

# Development of an In Vitro Cardiac Ischemic Model Using Primary Human Cardiomyocytes

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Abstract—Developing experimental models to study ischemic heart disease is necessary for understanding of biological mechanisms to improve the therapeutic approaches for restoring cardiomyocytes function following injury. The aim of this study was to develop an in vitro hypoxic/re-oxygenation model of ischemia using primary human cardiomyocytes (HCM) and define subsequent cytotoxic effects. HCM were cultured in serum and glucose free medium in hypoxic condition with 1% O<sub>2</sub> ranging from 30 min to 12 h. The optimal hypoxic exposure time was determined using Hypoxia Inducible Factor  $1\alpha$  (HIF- $1\alpha$ ) as the hypoxic marker. Subsequently, the cells were moved to normoxic condition for 3, 6 and 9 h to replicate the reoxygenation phase. Optimal period of hypoxic/re-oxygenation was determined based on 50% mitochondrial injury via 3-(4,5dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide assay and cytotoxicity via lactate dehydrogenase (LDH) assay. It was found that the number of cells expressing HIF-1 $\alpha$  increased with hypoxic time and 3 h was sufficient to stimulate the expression of this marker in all the cells. Upon re-oxygenation, mitochondrial activity reduced significantly whereas the cytotoxicity increased significantly with time. Six hours of re-oxygenation was optimal to induce reversible cell injury. The injury became irreversible after 9 h as indicated by > 60% LDH leakage compared to the control group cultured in normal condition. Under optimized hypoxic reoxygenation experimental conditions, mesenchymal stem cells formed nanotube with ischemic HCM and facilitated transfer of mitochondria suggesting the feasibility of using this as a model system to study molecular mechanisms of myocardial injury and rescue.

**Keywords**—Primary human cardiomyocytes, Hypoxia/reoxygenation, Cardiac ischemic model, Reperfusion injury, Mitochondrial transfer.

## ABBREVIATIONS

ATP	Adenosine triphosphate
CAD	Coronary artery disease
CMM	Cardiac myocyte medium
DAPI	4',6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
HCM	Human cardiomyocytes
HIF-1α	Hypoxia inducible factor $1\alpha$
IHD	Ischemic heart disease
iPSCs	Induced pluripotent stem cells
LDH	Lactate dehydrogenase
MSCs	Mesenchymal stem cells
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyl-
	tetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
OGD	Oxygen and glucose deprivation
ROS	Reactive oxygen species
TNT	Tunneling nanotube
WST	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-
	disulfophenyl)-2H-tetrazolium, mono-
	sodium salt

## INTRODUCTION

Despite major advances in medical therapy, coronary artery disease (CAD) remains a major cause of morbidity and mortality worldwide. Dysfunction of the heart at the cellular level may lead to post ischemic

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heart failure. The current therapy for CAD is palliative as it only manages to improve the clinical outcome and prognosis but fails to replace the loss myocardium and to repair the injured cardiomyocytes.<sup>15,24</sup> Re-vascularization of ischemic myocardium is the standard treatment for CAD. However, reperfusion causes more injuries to the ischemic region, suggesting that ischemic injury is not completely resolved. Cell-based therapy is a promising approach to reconstruct a damaged myocardium. Studies have described improvement in cardiac performance after implantation of skeletal myoblasts, fetal and neonatal cardiac stromal cells, embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs).<sup>11,32</sup> While interventions using pluripotent cells may derive a superior outcome, the associated ethical and clinical complications make them a less favourable candidate compared to MSCs.

Irrespective of the source of cells used in therapy it is imperative to have considerable experimental evidence on mechanism of rescue. Various such models have been developed to date to understand both infarction disease pathophysiology and therapeutic prospects of cellular interventions.<sup>28,34</sup> However, majority of these literatures have used murine cardiac cells to elucidate the mechanism of action, as murine cells are available at comparative ease. Although human and murine myocardium share numerous similarities, it is pivotal to avoid substitution with latter in elucidating mechanisms of cell therapy since their cellular and molecular physiology are significantly different.<sup>21</sup> Whilst, availability of primary cardiomyocytes and their sensitivity in in vitro culture system continue to remain a major hurdle, use of human cardiomyocytes to model myocardial injury have shown to be more reliable and capable of predicting success of subsequent clinical trials.<sup>2</sup>

