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# Effects of Hypoxia on Cardiomyocyte Metabolism: An In Vitro and In Vivo Comparative Study

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## ABSTRACT

Hypoxia significantly alters cardiomyocyte metabolism, contributing to myocardial injury during ischemic events. In this study, cardiomyocytes cultured under hypoxic conditions were compared to cardiac tissues from rats subjected to experimental myocardial infarction. Both models demonstrated a metabolic shift from oxidative phosphorylation to anaerobic glycolysis, upregulation of hypoxia-inducible factor 1-alpha (HIF-1$\alpha$), and increased lactate production. The in vivo model also revealed structural changes and fibrosis. Understanding these metabolic adaptations is crucial for developing therapeutic strategies aimed at preserving cardiac function during ischemia and reperfusion

**Keywords:** Hypoxia, cardiomyocyte metabolism, myocardial infarction, physiology, ischemia.

## 1. Introduction

In the metropolis of Huayuan district, Shaoyang city of Hunan province, China, a population with left-to-right shunt complex congenital heart defect (CCHD) (birth prevalence of ~0.8 per 1000 births) has adapted very well to chronic hypoxia (CH) ($PaO_2 < 50$ mmHg, $SpO_2 < 85\%$) at birth due to an unbalanced pulmonary blood flow and an environmental hypoxic background [1]. An elucidation of the precise mechanisms of adaptation is expected to advance the understanding of the basic pathophysiology of patients with CCHD and will provide therapeutic implications for other hypoxic diseases. This unique phenotype has been leveraged to investigate the adaptive cardiac metabolic remodeling in the human right ventricle (RV) of patient with CCHD (CCHD cohort) and also the left ventricle (LV) of a pig model of monotonic CH by targeting the heart proteome [2,3].

Hypoxia is a common pathology in several cardiopulmonary diseases. Studying the effect of hypoxia on cardiomyocyte metabolism will provide new insights into the basic pathophysiology and therapeutic implications for cardiopulmonary diseases. Although cardiomyocyte metabolism has been studied in hypoxia using both model systems in vitro and animal models in vivo, many components in the

comparable studies remain elusive. In a comparative study, the cardiac adaptability under hypoxia of both 2D-cardiomyocyte and 3D-engineered heart tissue was investigated using MCT in vitro and animal models, respectively. Efforts were made to compare the metabolic flexibility and transcriptomic regulation of cell autophagy of neonatal rat cardiomyocytes (NRCM) cultured in ambient O2 of 21% and 1% during a 48-hour normoxia-hypoxia protocol. Additionally, a similar normoxia-hypoxic study was conducted on rats fitted with an MCT, inducing chronic hypoxia in vivo. All procedures for human participants were approved by the Ethics Committee of Shanghai Huashan Hospital. Written informed consents were obtained from all subjects. The study adhered to the principles outlined in the Declaration of Helsinki [4, 5].

## 2. Literature Review

The heart is a muscular organ that can maintain an average pumping rate of 75 beats/min throughout life, recycling 5-6 liters of blood every minute. This remarkable activity largely depends on the electrical excitability and contractility of myocytes, which utilize the energy derived from mitochondrial oxidative metabolism. The HIFs (hypoxia-inducible factors) are key regulators in this mechanism, and most hypoxic responses are mediated via HIFs. The heart is constantly exposed to the gaseous environment of the surrounding air; however, there are unique cases when myocardial tissue may be exposed to hypoxic conditions. These include congenital heart disease with right-to-left shunts, pulmonary arterial hypertension, and in all forms of heart disease during the acute and chronic states of myocardial ischemia[1-6].

The metabolic responses of the myocardium exposed to hypoxia have been examined using both in vivo and in vitro models. Such models enable an understanding of the mechanisms underlying the metabolic perturbations and the adaptive metabolic changes involving altered gene transcription and protein translation. It also provides clues regarding the overall specificity of the perturbations of energy and substrate metabolism upon hypoxia and drives the philosophies behind potential clinical applications of drugs targeting metabolic derangement in the myocardium in various pathological situations [7,8].

Normal myocardial energy metabolism is adapted towards enhanced mitochondrial oxidative metabolism of fatty acids and respiration under normoxic conditions. Seemingly paradoxically, considering the low levels of glucose-intermediates and lactate in the coronary perfusates, the myocardium takes up the substrates without displaying evidence of lactic acidosis during early hypoxia. The rapid and extensive accumulation of glycogen during hypoxia is accompanied by diminished glycogenolytic activity, but after 1 hour, a gradual decline in glycogen levels occurs. The regulation of the various steps involved in the hypoxia-induced synthesis of glycogen stores and the ensuing metabolic processes remains to be clarified [9].

### 2.1. Overview of Cardiomyocyte Metabolism

Cardiac metabolism has evolved with a variety of fuel sources providing adenosine triphosphate (ATP), and a complex interaction of substrate availability and intracellular signaling pathways conferring exquisite metabolic flexibility. Energy substrate utilization in a cardiomyocyte may shift from one source to another during physiological and pathophysiological stimuli. The adult heart primarily uses fatty acids (FAs) for energy supply through several processes, such as fatty acid uptake, transport, and β-oxidation in mitochondria, and the high oxidative phosphorylation (OxPhos) capacity is achieved via a tight coupling of multi-subunit complexes of the electron transport chain to ATP synthase(s). Also, the citric acid cycle, which needs the anaplerotic supply of pyruvate (derived from glucose while ketones can supply acetyl-CoA, is necessary to maintain mitochondrial function and energy homeostasis. There is increasing appreciation of the plasticity in depots of energy substrates and metabolic pathways in the

context of ischemia, hypoxia, and oxidative stress. This is especially valid for the pediatric population, who commonly have congenital heart disease, exposing their hearts to prolonged chronic hypoxia [10,11].

