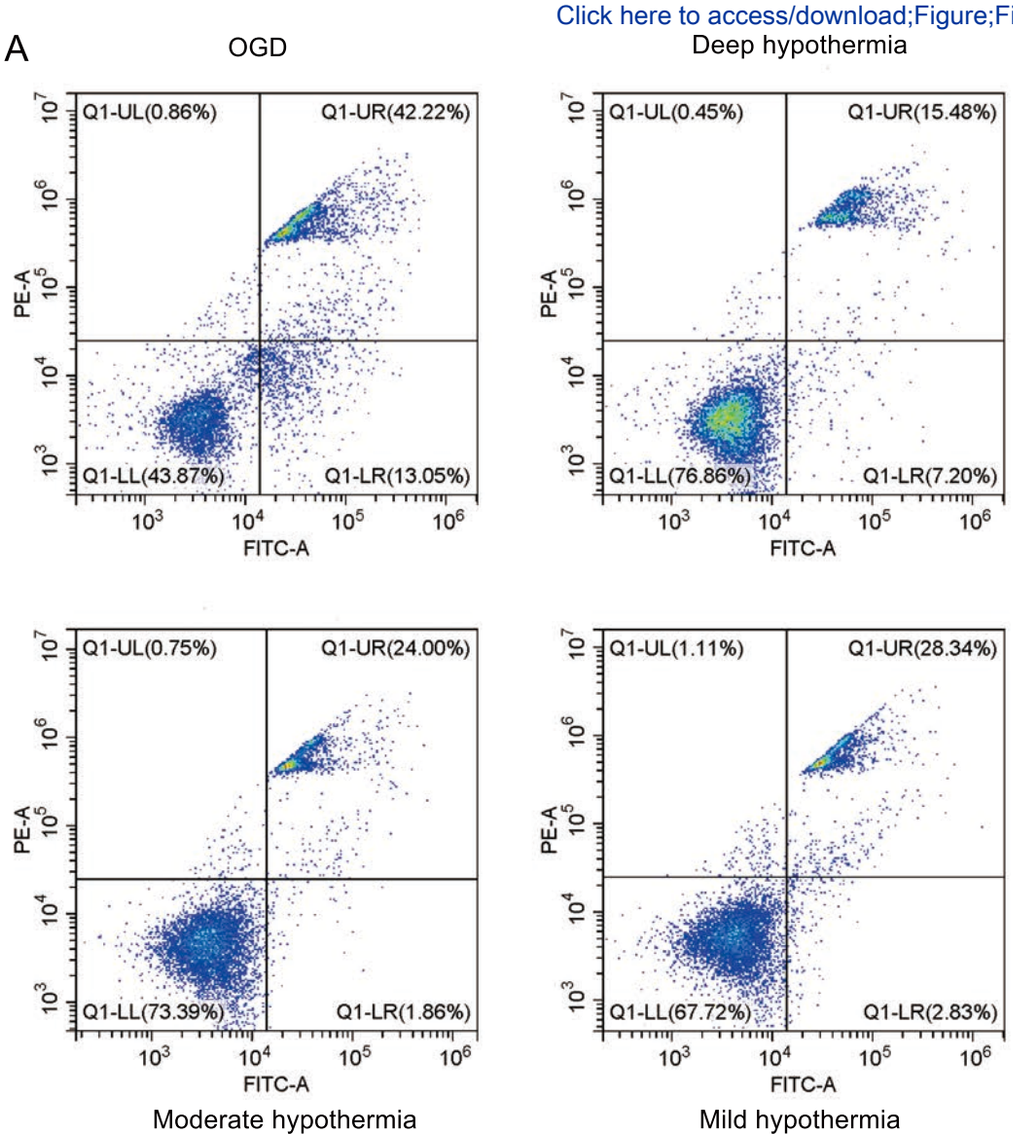

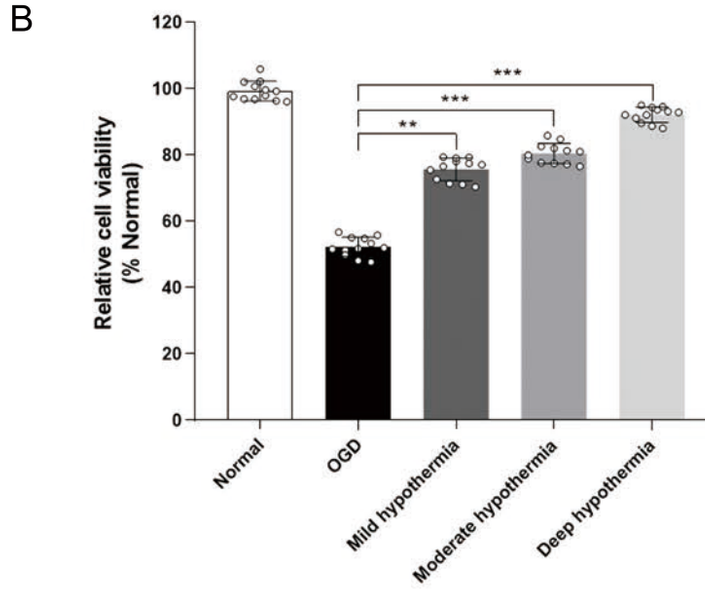

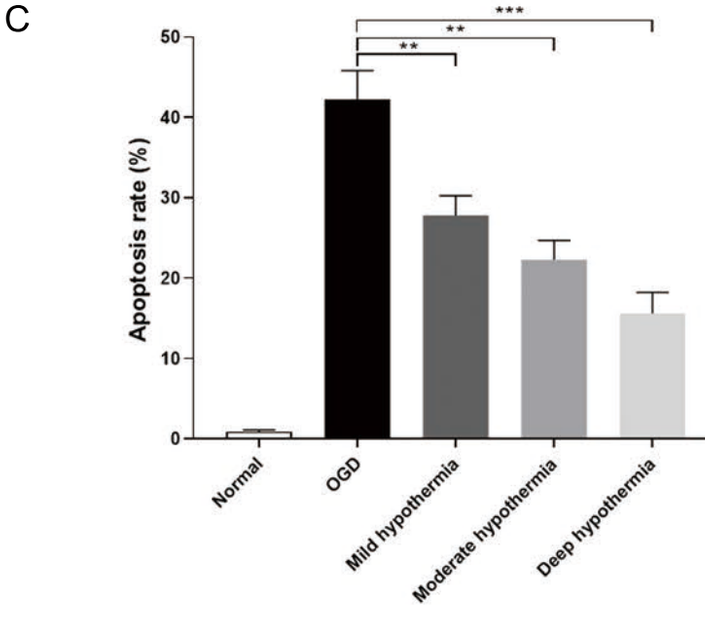

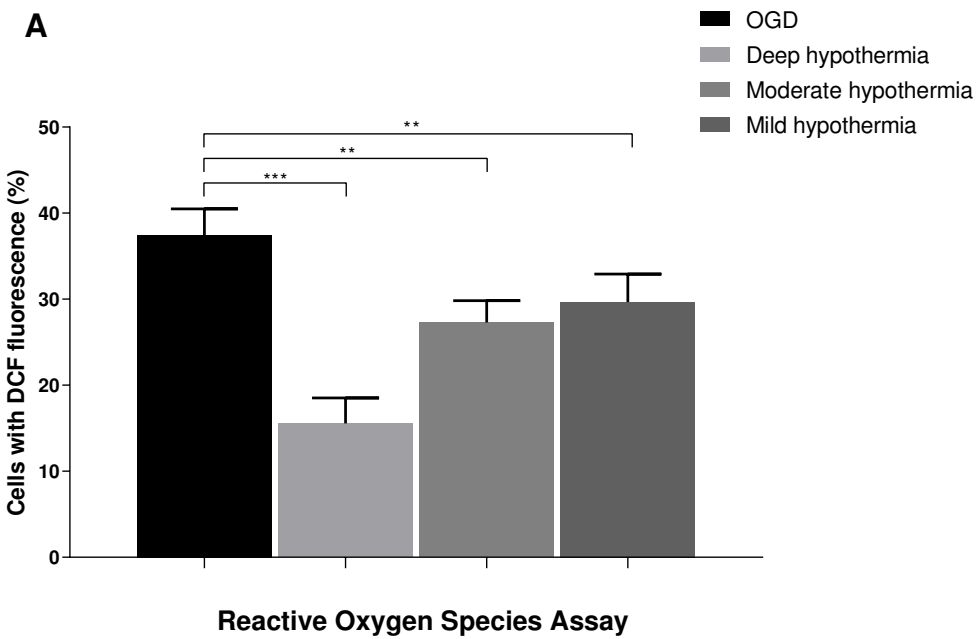

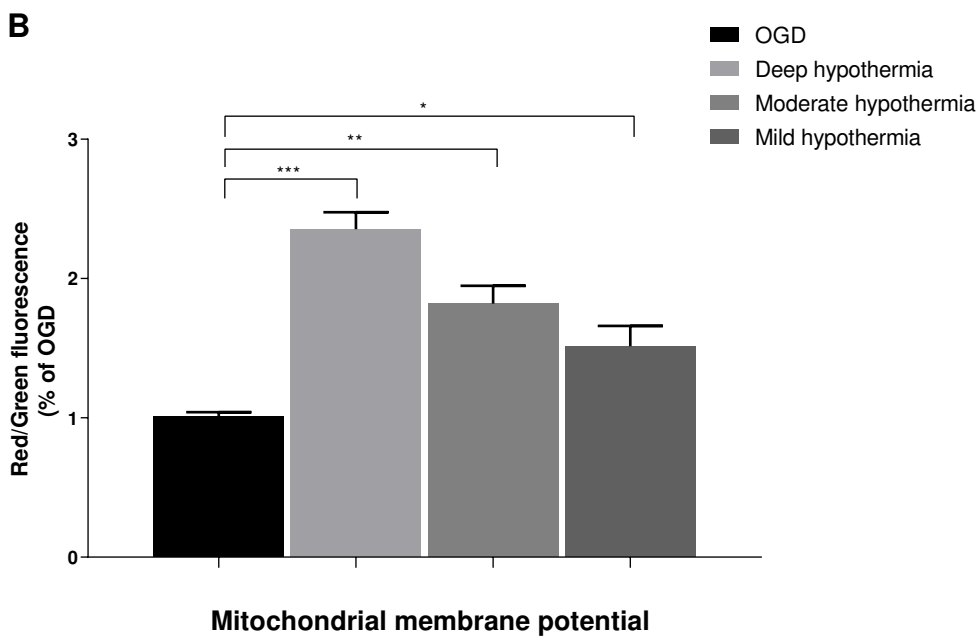

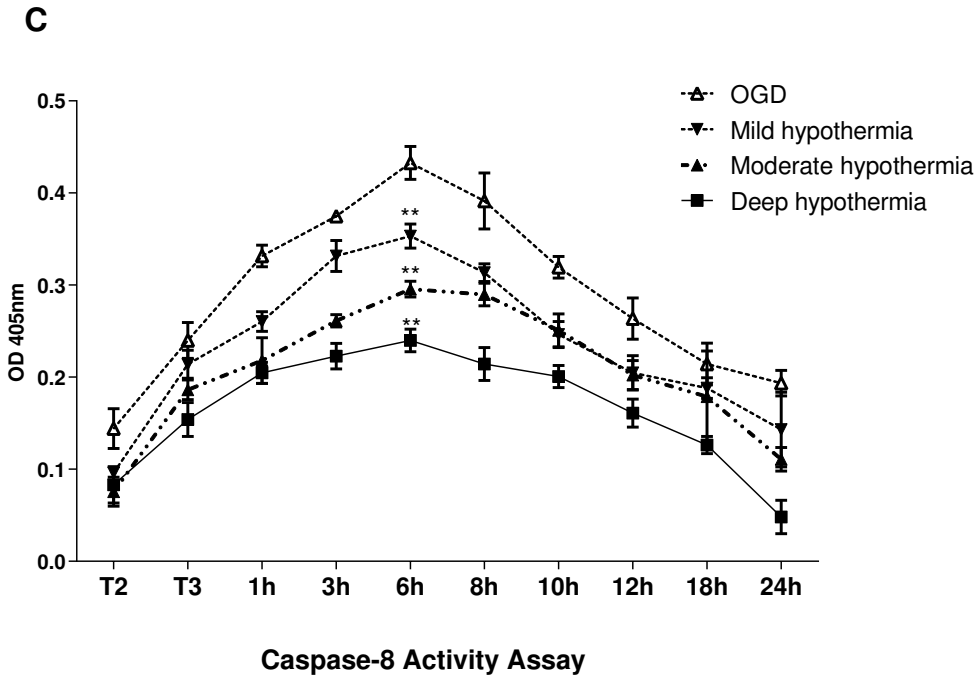

