IGF1 Knockdown Hinders Myocardial Development through Energy Metabolism Dysfunction Caused by ROS-Dependent FOXO Activation in the Chicken Heart

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Received 16 May 2019; Revised 16 November 2019; Accepted 20 November 2019

Academic Editor: Franco J L

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Insulin-like growth factor 1 (IGF1) is a multifunctional cellular regulatory factor that can regulate cell growth and development by mediating growth hormone stimulation. However, the mechanism of IGF1 dysfunction in cardiomyocyte development is seldom reported. To study this, we employed the models of IGF1 knockdown in chicken embryo in vivo and in cardiomyocytes in vitro. We detected the antioxidant capacity, PI3K/Akt pathway, energy metabolism-related genes, and myocardial development-related genes. Our results revealed that the low expression of IGF1 can significantly suppress the antioxidant capacity and increase the ROS (P < 0.05) levels, activating the AMPK and PI3K pathway by inhibiting the expression of IRS1. We also found that myocardial energy metabolism is blocked through IGF1, GLUT, and IGFBP inhibition, further inducing myocardial developmental disorder by inhibiting Mesp1, GATA, Nkx2.5, and MyoD expression. Altogether, we conclude that low IGF1 expression can hinder myocardial development through the dysfunction of energy metabolism caused by ROS-dependent FOXO activation.

1. Introduction

Insulin-like growth factors (IGFs) are a group of polypeptides with growth-promoting function. The secretory cells are widely distributed in tissues such as the liver, kidney, lung, heart, brain, and intestine [1]. IGFs play an important role in cell proliferation, differentiation, individual growth, and development [2]. The IGF family has two subtypes: insulin-like growth factor 1 (IGF1) and insulin-like growth factor 2 (IGF2). The production of IGF1 is dependent on the growth hormone (GH), which is an important growth factor in life processes. Myocardial development is a complex process that is regulated by complex molecular networks composed of many development-related factors. Many studies have shown that various signal pathways are involved in the development of vertebrate hearts, including the bone morphogenetic protein (BMP), Wnt, Notch, and fibroblast growth factor 4 (FGF 4) signal transduction pathways. The BMP and Wnt signaling pathways play an important role in the development of early mesoderm cells into cardiomyocytes; they act on the cardiac-specific transcription factor GATA4 and Nkx2.5 through a signal cascade process, promoting the differentiation of cardiac precursor cells into cardiomyocytes [3, 4]. Musarò et al. demonstrated that localized synthesis of IGF1 is closely related to skeletal muscle hypertrophy, the molecular pathways of which are similar to those responsible for cardiac hypertrophy [5].

Insulin is a hormone secreted by islet β cells, and it is the only hormone that reduces blood sugar and promotes the synthesis of glycogen, fat, and protein in animals [6]. Insulin has been proven to regulate metabolism and growth in the body [7]. The insulin receptor (IR) is a tetramer formed by
two alpha subunits and two beta subunits linked by disulfide bonds. The two alpha subunits are located on the outer side of the plasma membrane and have a binding site for insulin; the two beta subunits are transmembrane proteins that play a role in signal transduction. The IR family contains IR, insulin-like growth factor receptor (IGFR), and insulin receptor-related receptor (IRR). Intracellular signaling is initiated by activating intracellular tyrosine kinases through a series of structural conformational changes after IR binding to ligands, which exerts important physiological functions in the body [8]. The cardiac cell membrane is rich in IR, making cardiomyocytes a very important target organ for insulin action. Insulin plays a key role in the regulation of various aspects of cardiovascular metabolism through glucose metabolism, protein synthesis, and vascular tone. The IGF family can regulate cardiac lineage induction by expanding the mesodermal cell population [9]. Bisping et al. demonstrated that although IGF1 is unnecessary for cardiac structure and function, GATA4 must be activated by the IGF1 pathway to exert its function [10].

Conformational changes occur in the beta receptor subunit when insulin binds to IR to form a complex, and this leads to autophosphorylation and activation of tyrosine kinase (TK). The complex phosphorylates insulin receptor substrate (IRS) and activates the phosphatidylinositol 3-kinase (PI3K) pathway and mitogen-activated protein kinase (MAPK) pathway. Insulin augments cardiomyocyte contraction, increases ribosomal biogenesis and protein synthesis, stimulates vascular endothelial growth factor (VEGF), and thereby suppresses apoptosis, promoting cell survival and increasing blood perfusion of the myocardium principally through the PKB/Akt signaling pathway [11]. IGF1 can regulate the process of membrane assembly at the axonal growth cone by activating the PI3K pathway [12]. Zhu et al. found that IGF1 can upregulate VEGF-C in breast cancer by mediating the PI3K/Akt and MAPK/ERK1/2 signaling pathways [13]. Treating the smooth muscle cells of the saphenous vein with IGF1 can induce phosphorylation of PI3K-Akt/PKB and promote proliferation of saphenous vein smooth muscle cells [14].

Organisms can produce free radicals during normal metabolism, and excessive oxygen free radicals can cause damage to human tissues and cellular structures [15, 16]. The free radical balances can be maintained depending on the antioxidant system. The body can mediate the accumulation of excess reactive oxygen species (ROS) through some cell signal transduction, which enhances the expression of many protective proteins in the cell. IGF can sense the changes in ROS levels and thus affect the insulin pathway [17]. Papaiahgari et al. demonstrated that ROS can mediate the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) through the PI3K/Akt pathway [18].

In our previous study, we demonstrated that selenium deficiency disrupted insulin responsiveness through inhibition of the PI3K/Akt pathway by producing excessive oxygen free radicals [19, 20]; meanwhile, selenium deficiency can downregulate the expression of IGF1. However, the role of IGF1 in myocardial development is still less reported; in our present study, we developed models for IGF1 knockdown in cardiomyocyte cultures (siRNA) in vitro and IGF1 knockdown in a chicken embryo model in vivo to detect the effect of IGF1 suppression on energy metabolism, insulin pathways, and myocardial development.

2. Materials and Methods

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (SRM-11).

2.1. Primary Cardiomyocyte Culture. Twelve-day-old chicken embryos were used to obtain primary cardiomyocytes for culture. Subsequent to surface disinfection (using 75% alcohol), the chest was dissected to collect the apical portion of the pericardium (approximately 1/3 of the heart), which was immediately transferred to phosphate-buffered solution (PBS) (4°C) and washed to remove fat, connective tissue, and blood clots. Subsequently, the myocardial tissue was cut into small pieces and washed 3 times with PBS. After enzymatic digestion with collagenase-II (0.1%) for 15 minutes on a constant temperature magnetic stirrer (37°C, 100 r/min) for acclimatization and centrifugation, an equal volume of Dulbecco’s Modified Eagle’s Medium (DMEM)/F12w containing 10% fetal bovine serum and 1x mycillin was added to terminate digestion. The pellet was digested until the small tissue fragments were completely digested. All supernatants were collected with 300 mesh and 500 mesh filters. The cell suspension was centrifuged at 600 rpm for 5 minutes and resuspended in DMEM/F12w twice in disposable Petri dishes for differential adhesion (the first was 1 h; the second was 1.5 h). Nonadherent cells (cardiomyocytes) were collected, centrifuged at 600 rpm, counted, plated in 6-well plates at 2 × 10⁵ cells/mL, and incubated at 37°C, 5% CO₂, in an adherent culture incubator for 48 h [21].

2.2. Establishment of the IGF1 Knockdown Model In Vitro. Cardiomyocytes attain 80% confluence after approximately 48 h of incubation. In chicken cardiomyocyte primary cultures, which are the same as described in our previous experimental method, IGF1 was knocked down using siRNA (sense 5′-GTTGTATGGAGACAGA-3′, antisense 5′-TCTGTCTCAGAAGAGAAC-3′) subsequent to two washes with Opti-MEM (prewarmed). All cells were randomly divided into two groups: C (control group) and KD (knockdown group). For each group, 3 replicates were prepared with 6 × 10⁵ cardiomyocytes per replicate (n = 3). Cells in the knockdown group were transfected with 3 μL of 20 μM siRNA and 3 μL of Lipofectamine RNAi MAX Reagent (Invitrogen) in 2 mL of Opti-MEM. Cells in the control group were treated with 2 mL of Opti-MEM (Invitrogen), which only contain the same volume of Lipofectamine RNAi MAX Reagent. Approximately 48 h posttransfection, the cells were harvested for analysis.

2.3. Establishment of the IGF1 Silence Model In Vivo. First, 50 μg of nucleic acid was diluted with pure water without endotoxin to a concentration of 1 μg/μL; the final concentration of glucose was 5%, and the final volume was 100 μL.
Then, 25 μL of Entranster™ in vivo reagent was diluted with 50 μL of 10% glucose solution and supplemented with pure water. The final concentration of running glucose was 5%, and the final volume was 100 μL of liquid.

The diluted transfection reagent was added to the diluted nucleic acid solution to form a transfection complex, which was then left at room temperature for 15 min. Ninety hatching eggs were randomly divided into two groups (45 per group), viz., the normal group (N) and siRNA group (Si). The subgerminal cavity of each egg was injected with 1 μg siRNA and sealed with a sealing film. All of the eggs were incubated in a constant temperature incubator. The hearts were taken at 6, 8, and 10 days for subsequent experiments.

2.4. Detection of Intracellular ROS Accumulation. Posttransfection, ROS activities were measured using an ROS assay kit (Nanjing Jiancheng Bioengineering Institute, China). First, 10 μM DCFH-DA (2,7-dichlorofurescin diacetate) was added to the culture medium, containing the cell samples to be tested, which was then incubated at a constant temperature (37°C) for 45 min. Then, the medium was discarded, and PBS (37°C preheat) was used to wash the cells three times. Finally, the cells were collected for detecting the activities of ROS at the excitation wavelength of 500 ± 15 nm and emission wavelength of 530 ± 20 nm. The cardiomyocytes were visualized using fluorescence microscopy.

2.5. Determination of Oxidative Stress Markers. Cells were grown on 6-well plates at a density of 2.5 × 10^5 mL^{-1}, collected with jets of saline, and centrifuged at 700 × g; then, the supernatant was collected. The hearts of chicken embryos were taken for homogenization in saline solution and centrifuged at 700 g for 20 min, and the supernatants were collected. The hydrogen peroxide (H₂O₂), glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), malondialdehyde (MDA), induced nitric oxide synthase (iNOS), superoxide dismutase (SOD), and total antioxidant capability (T-AOC) contents were measured by detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The reactions were conducted in a 20 μL reaction mixture containing 10 μL of 2x SYBR Green I PCR Master Mix (Roche, Basel, Switzerland), 2 μL of cDNA, 0.4 μL of each primer (10 μM), 0.4 μL of 50x ROX reference Dye II, and 6.8 μL of PCR-grade water. The PCR procedure for IGFI and AMPK, PI3K, JNK, Akt, FOXO, IGF1R, GLUT1, GLUT3, GLUT8, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5, IGFBP7, IR, IRS1, MyoD, MyoG, Mesp1, MYF5, MYF6, GATA4, GATA6, Nkx2.5, and GAPDH consisted of 95°C for 30 s followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 60°C for 30 s. For each PCR, Dissociation Curve 1.0 Software (Applied Biosystems) was used to analyze the dissociation curves in order to detect and eliminate possible primer dimers and nonspecific amplification.

2.6. Determination of the mRNA Expression of the Genes Related to IGFI, the PI3K/Akt Pathway, Insulin, and Cardiac Differentiation. Total RNA was isolated from heart tissues of three points in time and cardiomyocytes by using the TRIzol reagent according to the manufacturer’s instructions (Roche, Basel, Switzerland). The dried RNA pellets were resuspended in 50 μL of diethyl pyrocarbonate-treated water. The concentration and purity of the total RNA were determined using a spectrophotometer. cDNA was synthesized from 5 μg of the total RNA using oligo-dT primers and Superscript II reverse transcriptase according to the manufacturer’s instructions (Promega, Beijing, China). cDNA was diluted at a ratio of 1:5 with sterile water and stored at −80°C.