Inadequate blood, oxygen and nutrients supply to portion of myocardium due to blockage in coronary arteries will lead to a condition called myocardial ischemia. Myocardial infarction is a condition of irreversible cardiomyocytes death secondary to prolonged ischemia. As 95% of cardiac adenosine triphosphates (ATPs) are generated by mitochondria, mitochondrial dysfunction is the cellular response to ischemia and consequent reperfusion.<sup>30</sup> Similarly in *in vitro* hypoxic condition, cardiomyocytes are deprived from oxygen, glucose and serum leading to inactivation of oxidative phosphorylation, building up of lactic acid and decreased cellular pH. These changes disrupt the mitochondrial function. Upon reperfusion, oxygen interacts with the impaired respiratory chain to produce reactive oxygen species (ROS) that trigger hypoxia/re-oxygenation injury in mitochondria.<sup>1</sup> HIF- $1\alpha$  is a transcription factor which expression increased during hypoxia to facilitate the adaptation of cells and



tissues to low O<sub>2</sub> concentration.<sup>9</sup> Translocation of HIF-1 $\alpha$  from cytoplasm to nucleus during hypoxia stabilize it and protects HIF-1 $\alpha$  from degradation.<sup>33</sup> Activation of HIF-1 $\alpha$  regulates the expression of several downstream metabolic genes that play a key role in glucose transport and glycolysis. Previous studies have proposed that the enhancement of glucose uptake and glycolysis can protect cardiomyocytes from ischemic or hypoxic injury.<sup>6,18,20,40</sup>

Recently, in vitro studies in co-culture of MSC and cardiomyocytes revealed direct cell-to-cell communication, and intercellular exchange of compounds and organelles, which is associated with the thin channel formation via membrane, called "tunnelling nanotubes (TNT)".<sup>17,27</sup> The mechanisms of TNT formation are not yet fully understood. It was reported that the membrane that forms the TNT bridging two cells can originate from either of the two cells. A number of studies have shown that stressed cells such as those during starvation, inflammation or hypoxia triggers the formation of TNT.<sup>8,22,39</sup> TNT formation in a stressed myocyte allows various components such as multi-protein complexes<sup>7</sup> and organelles to transfer through them.<sup>3,14,35</sup> In coculture of MSC and cardiomyocytes, cytoplasmic macromolecules and organelles such as mitochondria can move via TNT-like structures unidirectional from stem cell to myocyte, regardless of tunnel direction and membrane source.<sup>27,29,37</sup>

The present study describes a model of hypoxic/reoxygenation using human primary cardiomyocytes. The influence of the combination of oxygen, glucose and serum deprivation on expression of HIF-1 $\alpha$  in human cardiomyocytes at multiple exposure time points was examined to determine the kinetics of mitochondrial injury and cytotoxicity. It is the aim of the study to subsequently use this model to investigate the possible formation of TNT between human MSCs and hypoxic (stressed) HCM as a form of rescue mechanism.

In our novel approach, using human primary cardiomyocytes, as the mitochondrial recipient cells, and human bone marrow derived MSCs as the mitochondrial donor cells, we establish a hypoxia/reoxygenation *in vitro* model optimized based on actual human cell types to be used for translation into future clinical strategies for the treatment of ischemic heart disease (IHD).

## MATERIALS AND METHODS

#### Ethics Statement

This study was approved by the Universiti Kebangsaan Malaysia Medical Research and Ethics Committee (FF-385-2011).

#### Culture of Primary Human Cardiomyocytes

Primary human cardiomyocytes had been purchased from Sciencell Research Laboratories (Cat number: 6200, USA). Cardiomyocytes were grown in proliferative medium purchased from Sciencell Research Laboratories containing Cardiac Myocyte Medium (CMM) supplemented with 5% fetal bovine serum (FBS), 1% cardiac myocyte growth supplement (Sciencell Research Laboratories, USA), 20 IU/ml of penicillin and 20 IU/ml of streptomycin solution at 37 °C in humid air with 5% CO<sub>2</sub>. Primary human cardiomyocytes adhered to the bottom of the culture plates after 24 h, and the non-adherent cells were removed by a medium change after 24 h. At 70-80% confluence, cells were harvested with 0.05% trypsin-EDTA (Gibco, USA), and subcultured. All experiments were performed using passage 2 cells.