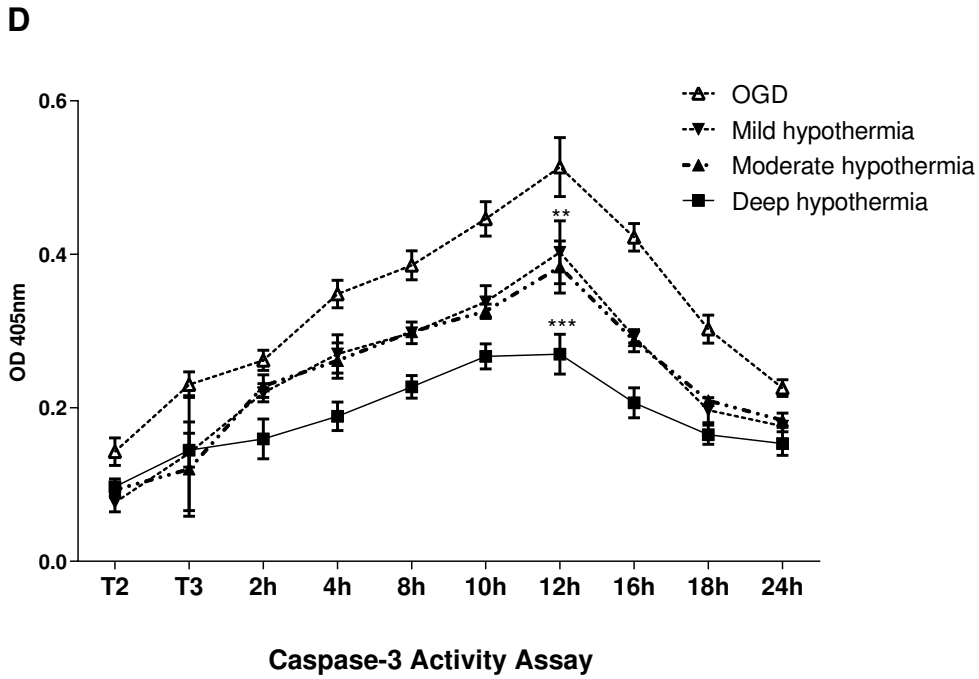

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company
Annexin V-FITC cell apoptosis detection kit	Bio-Technology,China
Cardiac myocyte growth supplement	Sciencell,USA
Caspase 3 activity assay kit	Bio-Technology,China
Caspase 8 activity assay kit	Bio-Technology,China
DMEM, no glucose	Gibco,USA
Dulbecco's modified eagle medium	Gibco,USA
Fetal bovine serum	Gibco,USA
Flow cytometry	CytoFLEX,USA
Human myocardial cells	BLUEFBIO,China
Mitochondrial membrane potential assay kit with JC-1	Bio-Technology,China
Penicillin/Streptomycin solution	Gibco,USA
Reactive oxygen species assay kit	Bio-Technology,China
Three-gas incubator	Memmert,Germany
Trypsin-EDTA (0.25%)	Gibco,USA