2.7. Determination of the Protein Expression of the Proteins Related to IGFI, the PI3K/Akt Pathway, Insulin, and Cardiac Differentiation. For total protein extraction, protein lysates were subjected to 15% SDS-polyacrylamide gel electrophoresis under reducing conditions. The separated proteins were then transferred to a nitrocellulose membrane for 2 h at 100 mA in a transfer apparatus containing Tris-glycine buffer and 20% methanol. The membrane was blocked with 5% skim milk for 24 h and incubated overnight with diluted primary antibodies against IGFI (1:500, Proteintech, China), PI3K (1 : 1000, Santa Cruz Biotechnology, USA), Akt (1 : 500, Santa Cruz Biotechnology, USA), FOXO (1 : 1000, Santa Cruz Biotechnology, USA), P-FOXO (1 : 1000, Santa Cruz Biotechnology, USA), P-JNK (1 : 1000, Abcam, Cambridge, UK), P-AMPK (1 : 1000, Abcam, Cambridge, UK), P-IGF1R (1 : 500, Proteintech, China), IGFBP2 (1 : 500, Proteintech, China), MyoG (1 : 500, Proteintech, China), and IGFBP4 (1 : 500, Proteintech, China). Primer Premier Software (PREMIER Biosoft International, USA) was used to design specific primers for IGFI and AMPK-activated protein kinase (AMPK), phosphatidylinositol 3-kinase (PI3K), c-Jun N-terminal kinase (JNK), threonine-protein kinase (Akt), forkhead box protein (FOXO), insulin-like growth factor 1 receptor (IGF1R), glucose transporter-1 (GLUT1), glucose transporter-3 (GLUT3), glucose transporter-8 (GLUT8), insulin-like growth factor-binding protein-1 (IGFBP1), insulin-like growth factor-binding protein-2 (IGFBP2), insulin-like growth factor-binding protein-3 (IGFBP3), insulin-like growth factor-binding protein-4 (IGFBP4), insulin-like growth factor-binding protein-7 (IGFBP7), insulin receptor (IR), insulin receptor substrate-1 (IRS1), MyoD, myogenin (MyoG), cardiac transcription factor mesoderm posterior 1 (Mesp1), myogenic factor-5 (MYF5), myogenic factor-6 (MYF6), GATA-binding protein 4 (GATA4), GATA-binding protein 6 (GATA6), Nkx2.5, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) based on known chicken sequences (Table 1). First, general PCR was performed to confirm the specificity of the primers. Quantitative real-time PCR (qPCR) was then performed with a Roche detection system (Applied Biosystems, Foster City, CA). The reactions were conducted in a 20 μL reaction mixture containing 10 μL of 2x SYBR Green I PCR Master Mix (Roche, Basel, Switzerland), 2 μL of cDNA, 0.4 μL of each primer (10 μM), 0.4 μL of 50x ROX reference Dye II, and 6.8 μL of PCR-grade water. The PCR procedure for IGFI and AMPK, PI3K, JNK, Akt, FOXO, IGF1R, GLUT1, GLUT3, GLUT8, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5, IGFBP7, IR, IRS1, MyoD, MyoG, Mesp1, MYF5, MYF6, GATA4, GATA6, Nkx2.5, and GAPDH consisted of 95°C for 30 s followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 60°C for 30 s. For each PCR, Dissociation Curve 1.0 Software (Applied Biosystems) was used to analyze the dissociation curves in order to detect and eliminate possible primer dimers and nonspecific amplification.
### Table 1: The primers used in the present study.

| Target gene | Primer sequence (5′-3′) |
|-------------|-------------------------|
| IGF1       | Forward 5′-GCTTCTTGTAGTTCCCTTGAAGTGAA-3′ |
|             | Reverse 5′-CATACCCCTGGCCTACTGAGTA-3′ |
| AMPK       | Forward 5′-CCATCTGTCAATATTGCTTCTG-3′ |
|             | Reverse 5′-AGGCTTCAGCCATCAATCAT-3′ |
| PI3K       | Forward 5′-GTGGCTTACACCTCTGATG-3′ |
|             | Reverse 5′-TGGTGGCTAACAGTGTTGTT-3′ |
| Akt        | Forward 5′-AGGAGGAAGATGATGGAAT-3′ |
|             | Reverse 5′-GAATGGATGCCCGTGAGTT-3′ |
| JNK        | Forward 5′-CAGATAAGCAGTTAGTGAGAGAAT-3′ |
|             | Reverse 5′-GACAGATGACGACGAGAAT-3′ |
| FOXO       | Forward 5′-GGGAGAACAGCATAGAGTAC-3′ |
|             | Reverse 5′-TGGTGGCAGGTGATTAGT-3′ |
| IGF1R      | Forward 5′-GGTTCAGGAGATAGAGTAC-3′ |
|             | Reverse 5′-TGGTGGCAGGTGATTAGT-3′ |
| GLUT1      | Forward 5′-CAGAAGCAGGAGAGAACGCAGA-3′ |
|             | Reverse 5′-CGGCAAAGAATGGGATGAAAA-3′ |
| GLUT3      | Forward 5′-TCCCCAGACTCTTAC CCTAC-3′ |
|             | Reverse 5′-CAGCAAAGAAGCAAGACATCCAC-3′ |
| GLUT8      | Forward 5′-CCAAATGGGAAACACTCACTCA-3′ |
|             | Reverse 5′-GGCCAAACCAGCAGCAAA-3′ |
| IGFBP1     | Forward 5′-TGCTCTGGAGCTGCTGATG-3′ |
|             | Reverse 5′-ACCCAGACCCAGGGAATC-3′ |
| IGFBP2     | Forward 5′-TGGTGAACAGCTGCTGTGAGCAG-3′ |
|             | Reverse 5′-TCTCCACGCTGCCCATAC-3′ |
| IGFBP3     | Forward 5′-ATGGTCCCTGTCGTAAGG-3′ |
|             | Reverse 5′-ATCCAGGAAGGCGTGTG-3′ |
| IGFBP4     | Forward 5′-TGTTGGTGTCGGAGGAGA-3′ |
|             | Reverse 5′-AGGCGATGGGCTGAAAG-3′ |
| IGFBP5     | Forward 5′-TGTTGGTGTCGGAGGAGA-3′ |
|             | Reverse 5′-ACCAAGCAGCAGCCTCCG-3′ |
| IGFBP7     | Forward 5′-TGTTGGTGTCGGAGGAGA-3′ |
|             | Reverse 5′-CCTCTCCTTTGGCATTG-3′ |
| IR         | Forward 5′-CAAACGCGTGACCAACCGCTCA-3′ |
|             | Reverse 5′-CATCCTGCCCATCAACCTCC-3′ |

### Table 1: Continued.

| Target gene | Primer sequence (5′-3′) |
|-------------|-------------------------|
| IRS1       | Forward 5′-TCACCCACACACACCATCCAC-3′ |
|             | Reverse 5′-ACACGGACCCGCGCTAC-3′ |
| MyoD       | Forward 5′-CGGCCGATAGCTTCTATG-3′ |
|             | Reverse 5′-GTTGTTGGCTTCCTCTTCTG-3′ |
| MyoG       | Forward 5′-AGGAGGAGAGGCTGAGAC-3′ |
|             | Reverse 5′-GTCGATGTACTGATGATCG-3′ |
| Mesp1      | Forward 5′-GCTTACACCCCTCTCACA-3′ |
|             | Reverse 5′-CCATCTGCTACATCAAC-3′ |
| MYF5       | Forward 5′-GGAGGAGGGCTGAGAAGA-3′ |
|             | Reverse 5′-CGGCCGATGAGTATAGTTCG-3′ |
| MYF6       | Forward 5′-GGAGGAGGGCTGAGAAGA-3′ |
|             | Reverse 5′-CTCTCGATGAGTATAGTTCG-3′ |
| GATA4      | Forward 5′-TCAGAAGGAAAGGCTAA-3′ |
|             | Reverse 5′-ATGCGAAGGACCCGAAT-3′ |
| GATA6      | Forward 5′-CCGACCACCTGCTATGAA-3′ |
|             | Reverse 5′-TGTCACAGTCTCAGTTG-3′ |
| Nkx2.5     | Forward 5′-GACAAGGAAAGGAGAGA-3′ |
|             | Reverse 5′-CTCTCGATGAGTATAGTTCG-3′ |
| GAPDH      | Forward 5′-AGAACATCATCCACCGGCTG-3′ |
|             | Reverse 5′-AGCCCTCATACTCCTTTG-3′ |

(1:1000, Abcam, Cambridge, UK), and MyoD (1:1000, Abcam, Cambridge, UK) followed by a horseradish peroxidase- (HRP-) conjugated secondary antibody against rabbit (IGF1, PI3K, Akt, P-Akt, FOXO, P-AK, JNK, P-JNK, 14-3-3, GLUT, IGF1R, IGBP2, MyoG, and MyoD) IgG (1:5000, Santa Cruz Biotechnology, USA). To verify equal loading of the samples, the membrane was incubated with a monoclonal GAPDH antibody (1:1500, Santa Cruz Biotechnology, USA), followed by an HRP-conjugated goat anti-mouse IgG (1:3000) secondary antibody. The signal was detected with X-ray films (TransGen Biotech Co., Beijing, China). The optical density (OD) of each band was determined using an Image VCD gel imaging system, and the relative abundance of IGF1, PI3K, Akt, P-Akt, FOXO, P-AKT, JNK, P-JNK, 14-3-3, GLUT, IGF1Rβ, IGBP2, MyoG, and MyoD proteins was calculated and presented as the ratios of OD of each of these proteins to that of GAPDH.

2.8. Measurement of ATP. Cardiomyocytes were grown on 6-well plates at a density of 2 × 10^5 cells/mL, gathered with the lysis solution, and centrifuged at 700 × g. The supernatant was collected, resuspended by salt water and incubated for...
35 min at 25°C. The level of adenosine triphosphate (ATP) in the cardiomyocytes was measured by using an ATP detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. The detection was carried out using an ultraviolet spectrophotometer (Synergy NEO, BioTek Instruments) with a detection wavelength of 636 nm.

2.9. Histopathological Examination. Cardiomyocytes were grown at a density of $2 \times 10^5$ cells/mL and then washed with PBS three times; 4% paraformaldehyde solution was added in a 24-well plate for cell fixing. After 12 h, the 4% paraformaldehyde solution in the 24-well plate was removed and 0.01 M PBS was added; then, the wells were soaked for 5 min $\times 3$ times. Hematoxylin staining solution was added to the wells and immersed for 1 min. The staining solution was removed, and distilled water was added to soak for 5 min. The distilled water was then removed and placed in 1% hydrochloric acid alcohol. After 1-3 s, it was aspirated. Tap water was added to soak for 5 min to return the cells to blue. Then, the tap water was removed, and Yihong dye solution was added to soak for 1 min. The eosin staining solution was aspirated and soaked in distilled water for 1 min. The staining pieces were removed, and glycerol ethanol was added dropwise. The staining effect was observed under a microscope and photographed under a 200x microscope [22].

The myocardial tissues were rapidly fixed in 10% formaldehyde for at least 24 h and embedded in paraffin for microscopic examination. From the prepared paraffin blocks, sections (5 μm thick) were cut, obtained, and stained with hematoxylin and eosin (H.E.) for light microscopic observation.

2.10. High-Resolution Respirometry of Mitochondrial Function. Cardiomyocytes were grown on 6-well plates at a density of $2 \times 10^5$ cells/mL. All cells were randomly divided into two groups: C (control group) and KD (knockdown group). The cells in the knockdown group were transfected with 3 μL of 20 μM siRNA and 3 μL of Lipofectamine RNAi MAX Reagent (Invitrogen) in 2 mL of Opti-MEM. The cells in the control group were treated with 2 mL of Opti-MEM (Invitrogen) containing the same volume of Lipofectamine RNAi MAX Reagent. Approximately 48 h posttransfection, the cells were harvested for analysis, gathered with the lysis solution, and centrifuged at 700 x g. The supernatant was closed and data were recorded using DatLab software 5.2 (Oroboros Instruments, Innsbruck, Austria). The cell suspension was transferred separately to oxygraph chambers at a final density of approximately $2 \times 10^5$ cells/mL. After a short stabilization period, the chambers were closed and data were recorded using the unpaired t-test, where $P < 0.05$ was considered a statistically significant difference.

3. Results

3.1. Development of an IGF1 Knockdown Model in Cells and Chicken Embryos. The mRNA and protein levels of IGF1 were significantly decreased ($P < 0.05$) in the KD group (Figures 1(a) and 1(b)). The results confirmed that we successfully established the model of IGF1 knockdown in vitro.

The mRNA levels of IGF1 were detected at 6, 8, and 10 days, and we found that the expression of IGF1 was significantly decreased at 10 days. For further verification, we took chicken embryos at 10 days to detect the protein level of IGF1. Compared with the N group, the mRNA levels in the Si group decreased ($P < 0.05$) at 8 days and 10 days (Figure 1(c)). The Si group exhibited significantly decreased protein levels compared with the N group (Figure 1(d)). The results confirmed that we successfully established the IGF1 silence model in vivo.

3.2. Detection of the Antioxidant Capacity in Cells and Chicken Embryos. To assess the relationship between oxidative stress and IGF1, the production of ROS, the levels of H2O2, MDA, and T-AOC and the activities of GSH, GSH-Px, SOD, CAT, and iNOS were measured in cardiomyocytes. As presented in Figure 2, the ROS activities were significantly increased ($P < 0.05$) compared with the C group. The levels of H2O2, MDA, and iNOS were significantly increased in the KD group ($P < 0.05$) (Figures 3(d)–3(f)). The levels of GSH, GSH-Px, CAT, SOD, and T-AOC in the KD group were significantly lower than those in the C group ($P < 0.05$) (Figures 3(a)–3(c), 3(g), and 3(h)).

To further demonstrate the antioxidant capacity of IGF1 silence in vivo, we also studied the antioxidant capacity of the myocardium. As shown in Figure 3, the levels of H2O2, MDA, and iNOS were significantly increased in the Si group ($P < 0.05$) at 6, 8, and 10 days (Figures 3(j)–3(n)). The levels of GSH, GSH-Px, CAT, SOD, and T-AOC in the Si group were significantly lower than those in the N group ($P < 0.05$) at 6, 8, and 10 days (Figures 3(i), 3(j), 3(l), 3(o), and 3(p)).