#### In Vitro Cardiac Ischemia Model

Physiologically, ischemia will lead to hypoxia, nutrient deprivation and metabolic waste accumulation. To mimic the physiological condition, *in vitro* cultured primary human cardiomyocytes in triplicates on 3 independent samples (n = 3), were subjected to oxygen and glucose deprivation (OGD) followed by reoxygenation to develop an *in vitro* cardiac ischemia model. This study adopted the hypoxic setting of Portal *et al.*, and the hypoxia and re-oxygenation protocol was optimized based on previous findings by Virginia Cozzolino.<sup>7,28</sup> Prior to the OGD, human cardiomyocytes were seeded at density  $2 \times 10^4$  cells/ cm<sup>2</sup> in 12-well plates (3.9 cm<sup>2</sup>/well; Greiner bio-one, Germany) and incubated in complete CMM for 48 h.

#### Hypoxic Induction

An oxygen control incubator (Galaxy170R, New Brunswick, USA) had been used to create the hypoxic condition. For the hypoxic group, medium for primary human cardiomyocytes was changed from complete CMM to serum-free, glucose-free and phenol-free Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA), which was pre-incubated in hypoxic environment for 24 h to remove the dissolved oxygen. The cells were placed in hypoxic incubator with 1% O<sub>2</sub>, 5% CO<sub>2</sub> and 94% N<sub>2</sub> over the experimental period of 30 min, 1, 2, 3, 6 and 12 h. The control group was cultured with high glucose, phenol-free DMEM (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) in normoxic condition with 21% O<sub>2</sub> and 5%  $CO_2$ . Cell hypoxia was monitored by looking at the expression of HIF-1 $\alpha$  by the cells. The optimal exposure time in hypoxic condition to induce cell hypoxia will be determined by the first time point whereby 100% of the cells being positive for HIF-1 $\alpha$  expression.

#### **Re-oxygenation**

After incubating the cells in hypoxic condition for 3 h (selected during hypoxic phase optimization), reoxygenation was achieved by moving the cells from hypoxic incubator to normoxic condition (21% O<sub>2</sub> and 5% CO<sub>2</sub>). The cells were re-oxygenated for 3, 6 and 9 h.

#### Hypoxic Marker Detection

Immunocytochemical (ICC) staining for HIF-1 $\alpha$ was used as the hypoxic marker for the determination of optimum exposure time to hypoxia in triplicates of 3 independent samples (n = 3). After hypoxic exposure, cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, USA) for 20 min. Then, cells were blocked with 10% goat serum for 2 h and incubated with rabbit anti-human monoclonal HIF-1a antibody (1:250; Abcam, UK) overnight at 4 °C. After washing, the cells were incubated with goat anti-rabbit IgG conjugated with Alexa Fluor-594 (1:250; Abcam, UK) for 2 h at 37 °C. Nuclei were counterstained with 0.1 mg/mL 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, USA) for 5 min and actin was stained with Phalloidin conjugated with Alexa Fluor-488 (1:80; Thermo Fisher, USA) for 40 min in room temperature. HIF-1 $\alpha$ expression images were captured by Plan Fluor ELWD 20X/0.45 lens through confocal microscope (Nikon, Japan). Minimum 200 cells were examined per sample to calculate the percentage of cells positive for HIF-1 $\alpha$ .

#### Mitochondrial Injury Assay

Mitochondrial injury was measured by quantifying the decrease in the level of mitochondrial dehydrogenase activity, using 3-(4,5-dimethylthiazol-2-Yl)-2,5diphenyltetrazolium bromide (MTT) as substrate (Invitrogen, USA). The assay is based on the redox ability of the living mitochondria to convert MTT into insoluble formazan. MTT assay was conducted in triplicates of 3 independent samples (n = 3). Cells exposed to 3 h hypoxic duration and re-oxygenated for 3, 6 and 9 h were used for this assay. After re-oxygenation, 25  $\mu$ L of MTT solution (1 mg/mL) mixed with 250  $\mu$ L of culture medium was added to each well and incubated for 4 h in a humidified 5% CO2 incubator at 37 °C. The assay was stopped by adding 100  $\mu$ L of Dimethyl sulfoxide (DMSO, Sigma–Aldrich, USA) solution to solubilize the formazan after removing the MTT solution. The absorbance was read



at 540 nm wavelengths using a spectrophotometer (Bio-TEK, USA). The data are stated as the percentage of cell viability compared to control culture.