Catalog Number	Comments/Description
C1062M	
6252	
C1115	
C1151	
11966025	
11960044	
16140071	
B49007AF	
BFN60808678	
C2006	
10378016	
S0033S	
ICO50	
25200056	

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Dear Prof. Nam Nguyen

Thank you for your letter on 14-Aug-2020, in which you encouraged us to revise our manuscript (ID: JoVE61837) entitled "In vitro assessment of myocardial protection following hypothermia-preconditioning in a human cardiac myocyte cell model".

We would like to thank the reviewers for their comments and the editor for the suggestions. These comments allowed us to significantly enhance the quality of this manuscript. The issues you reminded us of were carefully addressed.

We provided point-by-point responses to all the comments in the "Response to the Editor and Reviewers" section. We also addressed these issues in the highlighted revision. Two copies of the manuscript (one clean copy and one with highlighting to show revisions) have been submitted.

We would like to thank the editor and the reviewers for their comments and recommendations that have greatly improved the quality of this manuscript. We sincerely hope our responses are satisfactory. If you have further questions, please let us know by E-mail. Thank you again for your consideration, and we await a favorable response to the revision.

Yours sincerely,

Xuming Mo, MD, PhD

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NANJING MEDICAL UNIVERSITY

Response to the Editor:

Editor's Comments:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Thank you for the valuable comment. The manuscript has been polished by an English language editing company. In addition, we have also carefully checked the entire manuscript to correct the grammar and typos. Please find the corrections in the revised manuscript.

2. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please use original language throughout the manuscript. Please see lines: 235-242, 289-293, 320-323.

Response: We appreciate the valuable comment, which will significantly improve the quality of our manuscript. According to the editor's comment, we have revised overlapping sections throughout the revised manuscript.

3. Figure 1: Please unbold the Celsius abbreviation as the rest of the text in the labels is not bolded.

Response: We thank the editor for bringing this to our attention. We have corrected this mistake in the revised manuscript.

4. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Response: Thank you for the kind advice. We have added additional details to the revised manuscript. In the modified version, we primarily added the operating details associated with using the flow cytometer, and these additions did not affect the original experimental data. Thank you again for this kind suggestion.

5. 3.5: The details are in the note under step but should be presented here.

Response: Thank you for the kind suggestion. We have corrected this mistake in the revised manuscript.

6. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: Thank you for the kind suggestion. We have used yellow font to show the essential steps that will be shown in the video in the revised manuscript.

7.Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Response: Thank you for the valuable comment. We have added additional details to the revised manuscript to ensure that the highlighted steps form a coherent narrative and that they contain at least one action written in the imperative tense.

8. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: Thank you for the kind suggestion. We have corrected the methods section in the revised manuscript accordingly.

9. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.

Response: Thank you for the kind suggestion. We have corrected this mistake in the revised manuscript.

10. Please spell out all journal titles in the References.

Response: Thank you for the suggestion. We have added all journal titles in the References.

Other change

Because PC D and FC carried out literature search and data analysis, we would like to add these two authors in the manuscript. After consultations, all authors have agreed with the addition of these authors to this manuscript and with the rearrangement of the names. Furthermore, as this work was funded in part by the National Natural Science Foundation of China (81700288), in the revised version, we have added the funding number.

Response: Thank you for editor's the valuable comment. We have tried our best to improve the manuscript and made a number of changes in the revised manuscript. These changes will not influence the content and framework of the manuscript. The changes are not listed here but are rather marked in red in the revised manuscript. We profoundly appreciate the valuable comments provided by the editor, which have greatly improved the quality of our manuscript. Once again, thank you for your comments and suggestions.