3.3. Protein and mRNA Expression of the PI3K/Akt Pathway-Related Genes in Cells and Chicken Embryos. To examine whether IGF1 knockdown changed the expressions of the PI3K/Akt pathway-related genes, we detected the mRNA expression levels of AMPK, PI3K, JNK, Akt, and FOXO as well as the protein expression levels of FOXO, P-FOXO, JNK, P-JNK, PI3K, P-Akt, 14-3-3, and P-14-3-3. The effects of IGF1 knockdown on the mRNA abundance of PI3K-related genes in chicken cardiomyocytes are shown in Figure 4(a). Compared with the C group, the qPCR results revealed that the mRNA expression of AMPK, JNK, and FOXO was significantly increased ($P < 0.05$) in the KD group. However, the mRNA expression of PI3K and Akt was decreased ($P < 0.05$). The results revealed that compared with the C group, the protein expression of FOXO, P-FOXO, JNK, and P-JNK in the KD group was significantly increased ($P < 0.05$). However, the protein expression of PI3K, Akt, P-Akt, 14-3-3, and P-14-3-3 decreased in the KD group ($P < 0.05$) (Figure 4(b)).
Moreover, we detected the effects of IGF1 silencing on the mRNA abundance of PI3K-related genes (AMPK, PI3K, JNK, Akt, and FOXO) in the myocardium of chicken embryos as shown in Figure 4(c). The qPCR results revealed that the mRNA expression of AMPK, JNK, and FOXO was significantly increased \((P < 0.05)\) in the Si group at 6, 8, and 10 days. The mRNA expression of PI3K increased at 6 and 8 days but significantly decreased \((P < 0.05)\) at 10 days. The mRNA expression of Akt increased at 6 days, showed no significant change at 8 days, and significantly decreased \((P < 0.05)\) at 10 days. A western blot analysis was performed to determine the protein expression of PI3K-related genes. The results revealed that compared with the N group, the protein expression of FOXO, P-FOXO, JNK, and P-JNK in the Si group was significantly increased \((P < 0.05)\). However, the protein expression of PI3K, Akt, P-Akt, 14-3-3, and P-14-3-3 decreased in the Si group \((P < 0.05)\) (Figure 4(d)).

3.4. Protein and mRNA Expression of Insulin-Related Genes in Cells and Chicken Embryos. We also examined the effects of IGF1 knockdown on the mRNA abundance of insulin-related genes (IGF1R, GLUT1, GLUT3, GLUT8, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5, IGFBP7, IR, and IRS1) in chicken cardiomyocytes (Figure 5(a)); the qPCR results revealed that the mRNA expression of IGF1R, GLUT1, GLUT3, GLUT8, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP7, IR, and IRS1 was significantly decreased \((P < 0.05)\) in the KD group. A western blot analysis was performed to determine the protein expression of insulin-related genes. The results revealed that compared with the C group, the protein expression of GLUT3, IGF1R, and IGFBP2 in the KD group was significantly decreased \((P < 0.05)\) (Figure 5(b)).

Furthermore, we examined the effects of IGF1 knockdown on the mRNA abundance of insulin-related genes (IGF1R, GLUT1, GLUT3, GLUT8, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5, IGFBP7, IR, and IRS1) in the myocardium of chicken embryos as shown in Figure 5(c). The qPCR results revealed that the mRNA expression of IGF1R, GLUT1, GLUT3, GLUT8, IGFBP1, IGFBP2, and IGFBP7 increased in the Si group at 6 days, showed no significant change at 8 days, and significantly decreased \((P < 0.05)\) at 10 days. The mRNA expression of GLUT3, IGFBP3, IR, and IRS1 increased at 6 and 8 days, but significantly decreased \((P < 0.05)\) at 10 days. The mRNA expression of IGFBP4 increased at 6 days but significantly decreased at 8
and 10 days. The mRNA expression of IGFBP5 significantly decreased \( (P < 0.05) \) at 8 and 10 days. A western blot analysis was performed to determine the protein expression of insulin-related genes. The results revealed that compared with the N group, the protein expression of GLUT3, IGF1Rβ, and IGFBP2 in the Si group was significantly decreased \( (P < 0.05) \) (Figure 5(d)).

3.5. The Oxygen Consumption Rate and ATP Content in Myocardial Cells. To assess the effect of IGF1 knockdown on energy metabolism, the oxygen consumption rate and ATP content were detected. The results revealed that IGF1 knockdown significantly decreased the ATP content \( (P < 0.05) \) (Figure 6(a)). The results of the oxygen consumption rate revealed that the oxygen consumption rate of the IGF1 knockdown group was 15.166 while the oxygen consumption rate of the control group was 24.019, indicating that IGF1 knockdown significantly decreased the myocardial oxygen consumption rate under the same conditions and the same initial oxygen concentration (Figure 6(b)).

The instantaneous oxygen consumption rates of the cardiomyocytes for different groups are provided in Table 2.

3.6. Protein and mRNA Expression of Cardiac Differentiation-Related Genes in Cells and Chicken Embryos. The effects of IGF1 knockdown on the mRNA abundance of cardiac differentiation-related genes (MyoD, MyoG, Mesp1, MYF5, MYF6, GATA4, GATA6, and Nkx2.5) in chicken cardiomyocytes are shown (Figure 7(a)), and the qPCR results revealed that the mRNA expression of MyoD, Mesp1, MYF5, MYF6, GATA4, GATA6, and Nkx2.5 decreased \( (P < 0.05) \) at 8 and 10 days. A western blot analysis was performed to determine the protein expression of cardiac differentiation-related genes. The results revealed that compared with the C group, the protein expression of MyoG and MyoD in the KD group was significantly decreased \( (P < 0.05) \) (Figure 7(b)).

The effects of IGF1 knockdown on the mRNA abundance of cardiac differentiation-related genes (MyoD, MyoG, Mesp1, MYF5, MYF6, GATA4, GATA6, and Nkx2.5) in the myocardium of chicken embryos are shown in Figure 7(c). The qPCR results revealed that the mRNA expression of MyoD, Mesp1, and MYF5 increased in the Si group at 6 and 8 days, but significantly decreased \( (P < 0.05) \) at 10 days. The mRNA expression of MyoG, GATA4, and GATA6 increased at 6 days, but significantly decreased at 8 and 10 days. The mRNA expression of MYF6 increased at 6 days, showed no significant change at 8 days, and significantly decreased \( (P < 0.05) \) at 10 days. The mRNA expression of Nkx2.5 decreased \( (P < 0.05) \) at 6, 8, and 10 days. A western blot analysis was performed to determine the protein expression of cardiac differentiation-related genes. The results revealed that compared with the N group, the protein expression of MyoG and MyoD in the Si group was significantly decreased \( (P < 0.05) \) (Figure 7(d)).

3.7. Intracellular Morphological Observation and H.E. Stain in Cells and Chicken Embryos’ Myocardium. As observed under a microscope, normal cardiomyocytes were fusiform and tightly connected and the whole looked similar to paving stones accompanied by protruding pseudopodia that stretched out between cells, interweaving into a mesh in the control group (Figure 8(a)). In the KD group, as the density of cell growth decreased, the volume of the cardiomyocytes and intercellular junctions was evidently reduced. We observed that myocardial fibers and muscle fiber bundles were disintegrated, and the pseudopodia between cells did not interweave into a mesh in the KD group (Figure 8(b)). We observed myocardial cells stained by hematoxylin and eosin (H.E.). The cardiomyocytes in the control group displayed normal morphologies (Figure 8(c)). However, many slender cardiomyocytes appeared in the IGF1 knockdown group (Figure 8(d)), which indicated that cardiomyocyte development was blocked.
| Figure 3: (a–h) Oxidative stress markers of the GSH, GSH-Px, CAT, H2O2, MDA, iNOS, SOD, and T-AOC contents were measured in cardiomyocytes. (i–p) Oxidative stress markers of GSH, GSH-Px, CAT, H2O2, MDA, iNOS, SOD, and T-AOC contents were measured in the myocardium. C indicates the control group; KD indicates the knockdown group in vitro; N indicates the normal group; Si indicates the knockdown group in vivo. * shows a significant difference from the corresponding control (P < 0.05). n = 3. |
Figure 4: The effects of IGF1 knockdown on the mRNA levels (a) and protein levels (b) of PI3K-related genes in cardiomyocytes and the effects of IGF1 silencing on the mRNA levels at 6, 8, and 10 days (c) and on the protein levels (d) of PI3K-related genes in the myocardium. C indicates the control group; KD indicates the knockdown group in vitro; N indicates the normal group; Si indicates the knockdown group in vivo. GAPDH was selected as the reference. * shows a significant difference from the corresponding control ($P < 0.05$). $n = 3$. 

Oxidative Medicine and Cellular Longevity
Figure 5: The effects of IGF1 knockdown on the mRNA levels (a) and protein levels (b) of insulin-related genes in cardiomyocytes and the effects of IGF1 silencing on the mRNA levels at 6, 8, and 10 days (c) and on the protein levels (d) of insulin-related genes in the myocardium. C indicates the control group; KD indicates the knockdown group in vitro; N indicates the normal group; Si indicates the knockdown group in vivo. GAPDH was selected as the reference. * shows a significant difference from the corresponding control (P < 0.05). n = 3.
Myocardial injury and ultrastructural damage are shown in Figure 8(f). Blood vessel rupturing and increased tissue gaps were observed in the IGF1-deficient chicken heart group more than in the normal group (Figure 8(e)).

4. Discussion

IGF1 is a polypeptide neurotropic factor with a structure and function similar to insulin. IGF1 is a single-chain protein that promotes cell differentiation and proliferation, and it has a wide range of biological functions and participates in the regulation of various organs. IGF1 plays an important role in the development of human and vertebrate embryos [23]. In the present study, we established an IGF1 knockdown model in vivo and in vitro through transfection of small interfering RNA (siRNA) and the mRNA and protein levels of IGF1 were detected to support this point. Significant damage to myocardial tissue, rupturing of blood vessels, and increased tissue gaps were observed in IGF1-deficient chicken heart through histopathological observation, demonstrating that IGF1 suppression leads to dysplasia of cardiomyocytes and myocardial tissue.