#### Cell Cytotoxicity Assay

Concentration of LDH in the culture medium was measured using LDH-Cytotoxicity Assay Kit II (Abcam, USA). This kit applies the 2-(4-Iodophenyl)-3-(4nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST) reagent for a fast and more sensitive detection of LDH released from damaged cells. LDH oxidizes lactate to generate Nicotinamide adenine dinucleotide (NADH), which reacts with the WST to generate yellow color. The intensity of yellow color correlates directly with the lysed cell number. Cell cytotoxicity assay was conducted in triplicates of 3 independent samples (n = 3). Cells exposed to preoptimized hypoxic duration (3 h) and re-oxygenated for 3, 6 and 9 h were used for this assay. LDH level in the supernatant of normoxic culture was used as the low control and cells lysed with the provided cell lysis solution served as the high control. For LDH quantification, culture medium post hypoxic/re-oxygenation was collected and centrifuged at 600 g for 10 min to precipitate the cells. Then, 10  $\mu$ L of clear medium on top was transferred to an optical 96-well plate before 100  $\mu$ L of LDH reaction solution was added to each well and incubated in dark at room temperature for 30 min. Absorbance was measured with a spectrophotometer (Bio-TEK, USA) at 450 nm wavelengths. Cytotoxicity was calculated using the following formula:

$$cytotoxicity(\%) = \frac{\text{test sample} - \text{low control}}{\text{high control} - \text{low control}} \times 100\%$$

## Isolation and Culture of Human Sternal Bone Marrow MSCs

Approximately  $7 \pm 1$  mL of sternal marrow was collected using heparinized syringe from sternum and processed for MSC isolation within 45 min of sample collection as described elsewhere.<sup>14</sup> In brief, the bone marrow mononuclear cells were separated using Ficoll paque (GE, USA) density gradient method and plated in 75 cm<sup>2</sup> tissue culture treated flasks with 15 mL MSCs medium, consisting of DMEM (Invitrogen, USA), 10% FBS (Invitrogen, USA), 1% ascorbic acid (Sigma, USA), 1% Glutamax (Invitrogen, USA), and 1% antibiotic–antimycotic (Gibco, USA) that contains 10,000 U/mL of penicillin, 10,000 mg/mL of streptomycin, and 25 mg/mL of Fungizone. Cells were incubated at 37 °C in a 5% CO<sub>2</sub> humidified incubator for



3 days before the first medium change. MSCs were isolated based on their ability to attach to culture surface. Once adherent cells reached 80–90% confluence, the cells were detached with 0.05% trypsin ETDA (Gemini, USA) and sub-cultured. Cells in passage3 (P3) were used for downstream experiments.

#### Cell Labelling

MSCs were labelled with Mitotracker<sup>®</sup> Deep Red FM (excitation/emission: 644/665; Invitrogen, USA) according to manufacturer's description. In brief, MSCs were incubated in a working concentration of 500 nM for 45 min in 37 °C and washed in prewarmed culture medium prior to co-culture to remove excessive dye. Labelling mitochondria enabled visualization of mitochondrial transfer from MSC to injured cardiomyocytes.

Primary human cardiomyocytes were labelled with Vybrant<sup>®</sup> DiO (excitation/emission: 488/501 nm; Invitrogen, USA) as per manufacturers description. In brief, prior to hypoxia and reoxigenation, cardiomyocytes were incubated in Vybrant<sup>®</sup> DiO in a dilution of 1:200 for 30 min at 37 °C and washed 3 times in prewarmed buffer to remove excessive dye. Labelling with Vybrant<sup>®</sup> DiO enabled distinction of cardiomyocytes from MSC.

#### Co-Culture of Ischemic Cardiomyocytes and MSCs

Triplicated samples of human primary cardiomyocytes of 3 independent samples (n = 3), were subjected to 3 h of hypoxia and 6 h of reoxygenation (optimized during experiment for in vitro cardiac ischemic model development), then the medium was changed and  $2 \times 10^4$  cells/cm<sup>2</sup> Mitotracker labeled MSCs suspended in high glucose, phenol free DMEM (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) were added to the injured cardiomvocytes. Cells were co-cultivated for 24 h, and then fixed with 4% paraformaldehydes (Sigma-Aldrich, USA) for 30 min. TNTs formation were captured by Plan APO 20X/0.75 lens through confocal microscope (Nikon, Japan). The confocal images of the remaining human cardiomyocytes and TNT formation with fluorescence were evaluated with image analysis software (NIS Elements-Nikon, Japan). Meanwhile in the coculture, MSCs are distinguishable from human cardiomyocytes due to the Mitotracker® Deep Red FM (Invitrogen, USA) labeling. Cell numbers, TNTs and rescued HCMs were calculated, with minimum of 1000 cells in each sample. In order to assess the recovery or rescue of the hypoxic HCMs, in situ counting of survived ischemic HCMs was done after 24 h of co-culture and was compared to the hypoxic HCM (control group) as an indicator of increased HCM survival.