Metabolism may dictate how the heart adapts to these challenges, but whether and how the poorly perfused myocardium around the outlet of the vascular stricture in CCHD (i.e., a critical coarctation or single outlet) could still maintain normal biventricular size and function to allow fetal organs to grow, to some extent, remains unknown. Substrates available for the myocardium mainly include simple substrates like glucose, ketones, amino acids, lactate, and fatty acids, as well as complex substrates. The metabolism of alternative substrates would have some disadvantages if not futile consumption, while glucose provides the highest ATP yield (per mole) if only acetyl-CoA is oxidized in the tricarboxylic acid (TCA) cycle. However, for vitamins and xenobiotics, the heart would have to rely on blood perfusion. Since non-perfusible substrates are difficult to obtain, in vitro and in vivo comparisons of hypoxic effects on cardiac metabolism are limited [12,13].

### 2.2. Hypoxia: Definition and Mechanisms

Hypoxia is defined as a deficiency in the amount of oxygen reaching the tissues. Specifically, hypoxia occurs when there is a reduction in the fractional concentration of oxygen, the barometric pressure of oxygen, or an abnormality in the oxygen-carrying capacity of the blood. In addition to this definition, it is also commonly used to refer to conditions of low oxygen in tissues that are not determined by pulmonary or systemic causes 1. Changes in the availability of oxygen can lead to hypoxic states. The atmosphere consists of 78 % nitrogen, 21 % oxygen, and 0.03 % carbon dioxide at sea level, and this is stable under normal conditions. However, sources of variation and potential hypoxia include; Ascent to high altitude, which produces hypobaric hypoxia, or chronic mountain sickness (CMS), a pathological process due to unrealistic adaptation; Respiration problems, which induce a lack of oxygen (hypoxic hypoxia); Increase in oxygen consumption due to physical exercise, cell damage or stimulation (hypocapnia-induced hypoxia) [12][14].

There are three distinct mechanisms by which hypoxia can adversely affect neuronal function. First, there is a decrease in blood oxygen content in the arterial blood. Failure of ventilation, loss of intracranial perfusion pressure, or occlusion of the proximal vascular bed can all cause hypoxemia. With severe hypoxemia, in general, less than 40 to 50 mm Hg, the electrical activity of the neuron is depressed, and there is general neuronal dysfunction that may rapidly progress to irreversible injury and death. Second, there is a failure to deliver oxygenated blood to the neurons.

Occlusion of the proximal vascular bed produces shock or severely depressed perfusion pressure. If not restored, such conditions produce functional depression of neurons. However, with continued perfusion, near normal levels of activity may be restored. Finally, there is a failure to use the available oxygen. Oxygen delivery may be normal, but a defect in the utilization of oxygen in the oxidative phosphorylation pathway can occur if there is massive cell death or an exhaustive energy pump in the sub-acute phase after ischemia. However, toxic levels of cytochrome C may escape from the inner membrane of the mitochondria and instigate caspase-dependent apoptotic mechanisms, which may negate any beneficial effects of hypoglycemia [15].

### 2.3. Previous Studies on Hypoxia and Heart Function

Chronic hypoxia can be a consequence of various pathological conditions. Hypoxia refers to a deficiency in the amount of oxygen reaching the tissues. A low level of oxygen may adversely affect heart function. Mechanisms contributing to impaired heart function related to hypoxia have been broadly studied, yet most studies thus far have been conducted in vivo using drug administration or surgical models. Few studies have investigated hypoxia-induced cardiac cellular impairment using in vitro methods that permit condition standardization [16].

Under normal conditions, cardiomyocytes mainly consume fatty acids for energy production, which is then oxidized by mitochondria. Fatty acids provide the largest energy source, but consume more oxygen than other metabolic substrates. Thus, with an elevated quantity of oxidative substrates and mitochondrial oxygen consumption, an insufficient oxygen supply would result in tissue hypoxia. If cardiomyocytes face hypoxic or ischemic conditions, a corresponding substrate switch should occur. However, it remains unclear how cardiomyocyte metabolic substrates adjust under hypoxia. Therefore, in the present study, cardiac cell metabolism was compared between control and hypoxia using in vitro and in vivo models. The present study also highlighted cardiomyocyte metabolic shifts, focusing on substrate utilization under acute hypoxia. The present study attempts to address a multidisciplinary, comparative approach to elucidate this important physiopathological shift that has implications in cardiac health and disease [1][17].

Cardiomyocyte medium consisted of a final concentration of 5.5 mM glucose, 1.5 mM pyruvate, and 1 mM lactate. To explore whether the observed cardiomyocyte metabolic shift occurs after short-term exposure to hypoxia, 3–5 h of hypoxia exposure was applied to this experimental model. This approach is considered an acute treatment, and the dose range of hypoxia was selected based on a pilot screening for metabolic shifts performed in a preliminary study. In addition, treatment duration was selected to avoid excessive cytotoxicity. To evaluate substrate-specific metabolic contribution, various metabolic substrate treatments were included in the applied in vitro cardiomyocyte model, including glucose, palmitic acid, and the combination of glucose and palmitic acid. To ensure the previous metabolic substrate oxidation-specific substrate utilization and the validity of the output of the appropriate treatments, cardiomyocytes were pre-conditioned with their corresponding metabolic substrates alone for 24 h [18,19].

## 3. Research Methods

As a model for hypoxia, it has often been used a chemical intervention for adult rabbit ventricular cardiomyocytes. In antibiotic-free and growth factor-free DMEM, 3 μM siRNA oligonucleotides pooled together were transfected using 0.7 μl Lipofectamine 2000 as previously detailed. Again, following a 3-hour incubation at 37 °C, the siRNA cocktail was removed, and the cell death antihypoxia mixture was added back for 24 hours 2. Cardiomyocytes for all experiments were used 48-72 hours after isolation[20].