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Yours sincerely,

Xuming Mo, MD, PhD

Response to the Reviewers

Reviewer #1:

Dear reviewer 1:

Thank for your comments on our manuscript. We have revised our manuscript according to your comments:

The comments are as follows:

Comment 1

The paper by Zang et al. is clearly written with only minor English usage problems (most commonly letter capitalization). The objective is clear, the methods sound, and the figure data illustrate well the author's point. All the parameters measured are biologically meaningful, and the paper nicely illustrates the strengths of the model. The authors also appropriately discuss its limitations. Thus, I think it is suitable for turning into a video manual for future investigators wishing to us this setup. For the text, minor English proofing would be in order.

Response:
Thank you for the valuable comment. According to the reviewer's comment, we have revised spelling and grammar mistakes throughout the entire manuscript. In addition, the manuscript has been polished by an English language editing company. Please find the corrections in the revised manuscript. Thank you again for this kind suggestion.

Reviewer #2:

Dear reviewer 2:

I am very grateful for your comments regarding the manuscript. According to your advice, we have amended the relevant sections of the manuscript. Some of your questions are answered below.

Comment 1

L57-61

The authors say that there is no consensus on the classification of hypothermia in cardiac srugery. This is partially correct. However, there is an attempt to unify nomenclature. I suggest to quote this manuscript: TD Yan et al, 2013, Consensus on hypothermia in aortic arch surgery, Annals of Cardiothoracic Surgery

Further, the authors mix up articles on therapeutic hypothermia (e.g. after CPR tp prevent neurological injury) and hypothermia applied in the operating room. Therefore, I suggest not to reference #5 and #6, but quote more appropriate manuscripts in the field of cardiac surgery.

Response:
We appreciate the reviewer for the valuable comments. We apologize that we did not make this point clear in the original manuscript. We have carefully read the literature recommended by the reviewer, which describes the classification of hypothermia during cardiac surgery (Ann Cardiothorac Surg. 2013 Mar;2(2):163-8.). In the article, the classification of hypothermia therapy was discussed. According to the recommendations for aortic experts at high-volume aortic institutions, they divided TH into four categories: profound hypothermia (≤14 °C), deep hypothermia (14.1-20 °C), moderate hypothermia (20.1-28 °C), and mild hypothermia (28.1-34 °C). Standardization of the terminology will enhance the accuracy of scientific

discussions.

Besides, we whole-heartedly agree with the reviewer that the hypothermia setup for brain protection is different from that used for standard cardiac surgery. After reading articles related to hypothermia treatment, we found that most of the articles focused on brain injury. Also, the classification of temperature used in cardiac surgery lacked uniformity. Several articles have reported the use of different temperature settings. Leshnower et al. found that a temperature below 24℃ was considered deep hypothermia. (Ann Thorac Surg. 2015 Nov;100(5):1563-8). At the same time, 25 ℃ was also used as the dividing line between moderate and deep hypothermic(Ann Thorac Surg. 2015 May;99(5):1511-7). Also, some authors have divided temperature into two grades: moderate hypothermic (20 to 28 ℃) or deep hypothermia (12 to 20 ℃) (Ann Thorac Surg. 2018 Jan;105(1):54-61). There are no consensus exists regarding a definition of cooling during cardiac surgery. We have cited relevant literature in the revised manuscript according to the reviewer's insightful comments. Thank you again for this kind suggestion.

Comment 2

L96

Please give the full name first, before using abbreviations (FBS).

Response: We thank the reviewer for noticing this mistake, which we have corrected in the revised manuscript.

Comment 3

L98

Please give the full name first, before using abbreviations (HCM).

Response: We would like to thank the reviewer for noticing this mistake, which is now corrected.