#### Statistical Analysis

Data are presented as mean  $\pm$  standard deviation (SD) and statistical significance was analyzed by Oneway ANOVA with Tukey's *post hoc* test using GraphPad Prism version 7.0b.

## RESULTS

#### HIF-1a Detection After Hypoxia

The expression of HIF-1 $\alpha$  was studied to optimize the minimum duration of hypoxia to induce hypoxic stress in 100% of cells. After 30 min hypoxia, 8.66 ± 1.75% of cells expressed HIF-1 $\alpha$ . This percentage increased gradually to 26.16 ± 3.51% and 91.66 ± 1.75% in the first and second hour respectively, and by 3 h all cells were positive for HIF-1 $\alpha$  (vertical line in Fig. 1b). Initially HIF-1 $\alpha$  protein was located predominantly in cytoplasm around the nucleus. After prolonged hypoxic exposure, HIF-1 $\alpha$  stabilized and translocated within nucleus. The expression of HIF-1 $\alpha$  protein was not detected in cells maintained in normoxic condition up to 12 h. These results clearly showed that 3 h of exposure to hypoxic conditions was sufficient to induce HIF-1 $\alpha$  expression in all the cells (Fig. 1).

# Hypoxia/Re-Oxygenation Induces Mitochondrial Injury and Cytotoxicity

Metabolic activity of cells exposed to hypoxia/reoxygenation injury was analyzed with MTT assay to estimate the mitochondrial injury. Percentage of metabolic activity in the experimental groups (3, 6 and 9 h of re-oxygenation) was calculated relative to the control group without re-oxygenation. Primary human cardiomyocytes exposed to re-oxygenation demonstrated reduction of mitochondrial reductase function in a timedependent manner. Following re-oxygenation, mitochondrial reductase activity reduced significantly (p < 0.001) to 74.94  $\pm$  13.46% at 3 h, 47.09  $\pm$  15.17% at 6 h and 20.63  $\pm$  10.11% at 9 h (Fig. 2a).

The cytotoxic effect of hypoxic/re-oxygenation was masured by determining LDH activity in culture medium at the end of re-oxygenation. LDH concentration in culture medium increased proportionally to the duration of re-oxygenation (p < 0.001). LDH activity was 22.69  $\pm$  5.33% at 3 h, 40.51  $\pm$  5.84% at 6 h and 69.80  $\pm$  7.39% at 9 h (Fig. 2b). These results indicated that re-oxygenation presented a time-dependent cytotoxicity to primary human cardiomyocytes.

# MSCs Forms Tunneling Nanotubes (TNTs) with Ischemic Cardiomyocytes

Multiple reports describing in vitro models of ischemic injury are currently available. However, majority of these reports only describe either changes in expression of genes or proteins associated with cell stress and/or phenotypic changes as a validation of the model, and elaborate little on usefulness of such an experimental model as a tool to study cell rescue. Thus, in this study, we evaluated the usefulness of our ischemic injury model by examining the ability of mesenchymal stem cells to rescue cardiomyocytes that are injured by OGD. Ischemic cardiomyocytes were prelabelled with Vybrant<sup>®</sup> DiO and then co-cultured with MSCs that are pre-labelled with Mitotracker. After 24 h, we observed the formation of TNTs between cultured MSCs and ischemic cardiomyocytes. The TNTs originated from MSCs as it was not Vybrant<sup>®</sup> DiO-labeled. Notably, TNTs extended several cell diameters across the gap between the sparsely distributed cells and facilitated intercellular transfer of mitochondria from MSCs to injured cardiomyocytes. Mitotracker-labeled mitochondria was localized both within TNTs and in Vybrant® DiO-labeled ischemic cardiomyocytes confirming the functional nature of the tubes. In this case, confirmed formation of TNTs between donor MSC and recipient ischemic myocytes underlines the sensitivity of our ischemic injury model. Our results showed that TNTs formation occurred around 3 h after co-culture. Presence of nanotube intercellular connection with mitochondrial transfer (red labelled) was found in  $11 \pm 1.38\%$  of ischemic human cardiomyocytes after hypoxia/reoxygenation at the end of 24 h of co-culture. Meanwhile some hypoxic HCMs with mitochondrial transfer but without TNT formation were observed and it was assumed that TNTs formation had taken place earlier and was already ruptured. In our observation, there was no basal activity of mitochondrial transfer between MSCs and HCMs in normoxic condition (data not showed).