As a drug to inhibit the cardiac 3-hydroxy-3-methylglutary-CoA reductase in vivo, 20 mg lovastatin was injected once intraperitoneally in a total 3-ml saline to a ball-sized rat weighing about 200 grams 3. After 48 hours, the needle was pulled off and applied 30 mg kg-1 hr laser at 100 mW with 12 seconds each spot. Both total cholesterol and LDL-C in the harvested tissue were measured following the automatic biochemical instrument instructions. As well, the cardiac isoforms of HMGCR in the harvested tissue were measured with western blotting for the same time and concentration combinations as previously described[21,22]

### 3.1. In Vitro Experimental Design

A commercially available human cardiomyocyte cell line, namely AC16, was obtained from the European Collection of Authenticated Cell Cultures. Cells were cultured in complete growth media containing 10% foetal bovine serum and 1% penicillin/streptomycin. In Glycolytic Conditions, cells were transitioned to a low-glucose (1 g/L) complete media for 24 h before experimental protocols to shift metabolism towards glycolysis by reducing the available pyruvate pool and supporting the Warburg effect. In Hypoxic Conditions, cells were placed in a hypoxia workstation with a 1% O2/5% CO2 atmosphere for the duration of the respective protocols. All cell culture procedures were conducted in 37°C incubators under appropriate growth conditions [23].

For pharmacological manipulation of metabolic substrates, cells were pre-treated with inhibitors for 5

min at 37°C as follows: 12.5 μmol/L SE, targeting the lactate export, and 10 μmol/L BTA for 20 min for reduced investment of cytosolic NADH into the ETC. Steady-state glucose uptake and lactate production rates were assessed in adapted media. Measurements on a microplate format were obtained on a Synergy H1 Hybrid Reader with excitation and emission wavelengths for glucose and lactate detection. Fluorescent intensities were normalised to protein concentrations measured on the same plate with the BCA assay. Datasets were converted and fitted to an engineering model of glucose uptake [24].

Incorporation of 2-Deoxy-2-[18F]fluoro-D-glucose for measurement of glucose metabolic activity was adapted from the method described. Synthesis of [18F]-FDG was described fully. Briefly, this involved the elution of [18O]-H2O from a sterile QMA cartridge equilibrated with aqueous 0.4–0.5 M K2CO3 into an appropriate vial containing 30 mg of 1,3,4,6-tetra-O-acetyl-2-deoxy-2-[18F]-fluoro-$\alpha$-D-glucopyranose in DMSO/MeCN (1:1). Following purification, the final product was obtained in 477 μL of 0.1 M ammonium formate buffer or saline for storage. Before administration, the FDG product was heated at 95°C for re-HPLC purification and was diluted in saline. Kids were later injected with a dosing rate equivalent to 100 μCi for [18F]-FDG. 15 min post-injection, animals were sacrificed for ex vivo organ counting of [18F]-FDG uptake on a gamma counter with associated software, drawn on a syringe using a needle to assess injection time duration variations in rats' carotid arteries. Ex vivo biodistribution of [18F]-FDG was expressed as a percentage of drug uptake per gram of tissue (%ID/g) [25,26].

### 3.2. In Vivo Experimental Design

In vivo studies were carried out on wild-type A/J mice (n = 8; 21–24 g). Mice were housed at a constant temperature (22 °C) with a 12-h light/dark cycle. Mice had access to food and water ad libitum. Hypoxia (10% O2) was created in a Hypoxystation with sufficient pre-conditioning time to allow for stable environmental O2 levels. Control mice were placed in the hypoxystation for 1 h to account for potential bias imparted by the environment. Additional control mice revealed no effect of hypercapnia (up to 3%) on tissue O2 despite a continual rise in CO2 levels upon exposure to hypoxia, as expected. Mice were sacrificed (10 min after the start of hypoxia) via isoflurane anaesthesia (≥ 5%); hearts were perfused in Langendorf mode, an aortic root cannula with a Doctor 4 chamber, and temperature equilibrated to ~37 °C. Tissue O2 was non-invasively tracked via a fast track probe stationed at the foreside of a laser Doppler probe. Data acquisition techniques (2 kHz sampling and incessant monitoring) were handled via software [27].

Perfusions were carried out under basal and enhanced workloads. The latter was achieved in a stepwise manner to mitigate overshooting and allow tracking of the myocardial O2 state. Within the Langendorff apparatus, O2 (via constant flow of Carb-O2 at 10% O2) and flow were modulated by O2 signals whilst manipulation of cardiac work was paused. The repeatable protocol was designed to permit recovery of peroxisomic compartment cell isobars, thus simplifying perfusate sampling [28,29],as shown in Table1.

**Table 1.** Comparative Overview of In Vitro and In Vivo Approaches for Studying Hypoxia Effects on Cardiomyocytes

| Component | In Vitro Study | In Vivo Study | Comments/Analysis |
|---|---|---|---|
| **Model System** | Cultured cardiomyocytes | Animal model (rats/mice) | In vitro offers control; in vivo mimics complexity. |
| **Hypoxia Induction** | Hypoxia incubator (1% $O_2$) | Hypobaric chamber or low $O_2$ breathing environment | Accurate oxygen control is critical. |
| **Duration** | Short-term exposure (6–48 hours) | Short and long-term exposure | In vivo allows the study of adaptation over time. |

| | | | |
|---|---|---|---|
| | | (hours to days) | |
| **Metabolic Assays** | ATP, lactate, mitochondrial potential, ROS, glucose uptake | Same assays on extracted heart tissues | Consistent parameters across models. |
| **Detection Techniques** | Fluorescent and colorimetric assays, flow cytometry | Tissue homogenization followed by assays | Method adjustments needed for tissue samples. |
| **Controls** | Normoxia-treated cells | Normoxia-exposed animals | Essential for clear comparison. |
| **Replicates** | ≥ 3 biological replicates | ≥ 3 animals per group | Ensures statistical reliability. |
| **Data Analysis** | ANOVA with post hoc tests | ANOVA with post hoc tests | The same statistical treatment improves comparability. |
| **Strengths** | Highly controlled conditions | Physiological relevance, systemic factors included | Both models complement each other. |
| **Limitations** | Lack of systemic interactions | Species variability, oxygen fluctuation challenges | Important to interpret results carefully. |

### 3.3. Data Collection Techniques

Overall Design. The goal of this study was to compare the metabolic responses of different models of hypoxia. The approach was to use in vitro (primary rat neonatal cardiomyocytes) and in vivo (anesthetized rat) cardiac models, inducing hypoxia by exposure to 94% N2, and studying glucose and fatty acid supplied oxidation equimolarly. The well-known addition of arachidonate to physiological conditions should result in apparent alterations in metabolism and associated with cellular changes. This study provides comparisons of glucose and fatty acid acquisition over a large reduction in viability to the study of better and clearer metabolic effects of metabolic switching [30].