The concentration of free radicals is low under physiological conditions, which is important to cell signal regulation, metabolism, survival, and apoptosis [24, 25]. However, a large number of free radicals are induced when organisms are stimulated by physical factors or exogenous chemical substances; free radicals can covalently bind to biomacromolecules and peroxidize biofilm lipids to produce various toxic effects [26], which can lead to the occurrence of many diseases such as myocardial infarction [27, 28] and various cancers [29]. Oxygen free radicals can make lipid fatty acids into lipid peroxides and further decompose into a series of complex compounds, including MDA; therefore, the level of lipid oxidation can be detected by the level of MDA [30]. iNOS is an oxidative stress (free radical) that utilizes nitric oxide, which can be produced when cells are stimulated and activated. The cells can form a complex antioxidant enzyme defense system, which mainly includes SOD, CAT, GSH, and so on, to protect the body from peroxidative damage [26, 31]. SOD can eliminate free radicals in the body and protect cells from free radical damage. The level of SOD activity reflects the ability of antifree radicals. SOD can convert superoxide anion (O2−) into H2O2, and CAT converts H2O2 into water such that toxic O2− and H2O2 are converted into harmless water molecules [32, 33]. GSH is catalyzed to convert to oxidized glutathione (GSSH) with the assistance of GSH-Px, which can reduce oxidized substances and relieve their toxicity [34, 35]. In recent years, the antioxidant functions of IGF1 have been gradually discovered; Tumati et al. found that low IGF1 can induced excessive ROS, which will be further moderated by JNK-induced epithelial cytoprotection [36]. ROS, as an important endogenous stimulator in the body, can stimulate multiple pathways including myocardial development. Huk et al. found that ROS serves as secondary messengers to influence cardiac valve development [37]. ROS may be involved in adverse cardiac remodeling [38]. In our present study, we found that decreasing expression of IGF1 results in increasing ROS generation, suggesting that IGF1 may be involved in the regulation of ROS and the
| Time (min) | 1A: O$_2$ concentration (nmol/mL) | 1A: O$_2$ flux per V (pmol/(s∗mL)) | 1B: O$_2$ concentration (nmol/mL) | 1B: O$_2$ flux per V (pmol/(s∗mL)) |
|-----------|----------------------------------|-----------------------------------|----------------------------------|-----------------------------------|
| 0.03      | 134.4705                         | 134.4705                          | 222.2083                         | 222.2083                          |
| 0.07      | 134.361                          | 134.361                           | 222.1058                         | 222.1058                          |
| 0.1       | 134.3131                         | 134.3131                          | 222.0585                         | 222.0585                          |
| 0.13      | 134.2618                         | 134.2618                          | 222.1088                         | 222.1088                          |
| 0.17      | 134.1943                         | 134.1943                          | 222.157                          | 222.157                           |
| 0.2       | 134.1481                         | 134.1481                          | 222.0753                         | 222.0753                          |
| 0.23      | 134.0865                         | 134.0865                          | 221.9699                         | 221.9699                          |
| 0.27      | 134.0053                         | 134.0053                          | 221.9236                         | 221.9236                          |
| 0.3       | 133.9386                         | 133.9386                          | 221.8753                         | 221.8753                          |
| 0.33      | 133.8958                         | 133.8958                          | 221.8517                         | 221.8517                          |
| 0.37      | 133.8531                         | 133.8531                          | 221.8585                         | 221.8585                          |
| 0.4       | 133.8078                         | 133.8078                          | 221.8034                         | 221.8034                          |
| 0.43      | 133.7513                         | 133.7513                          | 221.7768                         | 221.7768                          |
| 0.47      | 133.694                          | 133.694                           | 221.7354                         | 221.7354                          |
| 0.5       | 133.6496                         | 133.6496                          | 221.6911                         | 221.6911                          |
| 0.53      | 133.5888                         | 133.5888                          | 221.6527                         | 221.6527                          |
| 0.57      | 133.5316                         | 133.5316                          | 221.6044                         | 221.6044                          |
| 0.6       | 133.4914                         | 133.4914                          | 221.5699                         | 221.5699                          |
| 0.63      | 133.4358                         | 133.4358                          | 221.5187                         | 221.5187                          |
| 0.67      | 133.3802                         | 133.3802                          | 221.499                          | 221.499                           |
| 0.7       | 133.3283                         | 133.3283                          | 221.4684                         | 221.4684                          |
| 0.73      | 133.2365                         | 133.2365                          | 221.431                          | 221.431                           |
| 0.77      | 133.1827                         | 133.1827                          | 221.3995                         | 221.3995                          |
| 0.8       | 133.1467                         | 133.1467                          | 221.365                          | 221.365                           |
| 0.83      | 133.1014                         | 133.1014                          | 221.3246                         | 221.3246                          |
| 0.87      | 133.0279                         | 133.0279                          | 221.2931                         | 221.2931                          |
| 0.9       | 132.9774                         | 132.9774                          | 221.2497                         | 221.2497                          |
| 0.93      | 132.9338                         | 132.9338                          | 221.2143                         | 221.2143                          |
| 0.97      | 132.8663                         | 132.8663                          | 221.1739                         | 221.1739                          |
| 1         | 132.8064                         | 132.8064                          | 221.1473                         | 221.1473                          |
| 1.03      | 132.7414                         | 132.7414                          | 221.1256                         | 221.1256                          |
| 1.07      | 132.6687                         | 132.6687                          | 221.0951                         | 221.0951                          |
| 1.1       | 132.6345                         | 132.6345                          | 221.0586                         | 221.0586                          |
| 1.13      | 132.5969                         | 132.5969                          | 221.0182                         | 221.0182                          |
| 1.17      | 132.5524                         | 132.5524                          | 220.9788                         | 220.9788                          |
| 1.2       | 132.4772                         | 132.4772                          | 220.9493                         | 220.9493                          |
| 1.23      | 132.4361                         | 132.4361                          | 220.9168                         | 220.9168                          |
| 1.27      | 132.3746                         | 132.3746                          | 220.8714                         | 220.8714                          |
| 1.3       | 132.3147                         | 132.3147                          | 220.836                          | 220.836                           |
| 1.33      | 132.2617                         | 132.2617                          | 220.7926                         | 220.7926                          |
| 1.37      | 132.189                          | 132.189                           | 220.7394                         | 220.7394                          |
| 1.4       | 132.124                          | 132.124                           | 220.7089                         | 220.7089                          |
| 1.43      | 132.089                          | 132.089                           | 220.6685                         | 220.6685                          |
| 1.47      | 132.024                          | 132.024                           | 220.6439                         | 220.6439                          |
| 1.5       | 131.9581                         | 131.9581                          | 220.6064                         | 220.6064                          |
| 1.53      | 131.9094                         | 131.9094                          | 220.5749                         | 220.5749                          |
| 1.57      | 131.8777                         | 131.8777                          | 220.5385                         | 220.5385                          |
| 1.6       | 131.8495                         | 131.8495                          | 220.4872                         | 220.4872                          |
| Time (min) | 1A: O$_2$ concentration (nmol/mL) | 1A: O$_2$ flux per V (pmol/(s*mL)) | 1B: O$_2$ concentration (nmol/mL) | 1B: O$_2$ flux per V (pmol/(s*mL)) |
|-----------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| 1.63      | 131.7649                        | 26.4118                         | 220.4695                        | 14.4177                         |
| 1.67      | 131.6828                        | 26.4994                         | 220.4242                        | 14.5174                         |
| 1.7       | 131.6366                        | 26.5005                         | 220.3641                        | 14.5189                         |
| 1.73      | 131.5896                        | 26.5017                         | 220.3326                        | 14.5197                         |
| 1.77      | 131.5374                        | 26.503                          | 220.3099                        | 14.5202                         |
| 1.8       | 131.5041                        | 26.5039                         | 220.2744                        | 14.5211                         |
| 1.83      | 131.4545                        | 26.4196                         | 220.2311                        | 14.5222                         |
| 1.87      | 131.3698                        | 26.4217                         | 220.2134                        | 14.5227                         |
| 1.9       | 131.2988                        | 26.4235                         | 220.1651                        | 14.6224                         |
| 1.93      | 131.2518                        | 26.4247                         | 220.1099                        | 14.6238                         |
| 1.97      | 131.1979                        | 26.426                          | 220.0469                        | 14.8224                         |
| 2         | 131.1304                        | 26.4277                         | 220.0232                        | 14.9215                         |
| 2.03      | 131.0833                        | 26.4289                         | 219.9888                        | 14.9223                         |
| 2.07      | 131.0269                        | 26.4303                         | 219.9523                        | 15.0217                         |
| 2.1       | 130.9645                        | 26.5173                         | 219.909                         | 15.1213                         |
| 2.13      | 130.9021                        | 26.5189                         | 219.8597                        | 15.2211                         |
| 2.17      | 130.8559                        | 26.5201                         | 219.8272                        | 15.3204                         |
| 2.2       | 130.802                         | 26.6069                         | 219.7829                        | 15.3215                         |
| 2.23      | 130.7396                        | 26.6085                         | 219.7464                        | 15.421                          |
| 2.27      | 130.6797                        | 26.61                            | 219.7228                        | 15.5201                         |
| 2.3       | 130.6318                        | 26.6112                         | 219.6735                        | 15.6198                         |
| 2.33      | 130.5899                        | 26.6122                         | 219.639                         | 15.7192                         |
| 2.37      | 130.5532                        | 26.6131                         | 219.71                           | 15.5204                         |
| 2.4       | 130.5147                        | 26.6141                         | 219.71                           | 15.3233                         |
| 2.43      | 130.4591                        | 26.53                            | 219.6095                        | 15.1288                         |
| 2.47      | 130.3608                        | 26.5324                         | 219.5297                        | 15.1308                         |
| 2.5       | 130.3129                        | 26.5336                         | 219.4824                        | 15.132                          |
| 2.53      | 130.2658                        | 26.4493                         | 219.444                         | 15.133                          |
| 2.57      | 130.2085                        | 26.4507                         | 219.3996                        | 15.1341                         |
| 2.6       | 130.1632                        | 26.3664                         | 219.3977                        | 15.0356                         |
| 2.63      | 130.1119                        | 26.3676                         | 219.3455                        | 15.0369                         |
| 2.67      | 130.0546                        | 26.2836                         | 219.2676                        | 15.0389                         |
| 2.7       | 129.9785                        | 26.371                          | 219.2115                        | 15.1388                         |
| 2.73      | 129.9281                        | 26.3722                         | 219.1632                        | 15.2385                         |
| 2.77      | 129.887                         | 26.3733                         | 219.114                         | 15.4368                         |
| 2.8       | 129.8391                        | 26.2889                         | 219.0834                        | 15.536                          |
| 2.83      | 129.781                         | 26.2904                         | 219.048                         | 15.6354                         |
| 2.87      | 129.7194                        | 26.2919                         | 219.0145                        | 15.6363                         |
| 2.9       | 129.6715                        | 26.2931                         | 218.979                         | 15.7357                         |
| 2.93      | 129.6159                        | 26.209                          | 218.9317                        | 15.7369                         |
| 2.97      | 129.5441                        | 26.2108                         | 218.8736                        | 15.8368                         |
| 3         | 129.4774                        | 26.2125                         | 218.8726                        | 15.8369                         |
| 3.03      | 129.4099                        | 26.2997                         | 218.8539                        | 15.9358                         |
| 3.07      | 129.368                         | 26.3007                         | 218.8224                        | 15.9366                         |
| 3.1       | 129.3235                        | 26.3018                         | 218.7642                        | 15.9381                         |
| 3.13      | 129.2585                        | 26.3035                         | 218.7248                        | 15.9391                         |
| 3.17      | 129.2055                        | 26.2193                         | 218.6795                        | 15.9402                         |
| 3.2       | 129.1627                        | 26.2203                         | 218.6342                        | 15.9413                         |
| Time (min) | 1A: O₂ concentration (nmol/mL) | 1A: O₂ flux per V (pmol/(s ∗ mL)) | 1B: O₂ concentration (nmol/mL) | 1B: O₂ flux per V (pmol/(s ∗ mL)) |
|-----------|-------------------------------|----------------------------------|---------------------------------|----------------------------------|
| 3.23      | 129.1157                      | 26.2215                          | 218.5977                        | 15.9422                          |
| 3.27      | 129.0456                      | 26.2233                          | 218.5652                        | 15.943                           |
| 3.3       | 128.9874                      | 26.2247                          | 218.5278                        | 16.0425                          |
| 3.33      | 128.9421                      | 26.2259                          | 218.4579                        | 16.1428                          |
| 3.37      | 128.9062                      | 26.2268                          | 218.4214                        | 16.2422                          |
| 3.4       | 128.8455                      | 26.2283                          | 218.3987                        | 16.2428                          |
| 3.43      | 128.7796                      | 26.2299                          | 218.3524                        | 16.3424                          |
| 3.47      | 128.7463                      | 26.2308                          | 218.316                         | 16.4418                          |
| 3.5       | 128.6693                      | 26.2327                          | 218.2786                        | 16.5413                          |
| 3.53      | 128.5992                      | 26.3199                          | 218.2559                        | 16.5419                          |
| 3.57      | 128.5308                      | 26.4072                          | 218.2234                        | 16.6412                          |
| 3.6       | 128.4803                      | 26.4939                          | 218.18                          | 16.6423                          |
| 3.63      | 128.4231                      | 26.5809                          | 218.1288                        | 16.7421                          |
| 3.67      | 128.3837                      | 26.5819                          | 218.0855                        | 16.8417                          |
| 3.7       | 128.3162                      | 26.5836                          | 218.0559                        | 16.6454                          |
| 3.73      | 128.2469                      | 26.5853                          | 218.0185                        | 16.3508                          |
| 3.77      | 128.1947                      | 26.5866                          | 217.9643                        | 16.2536                          |
| 3.8       | 128.1494                      | 26.6732                          | 217.9436                        | 16.2541                          |
| 3.83      | 128.1032                      | 26.6744                          | 217.9298                        | 16.0574                          |
| 3.87      | 128.0656                      | 26.6753                          | 217.8875                        | 15.96                            |
| 3.9       | 128.0075                      | 26.5913                          | 217.8421                        | 15.9611                          |
| 3.93      | 127.9288                      | 26.5932                          | 217.7978                        | 15.7652                          |
| 3.97      | 127.8724                      | 26.5947                          | 217.7387                        | 15.6682                          |
| 4         | 127.845                       | 26.5953                          | 217.6865                        | 15.768                           |
| 4.03      | 127.7877                      | 26.5968                          | 217.6372                        | 15.7692                          |
| 4.07      | 127.7099                      | 26.5987                          | 217.6225                        | 15.8681                          |
| 4.1       | 127.6654                      | 26.5998                          | 217.6008                        | 15.8686                          |
| 4.13      | 127.6081                      | 26.6013                          | 217.5614                        | 15.8696                          |
| 4.17      | 127.5363                      | 26.6031                          | 217.5259                        | 15.8705                          |
| 4.2       | 127.4713                      | 26.6047                          | 217.4767                        | 15.8717                          |
| 4.23      | 127.408                        | 26.6063                          | 217.4402                        | 15.8727                          |
| 4.27      | 127.3618                      | 26.6074                          | 217.3969                        | 15.8737                          |
| 4.3       | 127.3105                      | 26.6942                          | 217.3565                        | 15.9733                          |
| 4.33      | 127.2763                      | 26.6951                          | 217.325                         | 15.9741                          |
| 4.37      | 127.2233                      | 26.6964                          | 217.2836                        | 15.9751                          |
| 4.4       | 127.1626                      | 26.6979                          | 217.2422                        | 15.8776                          |
| 4.43      | 127.1216                      | 26.6989                          | 217.1742                        | 15.8793                          |
| 4.47      | 127.054                       | 26.7006                          | 217.1378                        | 15.9787                          |
| 4.5       | 126.9813                      | 26.7024                          | 217.0875                        | 15.98                            |
| 4.53      | 126.942                       | 26.7034                          | 217.0294                        | 16.08                            |
| 4.57      | 126.8941                      | 26.7046                          | 217.0038                        | 16.1791                          |
| 4.6       | 126.8342                      | 26.6206                          | 216.9585                        | 16.2788                          |
| 4.63      | 126.7932                      | 26.6216                          | 216.8984                        | 16.3788                          |
| 4.67      | 126.7145                      | 26.6236                          | 216.8846                        | 16.4776                          |
| 4.7       | 126.6461                      | 26.6235                          | 216.858                         | 16.4783                          |
| 4.73      | 126.5948                      | 26.5411                          | 216.8235                        | 16.5777                          |
| 4.77      | 126.5401                      | 26.5424                          | 216.7762                        | 16.5789                          |
| 4.8       | 126.4862                      | 26.5438                          | 216.7457                        | 16.6781                          |
Table 2: Continued.