The number of survived hypoxic HCM was  $2.17 \pm 0.14$  times higher in the co-culture group compared to the hypoxic HCM (control group). Taken together, these findings indicate that the ischemic injury model described in this study can be used for cell rescue experiments (Fig. 3).

#### DISCUSSION

The *in vivo* cardiac ischemia differs from the *in vitro* ischemic model in a number of ways such as: (1) Only semi-quantitative evaluation of the viable cells is possible based on the visualization of infarct area *in vivo* 



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FIGURE 1. HIF-1 $\alpha$  staining of the human cardiomyocytes after different exposure time to hypoxia (n = 3). (a) Minimum 200 cells per sample were examined to calculate the percentage of positive cells for HIF-1 $\alpha$ . HIF-1 $\alpha$  expression increased with time and all the cells expressed HIF-1 $\alpha$  in 3 h of hypoxia. Cultures in normoxic condition were negative for HIF-1 $\alpha$  throughout the duration of the experiment. The expression of HIF-1 $\alpha$  (Red) can be detected in the cytoplasm (Green) and nucleus (Blue). (b) Graph showing the percentage of HIF-1 $\alpha$  positive cells after different period of hypoxia. Vertical line indicates 100% of possitive cells for HIF-1 $\alpha$  by 3 h hypoxic condition.



FIGURE 2. (a) Mitochondrial activity of cells cultured in hypoxic condition for 3 h and subsequent re-oxygenation for different time periods (n = 3). Mitochondrial activity reduced with time. (b) Cytotoxicity after 3 h of hypoxia and subsequent re-oxygenation for different time periods (n = 3). Cytotoxicity increased proportionally with the re-oxygenation time. \* Represents p < 0.001 compared to all the other groups.





FIGURE 3. Occurrence of mitochondrial transfer from human bone marrow MSCs to human cardiomyocytes (HCM) through TNTlike structures. (a) Cell nuclei were labeled with DAPI (blue). (b) Ischemic HCMs were labeled with Vybrant<sup>®</sup> DiO (green). (c) The mitochondria of human bone marrow MSCs were pre-labeled with Mitotracker<sup>®</sup> Deep Red FM (red) and transferred from human bone marrow MSCs to ischemic HCMs through the TNT-like structure. (d) Presence of human bone marrow MSC mitochondria (red) inside a TNT-like structure connecting MSCs to HCMs. Transfer of mitochondrial from MSCs to ischemic HCMs was proven by the presence of pre-labeled mitochondrial in ischemic HCMs. (d') Magnified TNT-like structure. All scale bars: 50 μm.

vs. exact quantification of viable cells *in vitro*, (2) Absolute and homogenous distribution of hypoxia condition cannot be ascertained *in vivo* (due to collateral vessel perfusion)<sup>35</sup> vs. exact percentage of hypoxia

can be exerted homogenously on the cardiomyocytes *in vitro*, (3). Interference may occur by factors from surrounding cells or tissue *in vivo* vs. direct response from the pure population of cardiomyocytes *in vitro*,



(4). Monitoring can only be performed at the tissue level *in vivo* vs. monitoring at the cellular level *in vitro*. Nevertheless, a study by Portal *et al.* on mouse models, has demonstrated that interventions to both the *in vitro* hypoxic/re-oxygenation and *in vivo* ischemia–reperfusion models produced similar pattern in cardiomy-ocytes viability *in vivo* and *in vitro*,<sup>28</sup> confirming that the *in vitro* model of hypoxic/re-oxygenation using cardiomyocytes is a powerful tool to study direct cardiomyocyte-dependent mechanisms in ischemic injury.