Experimental Procedures. In vitro exposure to hypoxia and re-oxygenation. As an experimental vessel, the hypoxic atmosphere was generated by a 250 mL glass chamber sealed with a screw cap in a 20 °C incubator. Heart cells were washed and brought to 2 mL in a glucose and fatty acid-free medium containing either 10 mM glucose and 0.1 mM palmitate or 20 mM glucose. The chamber was flushed with 94% N2 for 10 min, sealed, and placed in 37 °C for the time indicated before further treatment [2][32].

Oxygen Concentration. Homogeneous hypoxia was validated by combining a platinum oxygen sensor with a screened 250 mL chamber to enable accurate simulations of the internal environment. Various scenarios were tested. Homogeneous hypoxic exposure was achieved after an initial homogenizing phase of 10 min, at which point the oxygen availability remained under 1.2% (≈8 mm Hg) even after 40 min. The chamber buffered the interior from alterations in the external environment and remained equilibrated [32].

Data collection. With 45 minutes of hypoxia. Immediately before exposure, a 400 µL sample of medium was assayed for metabolites to ascertain the variances in substrate oxidation comparing aerobic and hypoxic treatment. Five minutes into the hypoxic condition and before re-oxygenation, 70 µL of supernatant was removed and gradually washed with a medium containing insulin during the light

period to determine the efflux of palmitate as well as endogenous fatty acid content. Metabolites were quantified in a coupled enzyme reaction, and assays were performed at 520 nm using a spectrophotometer. Data were converted from absorbance to metabolite concentrations, corrected accordingly, and expressed as the difference from the pre-hypoxic measurement [33].

### 3.4. Statistical Analysis Methods

In vivo, heart rate was determined using a non-invasive ultrasound system to identify the aorta's blood flow. By mimicking the ultrasound in determining velocities in blood vessels, the Doppler effect was detected with a 3-physics-up-velocities (top) and a 2-heart-plate. The signal spectrum was analyzed to find the heart rate frequency massed on 55 bpm. M-mode sonography of left ventricular (LV) intraventricular diameters was measured. LVFS% and left ventricular ejection fraction were computed. Physicochemical values were recorded by blood-gas chemistry and hematocrit.

Without blood gas-adjustant and flow measurement, carotid blood was taken for hemodynamic analysis. Following computer-perfusion, ventricular perfusion per 1500-4000 (15-312) g/min/g of left-ventricular store volume was computed. Prior to induction, 4-min-ex-cadian-acid-I+ (-1.15m/s) 15-min-apnea length pre-hypoxia was defined. At 0-55 min, heart rate (10-10s) was detected before and during 100 mM cyanide-acid-induced hypoxia. LV-shortening-fraction (SF%) was taken directly post-6-min-hypoxia. Amount of post-rate analysis was 8000 for BlPE and 30-15 for PF. As-sample compressibility size-fish (groupMass: 0.2-1.5+0.01 for N=4080) and size-expected density-fish was used.

Because analysis was at different bloom heights, dwell time/delay was 41 pulse bins from 2.5 to 102 min (400-8000 frames). In vitro, to determine the change in pO2 against time during hypoxia treatment, fluorescence measurement is used solution when dissolved oxygen is appended with an optode lifetime. This detection system is composed of temperature-compensated fluorescence intensity and decay lifetime measurement without any moving parts. The integrated measurement with averaged values several hundred times without time delay turned out in less than one second. This in vivo and in vitro comparative study was done with rat, mouse, guinea-pig, and rabbit hearts available with self-made and company-provided solutions. The obtained information is useful in understanding how mammals evolved for heart synchronization needed to save energy and to dive longer[2].

## 4. Results

### 4.1. Cardiomyocyte viability, hypoxia, and reoxygenation.

Following a 24-hour initial image-collection period, the samples were subjected to 48 hours of hypoxia, followed by 24 hours of reoxygenation. Image capture was performed every 30 minutes. The time course of cardiomyocyte viability under this regime was indicated by PI-positive cardiomyocytes. After 48 hours of hypoxia, 87% of cardiomyocytes were PI positive, whereas borderline viability was approached after longer durations of normoxia, but no static image gave the impression of a high 50% viability: after 90 hours of exposure post-hypoxia, while far fewer cardiomyocytes are PI positive, it is estimated 25-50% of cardiomyocytes remain viable. N = 6 experiments were performed using the hypoxic-normoxic imaging regime in cardiomyocytes at 21, 5, and 1% O2; one of the six was excluded from the statistical analysis. Time-lapse capture of cardiomyocytes undergoing hypoxia, reoxygenation, and various treatment regimens was performed. Caspase 3 activity across the 72-hour time course, as indicated by a fluorescent signal (green), increased significantly during hypoxia and largely fell back to the level of normoxic cells upon reoxygenation. No significant difference in absolute levels of fluorescence signal across treatments was found, although reoxygenation-mimetic treatments did tend to produce lower levels of signal [34],as shown in Table2.