| Time (min) | 1A: O₂ concentration (nmol/mL) | 1A: O₂ flux per V (pmol/(s∗mL)) | 1B: O₂ concentration (nmol/mL) | 1B: O₂ flux per V (pmol/(s∗mL)) |
|------------|---------------------------------|----------------------------------|---------------------------------|----------------------------------|
| 4.83       | 126.4529                        | 26.4591                          | 216.7033                        | 16.6792                          |
| 4.87       | 126.3981                        | 26.4605                          | 216.6708                        | 16.68                            |
| 4.9        | 126.3494                        | 26.4617                          | 216.6294                        | 16.681                           |
| 4.93       | 126.2955                        | 26.3775                          | 216.5881                        | 16.6821                          |
| 4.97       | 126.2314                        | 26.3791                          | 216.5703                        | 16.6825                          |
| 5          | 126.1527                        | 26.3811                          | 216.5102                        | 16.684                           |
| 5.03       | 126.0963                        | 26.468                           | 216.4748                        | 16.6849                          |
| 5.07       | 126.0638                        | 26.4688                          | 216.4482                        | 16.6856                          |
| 5.1        | 126.0159                        | 26.47                            | 216.4009                        | 16.6868                          |
| 5.13       | 125.9492                        | 26.4717                          | 216.3457                        | 16.6881                          |
| 5.17       | 125.9039                        | 26.4728                          | 216.3388                        | 16.5989                          |
| 5.2        | 125.844                         | 26.3888                          | 216.4088                        | 16.2925                          |
| 5.23       | 125.7901                        | 26.3902                          | 216.3132                        | 16.0979                          |
| 5.27       | 125.726                         | 26.3918                          | 216.2078                        | 16.1005                          |
| 5.3        | 125.6944                        | 26.3071                          | 216.1861                        | 16.0025                          |
| 5.33       | 125.6525                        | 26.2226                          | 216.1142                        | 16.1028                          |
| 5.37       | 125.5875                        | 26.1387                          | 216.0659                        | 16.2026                          |
| 5.4        | 125.5413                        | 26.1399                          | 215.997                         | 16.3028                          |
| 5.43       | 125.4806                        | 26.0559                          | 216.0039                        | 16.2041                          |
| 5.47       | 125.4224                        | 25.9718                          | 215.9891                        | 16.106                           |
| 5.5        | 125.3745                        | 25.973                           | 215.9359                        | 16.1073                          |
| 5.53       | 125.3335                        | 25.974                           | 215.8955                        | 16.0098                          |
| 5.57       | 125.2753                        | 25.9755                          | 215.8601                        | 16.0107                          |
| 5.6        | 125.2163                        | 25.977                           | 215.8039                        | 16.0121                          |
| 5.63       | 125.1462                        | 25.9787                          | 215.7743                        | 15.9143                          |
| 5.67       | 125.0855                        | 25.9802                          | 215.7409                        | 15.9151                          |
| 5.7        | 125.0393                        | 25.8959                          | 215.6946                        | 15.8178                          |
| 5.73       | 124.9889                        | 25.8971                          | 215.6443                        | 15.819                           |
| 5.77       | 124.9119                        | 25.8991                          | 215.5921                        | 15.9189                          |
| 5.8        | 124.8709                        | 25.9001                          | 215.5665                        | 15.9195                          |
| 5.83       | 124.8264                        | 25.9012                          | 215.5192                        | 15.9207                          |
| 5.87       | 124.7777                        | 25.9024                          | 215.4818                        | 16.0201                          |
| 5.9        | 124.7204                        | 25.8183                          | 215.464                          | 16.1191                          |
| 5.93       | 124.6631                        | 25.8198                          | 215.4227                        | 16.1201                          |
| 5.97       | 124.5827                        | 25.8218                          | 215.3586                        | 16.2202                          |
| 6          | 124.5117                        | 25.9091                          | 215.3468                        | 16.3191                          |
| 6.03       | 124.4827                        | 25.9953                          | 215.3212                        | 16.3197                          |
| 6.07       | 124.4408                        | 25.9963                          | 215.2867                        | 16.3206                          |
| 6.1        | 124.4091                        | 25.9971                          | 215.2256                        | 16.3221                          |
| 6.13       | 124.3552                        | 25.9985                          | 215.1675                        | 16.422                           |
| 6.17       | 124.3014                        | 25.9143                          | 215.1261                        | 16.4231                          |
| 6.2        | 124.2501                        | 25.9156                          | 215.0995                        | 16.4237                          |
| 6.23       | 124.1782                        | 25.8319                          | 215.0641                        | 16.5231                          |
| 6.27       | 124.1115                        | 25.8335                          | 215.001                         | 16.5247                          |
| 6.3        | 124.0619                        | 25.8348                          | 214.9695                        | 16.624                           |
| 6.33       | 124.014                         | 25.9215                          | 214.9439                        | 16.6247                          |
| 6.37       | 123.9456                        | 26.0087                          | 214.9252                        | 16.6251                          |
| 6.4        | 123.8875                        | 26.0102                          | 214.8729                        | 16.6264                          |
Table 2: Continued.

| Time (min) | 1A: O₂ concentration (nmol/mL) | 1A: O₂ flux per V (pmol/(s∗mL)) | 1B: O₂ concentration (nmol/mL) | 1B: O₂ flux per V (pmol/(s∗mL)) |
|-----------|--------------------------------|----------------------------------|--------------------------------|----------------------------------|
| 6.43      | 123.8302                       | 26.0116                          | 214.8158                      | 16.6279                          |
| 6.47      | 123.7609                       | 26.0989                          | 214.7577                      | 16.7278                          |
| 6.5       | 123.7301                       | 26.1851                          | 214.7321                      | 16.7285                          |
| 6.53      | 123.684                        | 26.1863                          | 214.7045                      | 16.5321                          |
| 6.57      | 123.6215                       | 26.1878                          | 214.672                       | 16.3359                          |
| 6.6       | 123.5685                       | 26.2747                          | 214.6276                      | 16.337                           |
| 6.63      | 123.5138                       | 26.2761                          | 214.5715                      | 16.3384                          |
| 6.67      | 123.4497                       | 26.2777                          | 214.535                       | 16.3393                          |
| 6.7       | 123.4009                       | 26.2789                          | 214.4897                      | 16.439                           |
| 6.73      | 123.3778                       | 26.1939                          | 214.4395                      | 16.5388                          |
| 6.77      | 123.3077                       | 26.1957                          | 214.3794                      | 16.6388                          |
| 6.8       | 123.2479                       | 26.1972                          | 214.339                       | 16.7383                          |
| 6.83      | 123.2119                       | 26.1126                          | 214.3065                      | 16.7391                          |
| 6.87      | 123.1401                       | 26.1144                          | 214.2681                      | 16.8386                          |
| 6.9       | 123.1025                       | 26.0298                          | 214.2287                      | 16.8396                          |
| 6.93      | 123.0332                       | 26.0315                          | 214.1705                      | 16.9395                          |
| 6.97      | 122.987                        | 25.9472                          | 214.1252                      | 17.0392                          |
| 7         | 122.9255                       | 25.9487                          | 214.0888                      | 17.0401                          |
| 7.03      | 122.875                        | 25.95                             | 214.0799                      | 17.0403                          |
| 7.07      | 122.8015                       | 25.9518                          | 214.0504                      | 17.0411                          |
| 7.1       | 122.7596                       | 26.0384                          | 214.01                        | 17.0421                          |
| 7.13      | 122.7339                       | 25.9535                          | 213.9587                      | 17.1419                          |
| 7.17      | 122.663                        | 25.9553                          | 213.8967                      | 17.2419                          |
| 7.2       | 122.6134                       | 25.9565                          | 213.8632                      | 17.2428                          |
| 7.23      | 122.5544                       | 25.8725                          | 213.8346                      | 17.2435                          |
| 7.27      | 122.5013                       | 25.8738                          | 213.8248                      | 17.1452                          |
| 7.3       | 122.4304                       | 25.9611                          | 213.7834                      | 17.1462                          |
| 7.33      | 122.3731                       | 26.048                           | 213.7528                      | 17.0485                          |
| 7.37      | 122.3132                       | 26.0495                          | 213.7213                      | 16.9508                          |
| 7.4       | 122.279                        | 26.0504                          | 213.6662                      | 16.8536                          |
| 7.43      | 122.2243                       | 26.0518                          | 213.6041                      | 16.8552                          |
| 7.47      | 122.1619                       | 25.9678                          | 213.5509                      | 16.8565                          |
| 7.5       | 122.0986                       | 25.9694                          | 213.4987                      | 16.9563                          |
| 7.53      | 122.0456                       | 25.9707                          | 213.4484                      | 16.9576                          |
| 7.57      | 122.0139                       | 25.9715                          | 213.4031                      | 16.9587                          |
| 7.6       | 121.9806                       | 25.8868                          | 213.3824                      | 17.0578                          |
| 7.63      | 121.9173                       | 25.8029                          | 213.3529                      | 17.0585                          |
| 7.67      | 121.8626                       | 25.8043                          | 213.3036                      | 17.0597                          |
| 7.7       | 121.8078                       | 25.8056                          | 213.2652                      | 16.9622                          |
| 7.73      | 121.7326                       | 25.8075                          | 213.2327                      | 16.963                           |
| 7.77      | 121.6873                       | 25.8086                          | 213.213                        | 16.865                           |
| 7.8       | 121.6248                       | 25.8957                          | 213.1834                      | 16.7672                           |
| 7.83      | 121.5855                       | 25.8967                          | 213.1174                      | 16.7688                           |
| 7.87      | 121.547                        | 25.8122                          | 213.0642                      | 16.7702                           |
| 7.9       | 121.4957                       | 25.8134                          | 213.0258                      | 16.7711                           |
| 7.93      | 121.4495                       | 25.8146                          | 212.9894                      | 16.6735                           |
| 7.97      | 121.3803                       | 25.7308                          | 212.949                        | 16.6745                           |
| 8         | 121.3161                       | 25.8179                          | 212.9175                      | 16.5768                           |
Table 2: Continued.