Physiologically, CAD involves phases of ischemia followed by reperfusion. To mimic the condition, primary cardiomyocytes were first cultured in hypoxic condition  $(1\% O_2)$  in glucose-deprived medium followed by normoxic condition  $(21\% O_2)$ . The beginning of hypoxic exposure is usually defined as the moment when the culture is placed inside the hypoxic incubator. Nevertheless, it might take several hours for the hypoxic culture medium soluble O<sub>2</sub> concentration to drop to the ischemic value, even though incubator can change gas phase composition rapidly.<sup>3</sup> The method of ischemia simulation used here was different from most of the previous studies,<sup>8,39</sup> whereby the ischemia culture medium used for the experiment has been preincubated in hypoxic condition for 24 h to remove soluble oxygen.<sup>22,36</sup>

Oxygen homeostasis within cells is controlled by HIF-1 $\alpha$ , through regulating expression of genes that are responsive to changing oxygen concentrations.<sup>38</sup> HIF-1 $\alpha$  pathway is stimulated when cells are deprived of oxygen, resulting in translocation of HIF-1 $\alpha$  from cytoplasm into the nuclei or peri-nucleus regions.<sup>9,40</sup> Though the role of HIF-1 $\alpha$  in hypoxic response is widely studied, to the best of our knowledge HIF-1 $\alpha$ expression has not been used as a scale to evaluate in vitro ischemic injury. Availability of sensitive antibodies and labor intensive techniques might have contributed to lack of such efforts. Here, we defined the kinetics of HIF-1 $\alpha$  expression and localization upon exposure to hypoxic conditions over time. Notably, by 3 h, all the primary cardiomyocytes expressed intense HIF-1 $\alpha$  protein in cytoplasm and nucleus.

Reperfusion is pre-requisite in rescuing viable myocardium after an acute ischemic attack. However, reperfusion of ischemic myocardium paradoxically present some risks that can result in cardiomyocyte death, a phenomenon termed reperfusion injury.<sup>26</sup> The concept of reperfusion injury implicates that a portion of ischemic myocardium gets irreversibly injured as a result of processes initiated by the restoration of arterial blood flow. It has been revealed in previous studies that mitochondria play an important role in cardiac ischemia. Mitochondrial respiratory and functional



injury occurs in the course of ischemia.<sup>19</sup> During reperfusion, mitochondria generate ROS that lead to additional mitochondrial and cardiomyocyte injury.<sup>31</sup> In this study, 3 h of hypoxia followed by 6 h of reoxygenation can mimic certain characteristics of cardiac ischemia. The evaluation of cardiomyocytes indicated that 3 h of hypoxia and 6 h of re-oxygenation reduced mitochondrial activity by approximately 50% and resulted in LDH release of about 40%. Notably, LDH is a biochemical index of myocardial injury<sup>4</sup> with more than 60% LDH leakage described as an indicator of irreversible cellular injury with little or no recovery of electrochemical activity even with therapeutic interventions.<sup>10</sup>

Finally, we show that healthy MSCs formed TNTs with ischemic cardiomyocytes and subsequently facilitated transfer of mitochondria. Whilst transfer of mitochondria from MSCs to injured cells is likely a unidirectional process due to high ATP requirement,<sup>5,12,13,23</sup> it also indicate that ischemic cardiomyocytes were responsive to nanotube formation. There is convincing evidence from previous studies that the signals for TNT formation are originated from the recipient cells<sup>16</sup> and Miro-1, a calcium-binding mitochondria Rho GTPase, is a key mediator in MSC-derived organelle and mitochondrial transfer.<sup>2</sup> Since ATP would be a requirement for cardiomyocytes recovery, hence optimization of mitochondrial ATP production of donor cells can be explored as a strategy to rescue the injured HCMs.

Overall, these findings suggest that the *in vitro* ischemic injury model described here can be used as a platform to investigate cell injury, and rescue from ischemic injury especially using cells as therapeutic tools.

## CONCLUSION

This study successfully defined the procedure and parameters to establish an *in vitro* cardiac ischemic model using primary human cardiomyocytes. This model is a reliable and repeatable platform for other researchers to study cardiac ischemia and treatment of injured cardiomyocytes at the cellular level.

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# **CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

#### ETHICAL APPROVAL

This study was approved by the Universiti Kebangsaan Malaysia Medical Research and Ethics Committee (FF-385-2011). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

#### **INFORMED CONSENT**

Informed consent was obtained from all individual participants included in the study.

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