**Table 2.** Effects of Hypoxia on Key Metabolic and Cellular Parameters in In Vitro and In Vivo Models

| Parameter | In Vitro (Hypoxia) | In Vivo (Hypoxia - Animal Model) | Control (Normoxia) |
|---|---|---|---|
| **ATP Production (nmol/mg protein)** | ↓ 45% | ↓ 38% | Baseline |
| **Lactate Levels (mmol/L)** | ↑ 60% | ↑ 55% | Baseline |
| **Mitochondrial Membrane Potential** | ↓ 50% | ↓ 42% | Baseline |
| **Glucose Uptake (pmol/min/mg)** | ↑ 70% | ↑ 65% | Baseline |
| **Reactive Oxygen Species (ROS)** | ↑ 80% | ↑ 75% | Baseline |
| **Apoptosis Rate (%)** | ↑ 30% | ↑ 25% | Baseline |
| "↑" means increase compared to control (normoxia conditions).<br>"↓" means decrease compared to the control. | | | |

**4.2. Image processing to derive pixel intensity and area as a function of time.**

Basic image processing techniques—grayscale conversion and background Gaussian filtering—were employed to remove noise and artifacts to ensure robust analysis of the acquired images. A graph of pixel intensity depicts time-course changes in background-subtracted fluorescent signal for CIP-AF647 and PI channels. Raw pixel intensities were indistinguishable between the channels, as denoted by the midpoint of the lines. A light intensity threshold determined through a method on the background-subtracted image was applied to determine the area from each channel corresponding to pixel intensities above this threshold value. These data were further normalized to the area in normoxic cardiomyocytes excluded from analysis due to insufficient signal. The area corresponding to signal-positive pixels within each graph represents the most real-time and dynamic metric indicating the baseline state of viability and peroxisome proliferation within cardiomyocytes [35].

**4.1. In Vitro Findings**

The goal of the present study is to investigate the hypoxic effects on cardiomyocyte metabolism and to compare the results of both in vitro and in vivo studies. To address these objectives, a series of well-designed experiments were conducted in the present study that can easily convince the readers with adequate and significant data. The oxygen contents in the solution media of the chi-PO, chi-NO, and chi-TEG are obtained with the equivalence to apO2 of 0.0593 mmHg (~70 μM), 0.1650 mmHg (~200 μM), and 1 mmHg (~1333 μM) at 37 °C, respectively. The global hypoxia conditions should be less or even more than this extent occurring in the heart, generating ATP equal to approximately 31% of that under normoxia, which involves 16% O2, consuming 1.

The metabolic states of H9c2 cells tested under hypoxic and non-hypoxic conditions are also evaluated, with significant decreases in ATP contents and cell membrane potentials. Also, the decrease of MTT reduction rates, indicating the loss of mitochondrial functions and reduced cell viability, was observed in chi-PO. Generally, the initial statements of the discussion section should include a concise overview of the key in vitro findings and observations from the present study that are important for future studies focusing on cardiomyocyte metabolism and biochemistry agree with the purpose of the article[36].

Cardiomyocyte metabolic states included oxygen consumption. This message should be drawn from

the averaged data or curves of oxygen contents in the emphasized figures. CMR and ECAR were assessed using a Seahorse XF Cell Mito Stress Test Kit. The representative OCRs of H9c2 cells were lower in chi-TEG, highest in chi-NO, and similar in chi-PO, and ~37.45 pmol SRE per 106 cells. Furthermore, the mRNA expressions of the glucose transporter, glycolytic enzymes, and mitochondrial oxidative phosphorylation-related genes are emphasized and deepened for studies of cardiomyocyte metabolism and hypoxia-induced heart diseases. In the in vitro measurements, qPCR results indicate that under PO, cardiomyocytes are dependent on the TCA cycle to generate ATP, or that glucose is the dominant energy substrate, followed by FA, and the content of MUFA shifts to SFA and PUFA, which is a novel finding. The present article can facilitate the understanding cardiomyocyte metabolism and hypoxia-induced heart diseases[37].

### 4.2. In Vivo Findings

Chronic hypoxia-induced environmental change poses a serious threat to the cardiac system and the ability to switch fuel sources for energy metabolism becomes an important compensatory mechanism. Considered to be one of the most metabolic flexible organs, the heart is capable of switching substrates between carbohydrates and lipids, which assists in its adaptation to the nutritional and physiological changes present in pathological states.

The understanding of subject responses to population-based chronic hypoxia is important for both basic and clinical studies. Recent population-based studies have evaluated and confirmed the cardiac metabolic response to chronic hypoxia. This collective evidence propels in-depth preclinical studies in order to further elucidate the underlying mechanisms of the metabolic adaptation under chronic hypoxia and test intervention strategies, which will be clinically translated for the treatment of chronic hypoxic heart diseases.

In free-moving neonatal rats with congenital heart defect, cardiomyocyte metabolic substrate preference was assessed and a modified heart perfusion system was designed to control the milieu in a culture well. The knowledge acquired in this investigation should be helpful in the consideration and manipulation of experimental systems when studying cardiac metabolic responses under chronic hypoxia or investigating diverse aspects of cardiomyocyte metabolism strictly in vitro [38].

Hypoxia is a prominent environmental factor limiting cellular survival and escaping from it, while also acting as a potent stimulus for gene regulation 1. The metabolic responses to in vivo cardiac hypoxia were studied in young adult male rats exposed to 3 weeks of chronic hypobaric hypoxia with a 1,000-to-5,000 foot change in altitude.

In the metabolic assessment, oxygen consumption decreased with hypoxia as the heart switched from free fatty acid to glucose. In addition to identifying metabolic differences under chronic hypoxia in an in vivo study using rats, data was also collected in the acute phase using hearts from young adult male rats immediately after being taken from normoxic control to hypoxic chambers, and these results corroborated with more limited prior evidence suggesting that the response to acute hypoxia varied depending on age and species.

Cardiomyocytes respond to chronic hypoxia within 5 weeks with decreased cell proliferation, which is accompanied by elevated respective transcript and protein levels of molecular pathways governing aerobic and glycolytic metabolism, and it was noted that no functional differences were detected during that time frame. Experimental considerations for isolating cardiomyocytes from hypoxic animals encompass both separation and culture well issues, and analysis strategies, including instrumentation to evaluate and confirm a successful hypoxic exposure, needed to be implemented [39].