Comment 4 L98

Please provide more information on the cells used in this study. Where have the cells been purchased? Were the cells able to contract? Etc

Response: Thank you for the kind suggestion. We think that this is an important point, and we apologize if we did not make it clear in the text. The AC16 human cardiomyocyte cell line was derived from the fusion of primary cells from adult ventricular heart tissue with SV40-transformed human fibroblasts (J Mol Cell Cardiol. 2005 Jul;39(1):133-47) and was purchased from BLUEFBIO (Shanghai, China). AC 16 human myocardial cells do not have the ability to contract spontaneously and regularly. However, the cell line develops many biochemical and morphological features that are characteristic of cardiomyocytes (Cell Death Dis. 2019 Sep; 10(9): 668). In addition, AC16 human cardiomyocyte cells were selected for use in the present study as they are a common cell line used in myocardial damage research (Exp Biol Med. 2019 Jul; 244(10): 802–812) (Biochem Biophys Res Commun. 2019 Jun 30;514(3):826-834) (Cell Death Dis. 2019 Sep 11;10(9):668). We have revised the description of the reconstitution protocol to provide more details in the revised manuscript. Thank you again for this kind suggestion.

Comment 5

L114

I understand that PBS (as well as FBS) is a common abbreviation, but please give the full name first, before using abbreviations.

Response: According to the reviewer's comment, we have added the full name first, before using abbreviations. Thank you again for carefully reading our manuscript.

Comment 6

L123-124

I suggest to change this part to "as described previously by others".

Response: Thank you for the kind suggestion. The phrase "as described previously" has been changed to "as described previously by others" in the revised manuscript. In addition, we have carefully corrected this phrase throughout the manuscript according to your comment.

Comment 7

Figure 3

The picture quality is rather low and therefore hard to assess.

Response: We apologize for making this mistake in the original manuscript. We have carefully checked the revised manuscript, including each image, and all the modified portions are highlighted in red.

Comment 8

L286/287

Please provide reference for your statement.

Response: Thank you again for the thoughtful advice. Oxygen glucose deprivation (OGD) is a common model of myocardial ischemia in vitro. According to the reviewer's opinion, we cited relevant literature to support this view. For example, the roles and mechanisms of HIF-1α synchronization in the protection of cardiomyocytes were previously studied by constructing an oxygen-glucose deprivation injury model in vitro (Biomed Pharmacother. 2019 Dec;120:109464). In another study, pretreatment with IL-19 upregulated HO-1 expression in cultured neonatal mouse ventricular myocytes and attenuated OGD -induced injuries in vitro (Br J Pharmacol . 2019 Mar;176(5):699-710). To investigate the ischemic component, cells in these models are treated by hypoxia or oxygen/glucose deprivation. We have added these details in our revised manuscript. Thank you again for this kind suggestion.

Comment 9

L304

Please correct this typo: Established must read established.

Response: We thank the reviewer for bringing this typo to our attention, which has been corrected in the revised manuscript.

Response: We have tried our best to improve the manuscript and made a number of changes in the revised manuscript. These changes will not influence the content and framework of the manuscript. The changes are not listed here but are rather marked in red in the revised manuscript. We profoundly appreciate the valuable comments provided by the reviewer, which have greatly improved the quality of our manuscript. Once again, thank you for your comments and suggestions.

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We are grateful for your consideration of this manuscript, and we also very much appreciate your suggestions, which have been very helpful in improving the manuscript. We sincerely hope our responses are satisfactory. If you have further questions, please let us know by E-mail. Thank you again for your consideration, and we await a favorable response to the revision.

Yours sincerely,

Xuming Mo, MD, PhD

Editing Certificate

This document certifies that the manuscript

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

prepared by the authors

Xiaodong Zang1*, Di Yu1*, Zhaocong Yang1, Qinghui Hu1, Peicheng Ding1, Feng Chen1, Xuming Mo1

was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified native English speaking editors at AJE.

This certificate was issued on **August 26, 2020** and may be verified on the AJE website using the verification code **03FC-1BE6-E47F-1455-97E1** .

Neither the research content nor the authors' intentions were altered in any way during the editing process. Documents receiving this certification should be English-ready for publication; however, the author has the ability to accept or reject our suggestions and changes. To verify the final AJE edited version, please visit our verification page at aje.com/certificate. If you have any questions or concerns about this edited document, please contact AJE at support@aje.com.