| Time (min) | 1A: O₂ concentration (nmol/mL) | 1A: O₂ flux per V (pmol/(s∗mL)) | 1B: O₂ concentration (nmol/mL) | 1B: O₂ flux per V (pmol/(s∗mL)) |
|-----------|--------------------------------|----------------------------------|--------------------------------|----------------------------------|
| 8.03      | 121.2648                       | 25.8192                          | 212.9027                       | 16.5772                          |
| 8.07      | 121.2092                       | 25.8206                          | 212.8455                       | 16.4801                          |
| 8.1       | 121.1742                       | 25.736                           | 212.8081                       | 16.481                           |
| 8.13      | 121.1349                       | 25.6514                          | 212.7677                       | 16.5806                          |
| 8.17      | 121.0835                       | 25.5672                          | 212.7234                       | 16.5817                          |
| 8.2       | 121.0117                       | 25.569                           | 212.6643                       | 16.5831                          |
| 8.23      | 120.9604                       | 25.4848                          | 212.614                         | 16.6829                          |
| 8.27      | 120.8852                       | 25.572                           | 212.5707                       | 16.7825                          |
| 8.3       | 120.8227                       | 25.5737                          | 212.5559                       | 16.7829                          |
| 8.33      | 120.7919                       | 25.5745                          | 212.5165                       | 16.8824                          |
| 8.37      | 120.7458                       | 25.4901                          | 212.4958                       | 16.7844                          |
| 8.4       | 120.6979                       | 25.4913                          | 212.4791                       | 16.6863                          |
| 8.43      | 120.6346                       | 25.4929                          | 212.4111                       | 16.688                           |
| 8.47      | 120.5679                       | 25.4946                          | 212.4022                       | 16.5897                          |
| 8.5       | 120.5183                       | 25.4958                          | 212.4633                       | 16.3911                          |
| 8.53      | 120.4773                       | 25.4968                          | 212.3402                       | 16.2957                          |
| 8.57      | 120.4277                       | 25.4126                          | 212.2249                       | 16.3971                          |
| 8.6       | 120.355                        | 25.4144                          | 212.1825                       | 16.3982                          |
| 8.63      | 120.3105                       | 25.4155                          | 212.1116                       | 16.4984                          |
| 8.67      | 120.2857                       | 25.4161                          | 212.0653                       | 16.4996                          |
| 8.7       | 120.237                        | 25.4173                          | 212.0722                       | 16.4009                          |
| 8.73      | 120.166                        | 25.4191                          | 212.0702                       | 16.3025                          |
| 8.77      | 120.1292                       | 25.3345                          | 212.018                         | 16.3038                          |
| 8.8       | 120.0754                       | 25.3359                          | 211.955                         | 16.3053                          |
| 8.83      | 119.9967                       | 25.4233                          | 211.9087                       | 16.405                           |
| 8.87      | 119.9497                       | 25.4245                          | 211.8781                       | 16.5043                          |
| 8.9       | 119.9009                       | 25.4257                          | 211.8998                       | 16.4052                          |
| 8.93      | 119.8265                       | 25.4276                          | 211.8703                       | 16.3075                          |
| 8.97      | 119.7752                       | 25.4289                          | 211.7816                       | 16.3097                          |
| 9         | 119.7059                       | 25.5161                          | 211.7107                       | 16.41                            |
| 9.03      | 119.6717                       | 25.517                           | 211.6584                       | 16.5098                          |
| 9.07      | 119.617                        | 25.6039                          | 211.5944                       | 16.6099                          |
| 9.1       | 119.5503                       | 25.6055                          | 211.5461                       | 16.7096                          |
| 9.13      | 119.4981                       | 25.6923                          | 211.5245                       | 16.8087                          |
| 9.17      | 119.434                        | 25.7795                          | 211.4782                       | 16.8098                          |
| 9.2       | 119.3853                       | 25.7807                          | 211.4289                       | 17.0081                          |
| 9.23      | 119.3348                       | 25.8675                          | 211.3816                       | 17.1078                          |
| 9.27      | 119.2698                       | 25.8691                          | 211.3787                       | 17.2064                          |
| 9.3       | 119.2356                       | 25.8699                          | 211.3639                       | 17.2067                          |
| 9.33      | 119.2006                       | 25.8708                          | 211.3087                       | 17.2081                          |
| 9.37      | 119.1458                       | 25.8722                          | 211.354                         | 17.01                            |
| 9.4       | 119.1022                       | 25.8733                          | 211.3797                       | 16.8123                          |
| 9.43      | 119.0672                       | 25.7886                          | 211.2358                       | 16.7174                          |
| 9.47      | 119.0167                       | 25.7044                          | 211.1373                       | 16.7198                          |
| 9.5       | 118.9432                       | 25.6207                          | 211.0743                       | 16.8199                          |
| 9.53      | 118.8842                       | 25.6222                          | 211.0181                       | 17.0183                          |
| 9.57      | 118.8551                       | 25.5374                          | 211.0181                       | 17.1169                          |
| 9.6       | 118.8012                       | 25.5387                          | 210.9698                       | 17.2166                          |
| Time (min) | 1A: O₂ concentration (nmol/mL) | 1A: O₂ flux per V (pmol/(s∗mL)) | 1B: O₂ concentration (nmol/mL) | 1B: O₂ flux per V (pmol/(s∗mL)) |
|-----------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| 9.63      | 118.726                       | 25.5406                       | 210.9294                      | 17.3161                       |
| 9.67      | 118.6618                      | 25.6287                       | 210.8605                      | 17.4163                       |
| 9.7       | 118.6148                      | 25.5434                       | 210.8221                      | 17.5158                       |
| 9.73      | 118.5567                      | 25.5449                       | 210.7679                      | 17.5172                       |
| 9.77      | 118.5036                      | 25.5462                       | 210.7699                      | 17.5171                       |
| 9.8       | 118.4224                      | 25.6337                       | 210.8802                      | 17.2188                       |
| 9.83      | 118.3933                      | 25.72                         | 210.7452                      | 16.9267                       |
| 9.87      | 118.3591                      | 25.6353                       | 210.6369                      | 16.8309                       |
| 9.9       | 118.2958                      | 25.6369                       | 210.5807                      | 16.9308                       |
| 9.93      | 118.2266                      | 25.7241                       | 210.5226                      | 17.0307                       |
| 9.97      | 118.1958                      | 25.7249                       | 210.4714                      | 17.229                        |
| 10        | 118.1376                      | 25.6408                       | 210.4832                      | 17.4258                       |
| 10.03     | 118.0855                      | 25.6422                       | 210.428                        | 17.4272                       |
| 10.07     | 118.0299                      | 25.558                        | 210.3935                      | 17.428                        |
| 10.1      | 117.994                       | 25.4734                       | 210.3591                      | 17.4289                       |
| 10.13     | 117.9307                      | 25.3895                       | 210.3059                      | 17.4302                       |
| 10.17     | 117.8666                      | 25.3911                       | 210.294                       | 17.4305                       |
| 10.2      | 117.8016                      | 25.3927                       | 210.3728                      | 17.2315                       |
| 10.23     | 117.7708                      | 25.3935                       | 210.2822                      | 16.9382                       |
| 10.27     | 117.7272                      | 25.3946                       | 210.166                       | 16.8426                       |
| 10.3      | 117.6562                      | 25.3963                       | 210.1246                      | 16.7451                       |
| 10.33     | 117.5972                      | 25.3978                       | 210.0576                      | 16.8453                       |
| 10.37     | 117.5716                      | 25.3985                       | 210.0162                      | 16.9449                       |
| 10.4      | 117.51                        | 25.4                          | 210.0113                      | 16.945                        |
| 10.43     | 117.4536                      | 25.4014                       | 209.968                       | 17.0446                       |
| 10.47     | 117.4193                      | 25.4023                       | 209.9088                      | 17.1446                       |
| 10.5      | 117.3783                      | 25.4033                       | 209.8714                      | 17.244                        |
| 10.53     | 117.2954                      | 25.4054                       | 209.8419                      | 17.2448                       |
| 10.57     | 117.2355                      | 25.4924                       | 209.7877                      | 17.4432                       |
| 10.6      | 117.1962                      | 25.5789                       | 209.7512                      | 17.4441                       |
| 10.63     | 117.1551                      | 25.5799                       | 209.7059                      | 17.4452                       |
| 10.67     | 117.1004                      | 25.4958                       | 209.6665                      | 17.4462                       |
| 10.7      | 117.0474                      | 25.4971                       | 209.6143                      | 17.349                        |
| 10.73     | 116.9721                      | 25.499                        | 209.6833                      | 16.9532                       |
| 10.77     | 116.9345                      | 25.4999                       | 209.6232                      | 16.6592                       |
| 10.8      | 116.9046                      | 25.3296                       | 209.4882                      | 16.7611                       |
| 10.83     | 116.8396                      | 25.3313                       | 209.4616                      | 16.8602                       |
| 10.87     | 116.7891                      | 25.247                        | 209.3897                      | 16.9605                       |
| 10.9      | 116.7464                      | 25.1626                       | 209.3463                      | 17.0601                       |
| 10.93     | 116.6891                      | 25.0785                       | 209.3286                      | 17.1591                       |
| 10.97     | 116.6395                      | 24.9942                       | 209.3444                      | 17.0602                       |
| 11        | 116.5856                      | 24.9956                       | 209.2793                      | 17.1603                       |
| 11.03     | 116.5377                      | 24.9967                       | 209.2153                      | 17.2604                       |
| 11.07     | 116.4676                      | 24.9985                       | 209.1789                      | 17.3599                       |
| 11.1      | 116.4163                      | 24.9998                       | 209.1345                      | 17.361                        |
| 11.13     | 116.3522                      | 25.0869                       | 209.0971                      | 17.1649                       |
| 11.17     | 116.3248                      | 25.0021                       | 209.1651                      | 16.8676                       |
| 11.2      | 116.2735                      | 25.0034                       | 209.0853                      | 16.7711                       |
| Time (min) | 1A: O₂ concentration (nmol/mL) | 1A: O₂ flux per V (pmol/(s∗mL)) | 1B: O₂ concentration (nmol/mL) | 1B: O₂ flux per V (pmol/(s∗mL)) |
|-----------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| 11.23     | 116.2059                      | 25.005                        | 208.869                       | 16.8725                       |
| 11.27     | 116.1444                      | 25.0921                       | 208.9582                      | 16.8728                       |
| 11.3      | 116.0871                      | 25.0935                       | 208.9119                      | 17.071                        |
| 11.33     | 116.0229                      | 25.1806                       | 208.8528                      | 17.0725                       |
| 11.37     | 115.9836                      | 25.1816                       | 208.837                       | 17.0729                       |
| 11.4      | 115.946                       | 25.1826                       | 208.7878                      | 17.1726                       |
| 11.43     | 115.8827                      | 25.1841                       | 208.7444                      | 17.1737                       |
| 11.47     | 115.8254                      | 25.1856                       | 208.6873                      | 17.2736                       |
| 11.5      | 115.7809                      | 25.2722                       | 208.6548                      | 17.2745                       |
| 11.53     | 115.7219                      | 25.3592                       | 208.6193                      | 17.0783                       |
| 11.57     | 115.6792                      | 25.3603                       | 208.5819                      | 16.9807                       |
| 11.6      | 115.6125                      | 25.3619                       | 208.6223                      | 16.7827                       |
| 11.63     | 115.5646                      | 25.4486                       | 208.637                       | 16.5853                       |
| 11.67     | 115.5295                      | 25.4495                       | 208.5366                      | 16.4893                       |
| 11.7      | 115.4825                      | 25.4507                       | 208.4577                      | 16.4913                       |
| 11.73     | 115.4235                      | 25.4522                       | 208.442                       | 16.3932                       |
| 11.77     | 115.3713                      | 25.4535                       | 208.3848                      | 16.2961                       |
| 11.8      | 115.3294                      | 25.369                        | 208.311                       | 16.3964                       |
| 11.83     | 115.2867                      | 25.2846                       | 208.3159                      | 16.2978                       |
| 11.87     | 115.2268                      | 25.2861                       | 208.2676                      | 16.299                        |
| 11.9      | 115.1704                      | 25.2875                       | 208.2243                      | 16.3001                       |
| 11.93     | 115.1088                      | 25.3745                       | 208.1819                      | 16.3011                       |
| 11.97     | 115.0558                      | 25.2903                       | 208.1504                      | 16.3019                       |
| 12        | 114.9959                      | 25.2918                       | 208.0982                      | 16.3032                       |
| 12.03     | 114.9549                      | 25.2929                       | 208.0578                      | 16.3042                       |
| 12.07     | 114.8967                      | 25.3798                       | 208.0282                      | 16.108                        |
| 12.1      | 114.8258                      | 25.3816                       | 208.1248                      | 15.7115                       |
| 12.13     | 114.7727                      | 25.3829                       | 208.0134                      | 15.7143                       |
| 12.17     | 114.7326                      | 25.4694                       | 207.8992                      | 15.7171                       |
| 12.2      | 114.6787                      | 25.4708                       | 207.8736                      | 15.8163                       |
| 12.23     | 114.6334                      | 25.3864                       | 207.8223                      | 15.9161                       |
| 12.27     | 114.6043                      | 25.3016                       | 207.7583                      | 16.0162                       |
| 12.3      | 114.553                       | 25.3029                       | 207.7741                      | 16.0158                       |
| 12.33     | 114.4846                      | 25.2191                       | 207.7534                      | 15.9178                       |
| 12.37     | 114.4127                      | 25.2209                       | 207.6982                      | 15.9192                       |
| 12.4      | 114.3537                      | 25.2224                       | 207.6588                      | 15.9202                       |
| 12.43     | 114.3093                      | 25.309                        | 207.6095                      | 16.0199                       |
| 12.47     | 114.2426                      | 25.3107                       | 207.5268                      | 16.1205                       |
| 12.5      | 114.2015                      | 25.3117                       | 207.5051                      | 16.0225                       |
| 12.53     | 114.1733                      | 25.3124                       | 207.5603                      | 15.8241                       |
| 12.57     | 114.1314                      | 25.2279                       | 207.5573                      | 15.7257                       |
| 12.6      | 114.0604                      | 25.2297                       | 207.4293                      | 15.7289                       |
| 12.63     | 113.9963                      | 25.2313                       | 207.3593                      | 15.7306                       |
| 12.67     | 113.9535                      | 25.3179                       | 207.2973                      | 15.9292                       |
| 12.7      | 113.9056                      | 25.3191                       | 207.247                        | 16.029                        |
| 12.73     | 113.8509                      | 25.3205                       | 207.2224                      | 16.1281                       |
| 12.77     | 113.8184                      | 25.3213                       | 207.246                        | 16.226                        |
| 12.8      | 113.7663                      | 25.2371                       | 207.1948                      | 16.2273                       |
| Time (min) | 1A: O₂ concentration (nmol/mL) | 1A: O₂ flux per V (pmol/(s ∗ mL)) | 1B: O₂ concentration (nmol/mL) | 1B: O₂ flux per V (pmol/(s ∗ mL)) |
|-----------|-------------------------------|-----------------------------------|-------------------------------|-----------------------------------|
| 12.83     | 113.7141                      | 25.2384                           | 207.1377                      | 16.3273                          |
| 12.87     | 113.6551                      | 25.2398                           | 207.1002                      | 16.4267                          |
| 12.9      | 113.5833                      | 25.2416                           | 207.0451                      | 16.6251                          |
| 12.93     | 113.5285                      | 25.3285                           | 206.9948                      | 16.6264                          |
| 12.97     | 113.4841                      | 25.3296                           | 206.9603                      | 16.4302                          |
| 13        | 113.4413                      | 25.3307                           | 206.9298                      | 16.431                           |
| 13.03     | 113.3883                      | 25.332                            | 206.8579                      | 16.5313                          |
| 13.07     | 113.3327                      | 25.3334                           | 206.8756                      | 16.5308                          |
| 13.1      | 113.2848                      | 25.3346                           | 206.9396                      | 16.322                           |
| 13.13     | 113.2258                      | 25.2506                           | 206.8372                      | 16.3348                          |
| 13.17     | 113.1839                      | 25.2516                           | 206.7466                      | 16.337                           |
| 13.2      | 113.1506                      | 25.1669                           | 206.716                        | 16.3378                          |
| 13.23     | 113.0719                      | 25.0834                           | 206.651                        | 16.3394                          |
| 13.27     | 113.0155                      | 25.0848                           | 206.5899                      | 16.538                           |
| 13.3      | 112.9573                      | 25.0863                           | 206.5939                      | 16.5379                          |
| 13.33     | 112.918                       | 25.0872                           | 206.5353                      | 16.5389                          |
| 13.37     | 112.8684                      | 25.0885                           | 206.517                        | 16.6383                          |
| 13.4      | 112.8025                      | 25.0901                           | 206.4658                      | 16.6396                          |
| 13.43     | 112.7624                      | 25.1766                           | 206.4106                      | 16.4439                          |
| 13.47     | 112.7145                      | 25.1778                           | 206.3643                      | 16.4451                          |
| 13.5      | 112.6572                      | 25.1793                           | 206.3121                      | 16.5449                          |
| 13.53     | 112.5965                      | 25.1808                           | 206.2688                      | 16.6445                          |
| 13.57     | 112.5306                      | 25.268                            | 206.2363                      | 16.7438                          |
| 13.6      | 112.493                       | 25.1834                           | 206.2116                      | 16.843                           |
| 13.63     | 112.4554                      | 25.1843                           | 206.1821                      | 16.8437                          |
| 13.67     | 112.3998                      | 25.1002                           | 206.1545                      | 16.8444                          |
| 13.7      | 112.3425                      | 25.1871                           | 206.122                       | 16.7467                          |
| 13.73     | 112.2963                      | 25.1883                           | 206.0875                      | 16.7476                          |
| 13.77     | 112.2279                      | 25.2755                           | 206.053                        | 16.7484                          |
| 13.8      | 112.1638                      | 25.3626                           | 206.0176                      | 16.7493                          |
| 13.83     | 112.1219                      | 25.3637                           | 205.988                        | 16.75                            |
| 13.87     | 112.0791                      | 25.3648                           | 205.925                        | 16.6531                          |
| 13.9      | 112.0124                      | 25.3664                           | 205.9171                      | 16.3578                          |
| 13.93     | 111.962                       | 25.4532                           | 205.986                        | 16.0605                          |
| 13.97     | 111.9286                      | 25.454                            | 205.8836                      | 15.866                           |
| 14        | 111.8696                      | 25.4555                           | 205.7969                      | 15.9667                          |
| 14.03     | 111.826                       | 25.4566                           | 205.7732                      | 15.9673                          |
| 14.07     | 111.7644                      | 25.4581                           | 205.7102                      | 15.9689                          |
| 14.1      | 111.7251                      | 25.3736                           | 205.659                        | 15.9702                          |
| 14.13     | 111.6738                      | 25.3749                           | 205.6639                      | 15.8715                          |
| 14.17     | 111.6105                      | 25.291                            | 205.5861                      | 15.8735                          |
| 14.2      | 111.5566                      | 25.2923                           | 205.522                        | 15.8751                          |
| 14.23     | 111.4865                      | 25.3796                           | 205.4826                      | 15.9746                          |
| 14.27     | 111.4455                      | 25.3806                           | 205.4521                      | 16.0739                          |
| 14.3      | 111.4164                      | 25.3813                           | 205.4186                      | 16.0747                          |
| 14.33     | 111.36                        | 25.3827                           | 205.4018                      | 16.0751                          |
| 14.37     | 111.3009                      | 25.3842                           | 205.3506                      | 16.1749                          |
| 14.4      | 111.2325                      | 25.3859                           | 205.3201                      | 16.1757                          |
| Time (min) | 1A: O<sub>2</sub> concentration (nmol/mL) | 1A: O<sub>2</sub> flux per V (pmol/(s ∗ mL)) | 1B: O<sub>2</sub> concentration (nmol/mL) | 1B: O<sub>2</sub> flux per V (pmol/(s ∗ mL)) |
|-----------|-----------------------------------------|------------------------------------------|--------------------------------------|------------------------------------------|
| 14.43     | 111.1906                                | 25.387                                   | 205.2836                             | 15.9795                                 |
| 14.47     | 111.1479                                | 25.388                                   | 205.3152                             | 15.6832                                 |
| 14.5      | 111.0923                                | 25.3039                                  | 205.324                              | 15.486                                  |
| 14.53     | 111.0324                                | 25.3054                                  | 205.2265                             | 15.3899                                 |
| 14.57     | 110.9657                                | 25.3071                                  | 205.1408                             | 15.392                                  |