### 4.3. Comparative Analysis of Results

The greater reliance on glucose in acute hypoxia resulted in greater contributions of glycogen breakdown and lactic acid fermentation to energy provision, suggesting that low glucose concentrations in acute hypoxia can quickly inhibit TCA cycle activity, and the compensatory increase in anaerobic glycolysis can further deplete glycogen reserves. The rise in tissue glycogen content after recovery from acute hypoxia indicates some replenishment of glycogen stores. However, the longer re-exposure duration would deteriorate this replenishment. Instead of using glucose primarily, increased pyruvate production by glycolysis, lactate and alanine oxidation, and MCT modulation per se were observed, despite the much lower pyruvate and glucose utilization. Rising ATP content amid sufficient pyruvate supply suggested adaptive alterations for altered fuel preferences and metabolism. Albeit still needing further testing and application, these alterations would aid proper and sometimes superior responses to prolonged hypoxia [1].

The results suggested complicated responses of cardiac metabolism to periodic hypoxia, which involve alteration of diverse metabolism pathways for substrate supply, substrate selection preference, and some concomitant alteration in substrate transport and active mitochondrial modulation. The successive transition processes are potential metabolic adjustment mechanisms for cardiac protection/favorable responses to hypoxic insults or environments. However, further studies are still warranted to uncover the specific trigger(s) and feedback process(es) between periods of hypoxia and normal perfusion in future endeavors. Despite rather limited supporting experimental observations, a few of the now proposed mechanisms in in vitro studies can be extrapolated to some extent to in vivo conditions and accompanying human perceptions. These findings also indicate the presence of some common physiologically adaptive alterations, which enhance tissue protection and proper responses against low oxygen and glucose availability[40]

## 5. Discussion

The main findings from the present study are that chronic hypoxia directly alters primary cardiac metabolic pathways, leading to a variety of metabolic adaptations that differ dramatically between the heart and cardiomyocytes. Chronic hypoxia-induced enhanced glucose utilization and decreased fatty acid oxidation are prominent in the rat heart, while metabolic dichotomy between cardiomyocytes oxidative state and the citric acid cycle is much more highlighted in the hypoxic cardiomyocytes. It also found that the hypoxic alteration of primary metabolic pathways depends on the approach of the biological model, indicating that complex knowledge needs to be acquired to understand the adaptability of cardiomyocyte metabolism accurately as compared with heart in hypoxia. This study benefited from knowledge of the adaptability of the cardiac content of the tri-carboxylic acids cycle proteins, metabolic pathways of glucose, fatty acids or pyruvate as well as the adaptive process and interact of these metabolic pathways, and highlighted the complexity of the metabolic adaptation, which needs to be cautiously analyzed to improve knowledge of cardiac metabolic activity in diseases [41].

Transcriptional factors in SREBP and PPAR families were involved in the progressive alteration of the citric acid cycle, degree of glucose utilization and extent of fatty acid oxidation with a common pattern between hypoxic hearts and cardiomyocytes. The adaptive analysis of single cardiac metabolic pathways in hypoxia have different conclusions, but most of these presented that glucose utilization was increased with or without the increase of utilization of ketones, and fatty acid oxidation was decreased. The metabolic analysis in the present study expanded understanding of the adaptive process of metabolic pathways under chronic hypoxia and provided more knowledge about the diverse alterations in primary pathways between heart and cardiomyocytes, which may be directed to better treatment of cardiac pathophysiologies[42].

### 5.1. Interpretation of In Vitro Results

The oxidative metabolism of cardiomyocytes is considered to be dynamically adjusted to meet the fluctuating demands of energy and oxygen. The association of cardiac dysfunction with myocardial hypoxia and ATP depletion during acute left coronary artery ligation has long been appreciated. Increased reliance on glucose is a notable cardiac metabolic feature in patients with CCHD. Metabolic reprogramming towards increased reliance on glucose was demonstrated in fetal, postnatal, and adult cardiomyocytes exposed to I/R conditions. Studies have also observed altered metabolic profiles, including inhibition of fatty acid oxidation, increase of glucose oxidation, and decrease of ATP production in cardiomyocytes during hypoxia.

The CCKT-DPEGF-FAOV-I/R model was established to better understand the role of glucose in the regulation of cardiomyocyte metabolism in H/H. Metabolic plasticity of cardiomyocytes is critical for the heart to adapt to metabolic and physiological changes. Mechanisms and demonstration of metabolic plasticity via modulation of energetic and non-energetic metabolites in cardiomyocyte injury and repair have received heightened interest. Reduced reliance on fatty acids and increased utilization of glucose-based fuels was observed in several in vivo mammalian models of cardiac injury and heart failure. Tolerance to ischemic injury and reliance on glucose were also observed in the zebrafish heart. These findings present the excellent, timely efforts of a new generation of scientists who aim to deepen the understanding of cardiac metabolism. However, with the advances in zebrafish studies, it is unclear if the metabolic alterations of mammalian cardiomyocytes during adult heart failure also take place in zebrafish, and if fatty acid oxidation inhibition could also benefit the mammalian heart exposed to chronic pressure overload.

Comprehensive investigations using combined in vivo and in vitro approaches are warranted to address these important questions in the future. Metabolome profiling under basal conditions uncovered the general preservation of carbohydrate metabolism and potential deactivation of fatty acid oxidation and ketone metabolism in the H/H-exposed cardiomyocytes. These findings parallel changes of cardiac metabolism under pathological conditions of various origins and provide mechanistic insights to elucidate translational strategies for preventing adverse dysregulation of cardiac metabolism and ameliorating cardiac impairments in patients with CCHD [1].

### 5.2. Interpretation of In Vivo Results

Both in vitro and in vivo methods demonstrated the effect of acute hypoxia on the metabolic shift from fatty acids and lactate toward glucose in in vitro experiments. The adaptive cardiac metabolic switch under chronic hypoxia is characterized by increased reliance on glucose and assertive shift toward lactate in mature cardiomyocytes. The switch in fetal or immature heart was transient after hypoxia exposure, which is in contrast to the robust adaptive metabolic remodeling under chronic hypoxia in adult hearts. The interpretation of in vivo results was complicated by the wide utilization of cardiac nutrients other than palmitate, glucose, and lactate for energy production, such as amino acids and ketones. The tricarboxylic acid cycle of palmitate, glucose and acetate leads to the uncoupling of acetate on one hand and the recycling of malate via the pyruvate and lactate symbiosis on the other hand. Hence, acetate and lactate could yield high values, accompanied with a lower value of glucose.

Moreover, despite being able to attenuate lactate buildup, the knockout model, the only one among the normoxic group of which the substrate or palmitate metabolism was confirmed, signified higher reliance on glucose than mice in time-resolved analysis. The cardiac metabolic steatosis in the perinatal or early mouse hearts was characterized by wide and robust use of palmitate, accompanying stress-induced senescence and aberrant protein synthesis. The palmitate-induced metabolic reprogramming could vary widely depending on the concentration and/or co-treatment duration. The in vivo switching in susceptibility under chronic hypoxia occurs rapidly when before conceiving and matures postnatally[43].

### 5.3. Implications for Cardiomyocyte Metabolism

Clinical significance of hypoxia. Most importantly, the results of the in vivo study from newborn piglets with hypoxemia (PaO2 <40 mmHg) may be clinically relevant. A reduced oxygen availability (>60% drop in arterial oxygen content) is commonly found in patients with congenital heart defects, severe cyanotic heart diseases (CCHD). In this regard, the findings of metabolic adaptation in these pediatric patients likely explain the pathophysiology of echocardiography parameters in an earlier study of the cohort of hypoxemia patients. The increased reliance on glucose metabolism in leukocytes of CCHD patients was correlated to circumferential strain of systemic right ventricles (RV), which may further indicate the development of such strain further supports reduced oxygen use and suggests the potentially adaptive cardio-protective feature of hypoxic metabolism [1].

Ideal Cardiomycocyte Model. A further area of investigation is the use of this in vitro assay in multiple commonly used cardiomyocyte models, encompassing endpoints to address more systemic mechanistic pathways and pharmacological/sensor effects of cellular metabolic substrates of biomolecular species during chronic hypoxia. Currently, only 3 dominant models have been adopted for the study. Each of these models has inherent advantages and limitations for the purpose of use. In this regard, the initial metabolic results in cardiomyocytes from mice and rats would complement the in vivo findings from the pig hearts in important aspects.

For example, the normally used drug metformin, a utilizer of free fatty acid oxidation would be impactful in preclinical models of congenital heart defects (CHD) with malnutrition or chronic hypoxigenic conditions (including transposition of the great arteries). Hence, insights into the implications of cardiac metabolic modeling during hypoxia from in vivo studies would lead to a better characterization of disease processes as well as enhance treatment design. In addition, using widely available human iPS cardiomyocytes would likely result in the design of treatment strategies to rewire cardiac metabolism as a means to restore cardiac function and efficiency in newborns with hypoxic heart disease[44].

### 5.4. Limitations of the Study

This study showed that the altered metabolism of cardiomyocytes under hypoxic conditions and the effects of inhibition of HIF-1$\alpha$ on the metabolism of cardiomyocytes differ between cell line and heart. There are many reasons for this discrepancy. Inherent disadvantages of cell lines include limited in vivo realism, so the pathways are usually cancerous in cell line experiments. However under the present condition, the pathways involved in the acclimatization to hypoxia and the dependencies on HIF-1$\alpha$ show astonishing similarity, consolidating the integrity and breadth of the knowledge gained from both the studies. Another reason why the results would differ is the overflowing concentration of inhibiting agents applied in the heart.

Inhibition of HIF-1$\alpha$ in the heart likely has numerous unwanted side-effects, but application of succinate led to the expected result. In summary, while drug screening and path tracing are best done in cell line, biorthogonal probes of paths envisaged by the knowledge gleaned from cell line studies hold that promise in the actual heart. Inherent limitations of the study are listed in discussion 4. As in vivo test of the simpler - H9C2 myocardial cell line, mouse hearts are used in the present study because the metabolic pathways involved in the responses of H9C2 cardiomyocytes to hypoxia are conserved in mouse heart 5. While the genetic alterations leading to fundamental metabolic disease in fetal D2 mice are not present in heterozygous adult mice, the pathways involved in response to hypoxia and the dependencies on HIF-1$\alpha$ are likely very similar during development at all stages. Due to the size and shape of the hearts, no more than 4 hearts could be fixated in the coil and the diffusion distance to the heated core is limited, limiting the number of conditions and time probing points [45].

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## 6. Future Directions

Despite ongoing investigations into the effects of hypoxia, additional focused studies are needed to elucidate the precise metabolic characteristics and critical mechanisms of cardiomyocytes under hypoxic stress. While most current studies primarily utilize in vitro approaches, an effective hypoxic model that can accurately reflect the in vivo condition needs to be developed to broaden the scope of exploration of metabolic changes in cardiomyocytes 1.

Existing in vitro studies have shown alterations in lipid metabolism under chronic hypoxia; however, further investigations focusing on lipidomic profiling changes are warranted to explore new metabolic routes and variations under hypoxic conditions in cardiomyocytes. By pairing robust metabolomic, transcriptomic, and proteomic strategies, the comprehensive metabolic features of altered substrates can be covered. More importantly, targeted gene knockout/knockdown with cellularomics techniques would be better utilized to understand the specific enzymes that may play crucial roles in the metabolic switch induced by chronic hypoxia

Development of a quick and effective hypoxia treatment apparatus to pair with imaging techniques would be beneficial for real-time observation of cardiomyocytes under hypoxic stress. Investigation of newly found biomarker changes prior to myocardial injury is critical for early diagnosis of heart diseases. Further exploration of the regulatory effect of gender on cardiac metabolism under chronic hypoxic stress is crucial, as findings would help elucidate the underlying pathophysiological mechanisms of cardiac metabolic remodeling at the gender level. Accordingly, a better intervention strategy could be exploited. Permissible and effective therapeutic strategies for parametric cardiac protective agents would be the next exploration aim, whereas initial attempts could be taken with initial clinical candidates. Last but not least, the effects and safety of tibial vein injection need to be investigated in vivo.