| 14.6      | 110.9332                                | 25.3079                                  | 205.0945                             | 15.4917                                 |
| 14.63     | 110.876                                 | 25.3093                                  | 205.0373                             | 15.4931                                 |
| 14.67     | 110.811                                 | 25.3965                                  | 204.992                              | 15.4943                                 |
| 14.7      | 110.7622                                | 25.3977                                  | 204.9782                             | 15.4946                                 |
| 14.73     | 110.7118                                | 25.3989                                  | 204.9142                             | 15.5947                                 |
| 14.77     | 110.6485                                | 25.4005                                  | 204.8728                             | 15.6943                                 |
| 14.8      | 110.6066                                | 25.4016                                  | 204.8482                             | 15.7934                                 |
| 14.83     | 110.5844                                | 25.4021                                  | 204.8029                             | 15.8931                                 |
| 14.87     | 110.5262                                | 25.3181                                  | 204.7418                             | 16.0916                                 |
| 14.9      | 110.4715                                | 25.3194                                  | 204.7162                             | 16.2893                                 |
| 14.93     | 110.4108                                | 25.3209                                  | 204.6926                             | 16.3884                                 |
| 14.97     | 110.3586                                | 25.3223                                  | 204.6591                             | 16.4877                                 |
| 15        | 110.297                                 | 25.3238                                  | 204.6118                             | 16.5874                                 |
| 15.03     | 110.2474                                | 25.325                                   | 204.5763                             | 16.5883                                 |
| 15.07     | 110.203                                 | 25.3261                                  | 204.5605                             | 16.5887                                 |
| 15.1      | 110.1371                                | 25.3278                                  | 204.5162                             | 16.5898                                 |
| 15.13     | 110.079                                 | 25.4148                                  | 204.4719                             | 16.6894                                 |
| 15.17     | 110.0328                                | 25.4159                                  | 204.4098                             | 16.691                                  |
| 15.2      | 109.9926                                | 25.4169                                  | 204.3803                             | 16.7902                                 |
| 15.23     | 109.9302                                | 25.4185                                  | 204.3744                             | 16.6919                                 |
| 15.27     | 109.8883                                | 25.505                                   | 204.4561                             | 16.2958                                 |
| 15.3      | 109.8609                                | 25.4202                                  | 204.3576                             | 16.0027                                 |
| 15.33     | 109.8122                                | 25.3359                                  | 204.2778                             | 15.9062                                 |
| 15.37     | 109.7386                                | 25.3378                                  | 204.2266                             | 15.9075                                 |
| 15.4      | 109.6762                                | 25.3393                                  | 204.1566                             | 15.9092                                 |
| 15.43     | 109.63                                 | 25.255                                   | 204.1133                             | 16.0088                                 |
| 15.47     | 109.5796                                | 25.2562                                  | 204.1172                             | 15.8117                                 |
| 15.5      | 109.5377                                | 25.2573                                  | 204.0798                             | 15.8126                                 |
| 15.53     | 109.4975                                | 25.1728                                  | 204.0148                             | 15.9128                                 |
| 15.57     | 109.4633                                | 25.1736                                  | 203.9606                             | 16.0126                                 |
| 15.6      | 109.3966                                | 25.0898                                  | 203.933                              | 16.1118                                 |
| 15.63     | 109.3188                                | 25.0917                                  | 203.8966                             | 16.1127                                 |
| 15.67     | 109.264                                 | 25.0931                                  | 203.867                              | 16.1135                                 |
| 15.7      | 109.2221                                | 25.0086                                  | 203.8246                             | 16.2131                                 |
| 15.73     | 109.1512                                | 25.0959                                  | 203.7872                             | 16.214                                  |
| 15.77     | 109.1204                                | 25.0967                                  | 203.7557                             | 16.2148                                 |
| 15.8      | 109.0853                                | 25.012                                   | 203.7251                             | 16.117                                  |
| 15.83     | 109.0297                                | 24.9279                                  | 203.6769                             | 15.8227                                 |
| 15.87     | 108.9853                                | 24.929                                   | 203.6355                             | 15.7252                                 |
| 15.9      | 108.9297                                | 24.9304                                  | 203.5941                             | 15.7262                                 |
| 15.93     | 108.8775                                | 24.8462                                  | 203.5567                             | 15.7272                                 |
| 15.97     | 108.8151                                | 24.8478                                  | 203.5193                             | 15.7281                                 |
| 16        | 108.7647                                | 24.849                                   | 203.4917                             | 15.8273                                 |
| Time (min) | 1A: O₂ concentration (nmol/mL) | 1A: O₂ flux per V (pmol/(s∗mL)) | 1B: O₂ concentration (nmol/mL) | 1B: O₂ flux per V (pmol/(s∗mL)) |
|-----------|-------------------------------|---------------------------------|-------------------------------|---------------------------------|
| 16.03     | 108.7245                      | 24.85                           | 203.4651                      | 15.7295                         |
| 16.07     | 108.6757                      | 24.8512                         | 203.4207                      | 15.7306                         |
| 16.1      | 108.6364                      | 24.8522                         | 203.3498                      | 15.8309                         |
| 16.13     | 108.5765                      | 24.8537                         | 203.2897                      | 15.9309                         |
| 16.17     | 108.5167                      | 24.7697                         | 203.2722                      | 16.0298                         |
| 16.2      | 108.4594                      | 24.7711                         | 203.2375                      | 16.1292                         |
| 16.23     | 108.3978                      | 24.7727                         | 203.1922                      | 16.2289                         |
| 16.27     | 108.3499                      | 24.7739                         | 203.1518                      | 16.3284                         |
| 16.3      | 108.3037                      | 24.775                          | 203.137                       | 16.3288                         |
| 16.33     | 108.2576                      | 24.7762                         | 203.1055                      | 16.4281                         |
| 16.37     | 108.1986                      | 24.7777                         | 203.0612                      | 16.4292                         |
| 16.4      | 108.1558                      | 24.7787                         | 203.0011                      | 16.4307                         |
| 16.43     | 108.1131                      | 24.7798                         | 202.9705                      | 16.5299                         |
| 16.47     | 108.0446                      | 24.867                          | 202.9508                      | 16.5304                         |
| 16.5      | 107.9856                      | 24.8685                         | 202.9173                      | 16.5313                         |
| 16.53     | 107.9352                      | 24.9553                         | 202.8809                      | 16.6307                         |
| 16.57     | 107.8805                      | 25.0422                         | 202.8375                      | 16.5333                         |
| 16.6      | 107.8497                      | 25.0429                         | 202.7804                      | 16.3377                         |
| 16.63     | 107.8086                      | 24.9584                         | 202.7489                      | 16.2399                         |
| 16.67     | 107.7633                      | 24.8741                         | 202.7193                      | 16.1422                         |
| 16.7      | 107.7094                      | 24.8754                         | 202.6908                      | 16.0444                         |
| 16.73     | 107.6436                      | 24.8771                         | 202.6484                      | 16.1439                         |
| 16.77     | 107.6111                      | 24.8779                         | 202.6307                      | 16.0459                         |
| 16.8      | 107.5435                      | 24.8796                         | 202.6031                      | 15.948                          |
| 16.83     | 107.4939                      | 24.8808                         | 202.5489                      | 15.8509                         |
| 16.87     | 107.4486                      | 24.8819                         | 202.5046                      | 15.852                          |
| 16.9      | 107.3905                      | 24.7979                         | 202.4721                      | 15.8528                         |
| 16.93     | 107.3152                      | 24.7998                         | 202.409                        | 15.8544                         |
| 16.97     | 107.2827                      | 24.8861                         | 202.3755                      | 15.8552                         |
| 17        | 107.2391                      | 24.8872                         | 202.3469                      | 15.8559                         |
| 17.03     | 107.1724                      | 24.8888                         | 202.3184                      | 15.8567                         |
| 17.07     | 107.1126                      | 24.9758                         | 202.275                        | 15.7592                         |
| 17.1      | 107.0561                      | 25.0628                         | 202.2445                      | 15.76                           |
| 17.13     | 106.9997                      | 25.0642                         | 202.1962                      | 15.7612                         |
| 17.17     | 106.9544                      | 25.1508                         | 202.1489                      | 15.7624                         |
| 17.2      | 106.9202                      | 25.1517                         | 202.1135                      | 15.6647                         |
| 17.23     | 106.8783                      | 25.0672                         | 202.1834                      | 15.466                          |
| 17.27     | 106.8158                      | 25.0688                         | 202.1263                      | 15.3689                         |
| 17.3      | 106.7577                      | 25.1557                         | 202.0248                      | 15.3714                         |
| 17.33     | 106.7184                      | 25.1567                         | 201.9992                      | 15.2736                         |
| 17.37     | 106.6611                      | 25.1582                         | 201.946                        | 15.1764                         |
| 17.4      | 106.5995                      | 25.1597                         | 201.8967                      | 15.1776                         |
| 17.43     | 106.5499                      | 25.0754                         | 201.8524                      | 15.1787                         |
| 17.47     | 106.5011                      | 25.0766                         | 201.8202                      | 15.18                           |
| 17.5      | 106.4687                      | 25.0775                         | 201.7657                      | 15.1809                         |
| 17.53     | 106.4208                      | 25.0786                         | 201.7174                      | 15.2806                         |
| 17.57     | 106.3541                      | 25.0803                         | 201.6593                      | 15.3806                         |
| 17.6      | 106.3173                      | 25.0812                         | 201.6593                      | 15.3806                         |
| Time (min) | 1A: O₂ concentration (nmol/mL) | 1A: O₂ flux per V (pmol/(s∗mL)) | 1B: O₂ concentration (nmol/mL) | 1B: O₂ flux per V (pmol/(s∗mL)) |
|-----------|-------------------------------|-----------------------------------|-------------------------------|----------------------------------|
| 17.63     | 106.2609                      | 25.0826                           | 201.617                       | 15.4801                          |
| 17.67     | 106.1907                      | 25.0844                           | 201.5825                      | 15.4818                          |
| 17.7      | 106.1454                      | 25.0855                           | 201.547                       | 15.5804                          |
| 17.73     | 106.0924                      | 25.0869                           | 201.5155                      | 15.5812                          |
| 17.77     | 106.0445                      | 25.0881                           | 201.4692                      | 15.6809                          |
| 17.8      | 105.9975                      | 25.0892                           | 201.4396                      | 15.7801                          |
| 17.83     | 105.9376                      | 25.1762                           | 201.3845                      | 15.7815                          |
| 17.87     | 105.8923                      | 25.1774                           | 201.4436                      | 15.6815                          |
| 17.9      | 105.8376                      | 25.1787                           | 201.4495                      | 15.4843                          |
| 17.93     | 105.788                        | 25.18                             | 201.3254                      | 15.4874                          |
| 17.97     | 105.7521                      | 25.1809                           | 201.2899                      | 15.4883                          |
| 18        | 105.6973                      | 25.0967                           | 201.219                       | 15.5886                          |
| 18.03     | 105.6221                      | 25.0986                           | 201.151                       | 15.6888                          |
| 18.07     | 105.5785                      | 25.0997                           | 201.148                       | 15.7874                          |
| 18.1      | 105.5417                      | 25.0151                           | 201.1106                      | 15.7883                          |
| 18.13     | 105.475                        | 25.0168                           | 201.0584                      | 15.7896                          |
| 18.17     | 105.4186                      | 25.0182                           | 200.9953                      | 15.8897                          |
| 18.2      | 105.3724                      | 25.0193                           | 200.9648                      | 15.8905                          |
| 18.23     | 105.3262                      | 25.0205                           | 200.9313                      | 15.9898                          |
| 18.27     | 105.2826                      | 25.0216                           | 200.9047                      | 16.089                           |
| 18.3      | 105.2287                      | 24.9374                           | 200.8781                      | 16.0897                          |
| 18.33     | 105.168                        | 24.9389                           | 200.9707                      | 15.8903                          |
| 18.37     | 105.1201                      | 24.9401                           | 200.8722                      | 15.8928                          |
| 18.4      | 105.074                        | 24.9413                           | 200.7747                      | 15.8952                          |
| 18.43     | 105.0363                      | 24.9422                           | 200.7491                      | 15.8959                          |
| 18.47     | 104.973                        | 25.0293                           | 200.6919                      | 15.8973                          |
| 18.5      | 104.9235                      | 25.0306                           | 200.6338                      | 16.0958                          |
| 18.53     | 104.8781                      | 25.0317                           | 200.6141                      | 16.0963                          |
| 18.57     | 104.8123                      | 24.9478                           | 200.5757                      | 15.9987                          |
| 18.6      | 104.7413                      | 25.0351                           | 200.5382                      | 15.9012                          |
| 18.63     | 104.708                        | 25.036                            | 200.5028                      | 15.902                           |
| 18.67     | 104.6703                      | 25.0369                           | 200.4446                      | 15.9035                          |
| 18.7      | 104.631                        | 24.9524                           | 200.4141                      | 16.0028                          |
| 18.73     | 104.5805                      | 24.9536                           | 200.3727                      | 16.0038                          |
| 18.77     | 104.5181                      | 24.9552                           | 200.3323                      | 16.0048                          |
| 18.8      | 104.4651                      | 24.9565                           | 200.3195                      | 16.0051                          |
| 18.83     | 104.4044                      | 24.958                            | 200.3796                      | 15.8066                          |
| 18.87     | 104.3625                      | 24.9591                           | 200.2663                      | 15.7109                          |
| 18.9      | 104.3197                      | 24.8746                           | 200.1777                      | 15.8117                          |
| 18.93     | 104.2607                      | 24.8761                           | 200.1461                      | 15.8124                          |
| 18.97     | 104.2154                      | 24.8772                           | 200.1077                      | 15.9119                          |
| 19        | 104.1701                      | 24.7929                           | 200.0664                      | 16.0115                          |
| 19.03     | 104.1111                      | 24.7943                           | 200.0752                      | 16.0112                          |
| 19.07     | 104.0623                      | 24.7956                           | 200.0181                      | 16.1112                          |
| 19.1      | 104.0222                      | 24.7966                           | 199.9629                      | 16.1126                          |
| 19.13     | 103.9862                      | 24.712                            | 199.9215                      | 16.2121                          |
| 19.17     | 103.9221                      | 24.7136                           | 199.8821                      | 16.3116                          |
| 19.2      | 103.8853                      | 24.629                            | 199.8792                      | 16.2132                          |
| Time (min) | 1A: O₂ concentration (nmol/mL) | 1A: O₂ flux per V (pmol/(s∗mL)) | 1B: O₂ concentration (nmol/mL) | 1B: O₂ flux per V (pmol/(s∗mL)) |
|-----------|-------------------------------|---------------------------------|-------------------------------|---------------------------------|
| 19.23     | 103.8263                      | 24.6304                         | 199.823                       | 16.0175                         |
| 19.27     | 103.763                       | 24.632                          | 199.7767                      | 15.9202                         |
| 19.3      | 103.7194                      | 24.5476                         | 199.8575                      | 15.7211                         |
| 19.33     | 103.6767                      | 24.4632                         | 199.7807                      | 15.526                          |
| 19.37     | 103.6245                      | 24.4645                         | 199.6605                      | 15.6276                         |
| 19.4      | 103.5476                      | 24.5519                         | 199.6437                      | 15.628                          |
| 19.43     | 103.5022                      | 24.553                          | 199.5817                      | 15.728                          |
| 19.47     | 103.4595                      | 24.5541                         | 199.5324                      | 15.8278                         |
| 19.5      | 103.4107                      | 24.5553                         | 199.5166                      | 15.9267                         |
| 19.53     | 103.3654                      | 24.5564                         | 199.4851                      | 16.026                          |
| 19.57     | 103.3218                      | 24.5575                         | 199.4585                      | 16.0267                         |
| 19.6      | 103.2722                      | 24.4733                         | 199.4103                      | 16.1264                         |
| 19.63     | 103.2064                      | 24.4749                         | 199.3679                      | 16.1274                         |
| 19.67     | 103.1653                      | 24.4759                         | 199.3068                      | 15.9319                         |
| 19.7      | 103.12                        | 24.4771                         | 199.2645                      | 15.933                          |
| 19.73     | 103.0618                      | 24.4785                         | 199.2507                      | 15.9333                         |
| 19.77     | 103.0063                      | 24.4799                         | 199.3393                      | 15.6356                         |
| 19.8      | 102.9524                      | 24.4813                         | 199.2241                      | 15.6385                         |
| 19.83     | 102.9028                      | 24.4825                         | 199.1285                      | 15.6409                         |
| 19.87     | 102.8412                      | 24.5696                         | 199.0999                      | 15.7401                         |
| 19.9      | 102.8045                      | 24.5705                         | 199.0379                      | 15.8402                         |
| 19.93     | 102.7514                      | 24.6573                         | 198.9975                      | 15.8412                         |
| 19.97     | 102.6916                      | 24.6588                         | 198.9935                      | 15.9398                         |
| 20        | 102.6463                      | 24.7455                         | 198.9512                      | 15.9408                         |
| 20.03     | 102.5941                      | 24.7468                         | 198.8931                      | 16.0408                         |
| 20.07     | 102.5342                      | 24.7483                         | 198.8428                      | 16.1406                         |
| 20.1      | 102.4821                      | 24.7496                         | 198.8133                      | 16.2398                         |
| 20.13     | 102.4573                      | 24.7502                         | 198.7857                      | 16.339                          |
| 20.17     | 102.4111                      | 24.7513                         | 198.7561                      | 16.1427                         |
| 20.2      | 102.3624                      | 24.7525                         | 198.697                        | 16.1442                         |
| 20.23     | 102.2982                      | 24.7542                         | 198.6813                      | 16.1446                         |
| 20.27     | 102.2452                      | 24.841                          | 198.7532                      | 16.0443                         |
| 20.3      | 102.2127                      | 24.8418                         | 198.6172                      | 16.0477                         |
| 20.33     | 102.1597                      | 24.7576                         | 198.5364                      | 16.1482                         |
| 20.37     | 102.087                       | 24.8449                         | 198.5                           | 16.2477                         |
| 20.4      | 102.0477                      | 24.8459                         | 198.4488                      | 16.3474                         |
| 20.43     | 101.9964                      | 24.8472                         | 198.4094                      | 16.4469                         |
| 20.47     | 101.9553                      | 24.7627                         | 198.4143                      | 16.4468                         |
| 20.5      | 101.9134                      | 24.7638                         | 198.3493                      | 16.547                          |
| 20.53     | 101.8647                      | 24.6795                         | 198.3089                      | 16.548                          |
| 20.57     | 101.798                       | 24.6811                         | 198.2586                      | 16.6477                         |
| 20.6      | 101.7338                      | 24.6827                         | 198.2271                      | 16.747                          |
| 20.63     | 101.6817                      | 24.6841                         | 198.1926                      | 16.6494                         |
| 20.67     | 101.6372                      | 24.6852                         | 198.1808                      | 16.4527                         |
| 20.7      | 101.5928                      | 24.6863                         | 198.1335                      | 16.4538                         |
| 20.73     | 101.5508                      | 24.6873                         | 198.1197                      | 16.4542                         |
| 20.77     | 101.4953                      | 24.6887                         | 198.1936                      | 16.2553                         |
| 20.8      | 101.4431                      | 24.69                           | 198.0685                      | 16.1599                         |
occurrence of oxidative stress. IGF1 significantly decreased the CAT, SOD, GSH, and GSH-Px activities in vivo and in vitro. These changes were accompanied by reduced T-AOC, which is an important indicator for determining the body’s antioxidant capacity; meanwhile, the expression of iNOS was significantly increased. All of these results demonstrate that IGF1 suppression can significantly reduce the body’s antioxidant capacity and enrich many oxygen free radicals in the body; this may be one of the important causes of myocardial damage and dysplasia.