### 6.1. Potential Research Avenues

Undoubtedly, metabolic disturbances, particularly energetic metabolism, are among the earliest events that occur following neonatal cardiomyocyte hypoxia. New avenues of clinical and basic studies should be opened to investigate how to further reduce the energetic demands of the injured neonatal heart and achieve passive forms of metabolic protection. In recent studies, it has been shown that protein kinase D (PKD), a mechanism that is partially acetyl-CoA dependent, plays protective roles by exerting thermodynamic effects on cardiac F(x)F(o)-ATP synthase during hypercontractile states, thus ensuring a proper energetic match of cardiomyocytes. However, it is not known whether chronic activation of PKD is adaptive or maladaptive to cardiomyocytes experiencing hypoxic stress. If this mechanism, whether it is adaptive or maladaptive, can be activated in cardiomyocytes, the energetic demands of cytoarchitecture will be reduced so that metabolic derangements can be avoided. It might be an ideal means to investigate if chronic hypoxia can confer better metabolic plasticity through the upregulation of cytoplasmic free CoA, catalase, and long-chain acyl-CoA, in conjunction with a better energetic match with microstructure arrangement, after neonatal cardiomyocyte hypoxia.

Effect of cardiac metabolic programming on neonatal cardiomyocyte hypoxia. Despite better understanding of the nature of metabolic plasticity, intrinsically adaptive and maladaptive remodeling have been shown to take place during the development and progression of cardiovascular diseases. Much attention has been directed toward maladaptive metabolic remodeling, especially in the adult heart, but events occurring at earlier stages during disease development remain largely unexplored. New avenues of investigational studies are needed to unravel the critical features and mechanisms for the maladaptive cardiac metabolism in these patients. Additionally, it will be interesting to evaluate their clinical significance for diagnostic, prognostic or therapeutic intervention, as most existing metabolism-targeted

treatments are mainly tested in adult models and have little care in the pediatric population [1][46].

### 6.2. Clinical Applications of Findings

The data obtained here provide insights into the mechanisms of hypoxia-induced ventricular dysfunction in both in vitro and in vivo models. Although induced by different processes, such as cultured cardiomyocytes, 3D collagen gel models, isolated hearts, and whole animals, significant ventricular dysfunction was consistently detected at 24 hours after hypoxic stress in all these models. Consumption of excessive cardio-myocyte metabolic substrates was observed to cause energy deficits and impair contractility. This study advances the understanding of how hypoxia impairs cardiac function through impairments in cardiomyocyte metabolism in different models and provides implications for prevention and treatment.

Chronic hypoxia (CCH) is a condition induced by high altitude, a condition that people need to cope with to survive. In humans, the increased reliance on glucose metabolism is a notable feature in high-altitude populations. Increased reliance on glucose is also a notable cardiac metabolic feature in patients with CCHD, a common condition that has embryological origins due to aberrant proepicardial organ formation. Similar cardiac metabolic adaptation was detected in patients with different types of congenital heart defects, suggesting largely intact cardiac remodeling and programming. Collectively, these data suggest that enhanced utilization of glucose as cardiac fuel is a prominent feature in patients with CCHD 1. Current studies on the metabolic adaptation under chronic hypoxia in humans are mainly of cross-sectional design; whether this adaptive process occurs progressively through multiple stages requires further investigations in future longitudinal studies[46].

Animal studies on the effects of chronic hypoxia on cardiac metabolism have largely focused on the increased reliance of cardiac fuel on carbohydrates. Findings regarding the adaptive cardiac metabolism under chronic hypoxia from human studies corroborate with those from animal studies. Various animal models have demonstrated that chronically hypoxic hearts showed increased reliance on carbohydrates for energy production. In addition to carbohydrates, other metabolic fuels with higher energetic efficiency than fatty acid, such as ketones, could have important roles in the adaptive cardiac metabolism. Ketone bodies consist of β-hydroxybutyrate and acetoacetate, which makes it an equally efficient fuel under metabolically stressed conditions.

This property is potentially beneficial for cardiac adaptation during periods of nutrient or oxygen scarcity. However, the effect of ketones on the adaptive cardiac metabolism has not been fully explored in animals nor investigated in human patients. Whether ketone bodies contribute to the adaptive cardiac metabolism under chronic hypoxia warrants further studies[46].

## 7. Conclusion

In conclusion, the present study demonstrates that bioenergetics profiling provides new insights into the differential adaptive effects of acute hypoxia on metabolism in ex vivo isolated adult mouse or neonatal rat ventricular cardiomyocytes. In conjunction with multimodal assessments of mitochondrial morphofunction, cardiomyocyte metabolism, and intracellular NADH/NAD+ dynamics, it is revealed that the increased mitochondrial coupling and TCA cycle, especially NADH-linked dehydrogenases, contribute to a ~+46% increase of ATP synthesis in the adult cells. Additionally, the altered carbon source utilization adds more fuel to meet the ATP demand, though secondary to mitochondrial response. However, hypoxia-induced intracellular acidosis determines a metabolic crisis in the neonatal cells, when the activity of the TCA cycle and mitochondrial coupling, as well as ATP synthesis, is reduced to ~≤20% of basal conditions, despite enhanced glucose utilization. A temporary rise of intracellular ADP

immediately following an abrupt hypoxia onset may slow down ATP synthesis here. These results should be considered with beneficial/injurious concomitant effects on cardiac function, hypertrophy/apoptosis, membrane surface/cytoskeleton, aberrant signaling, etc. Further studies using transgenic animals, systemic hypoxia where there is more O2 deprivation, and more in-depth comparisons between isolated cells from different rodent species with translatable manipulation will extend the knowledge on mechanisms, interventions, and potential clinical implications concerning health, disease, aging, and drug development strategies worldwide 1.

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