Table 2: Continued.

| Time (min) | 1A: O₂ concentration (nmol/mL) | 1A: O₂ flux per V (pmol/(s×mL)) | 1B: O₂ concentration (nmol/mL) | 1B: O₂ flux per V (pmol/(s×mL)) |
|-----------|-------------------------------|----------------------------------|---------------------------------|----------------------------------|
| 20.83     | 101.3952                      | 24.6912                          | 197.9907                        | 16.1619                          |
| 20.87     | 101.3499                      | 24.6923                          | 197.968                          | 16.1624                          |
| 20.9      | 101.296                       | 24.6937                          | 197.901                          | 16.1641                          |
| 20.93     | 101.2285                      | 24.6954                          | 197.8577                        | 16.1652                          |
| 20.97     | 101.1797                      | 24.6966                          | 197.8616                        | 16.1651                          |
| 21       | 101.131                       | 24.6978                          | 197.8104                        | 16.1664                          |
| 21.03     | 101.0771                      | 24.6992                          | 197.7444                        | 16.2665                          |
| 21.07     | 101.0275                      | 24.7004                          | 197.6823                        | 16.3666                          |
| 21.1     | 100.9796                      | 24.7016                          | 197.6518                        | 16.1703                          |
| 21.13     | 100.9437                      | 24.7025                          | 197.64                          | 16.0721                          |
| 21.17     | 100.9027                      | 24.618                           | 197.6114                        | 16.0728                          |
| 21.2     | 100.8556                      | 24.6192                          | 197.571                          | 16.0739                          |
| 21.23     | 100.8095                      | 24.6203                          | 197.5286                        | 16.0749                          |
| 21.27     | 100.759                       | 24.5361                          | 197.5996                        | 15.9746                          |
| 21.3     | 100.7017                      | 24.5375                          | 197.505                          | 15.78                            |
| 21.33     | 100.6427                      | 24.539                           | 197.4085                        | 15.8809                          |
| 21.37     | 100.5948                      | 24.5402                          | 197.373                          | 15.8818                          |
| 21.4     | 100.5392                      | 24.6271                          | 197.3257                        | 15.9815                          |
| 21.43     | 100.4896                      | 24.6283                          | 197.2981                        | 15.9822                          |
| 21.47     | 100.4452                      | 24.6295                          | 197.2804                        | 15.9826                          |
| 21.5     | 100.3879                      | 24.6309                          | 197.2311                        | 15.9838                          |
| 21.53     | 100.3468                      | 24.5464                          | 197.1917                        | 15.9848                          |
| 21.57     | 100.2964                      | 24.5477                          | 197.1474                        | 16.0844                          |
| 21.6     | 100.2357                      | 24.6347                          | 197.1178                        | 15.7896                          |
| 21.63     | 100.1818                      | 24.5505                          | 197.0804                        | 15.7906                          |
| 21.67     | 100.1219                      | 24.6375                          | 197.0607                        | 15.7911                          |
| 21.7     | 100.0886                      | 24.6384                          | 197.1238                        | 15.5925                          |
| 21.73     | 100.039                       | 24.6396                          | 197.0646                        | 15.4954                          |
| 21.77     | 99.9937                       | 24.6407                          | 196.9642                        | 15.4979                          |
| 21.8     | 99.9338                       | 24.6422                          | 196.9326                        | 15.4987                          |
| 21.83     | 99.8817                       | 24.558                           | 196.8568                        | 15.5006                          |
| 21.87     | 99.8415                       | 24.559                           | 196.8016                        | 15.6005                          |
| 21.9     | 99.7902                       | 24.5603                          | 196.8026                        | 15.699                           |
| 21.93     | 99.7397                       | 24.5616                          | 196.7612                        | 15.7986                          |
| 21.97     | 99.6961                       | 24.5627                          | 196.7208                        | 15.7996                          |
| 22       | 99.6294                       | 24.5643                          | 196.6834                        | 15.8005                          |
| 22.03     | 99.5918                       | 24.5653                          | 196.6351                        | 15.9002                          |
| 22.07     | 99.5405                       | 24.5666                          | 196.5928                        | 15.9013                          |
| 22.1     | 99.4909                       | 24.5678                          | 196.5691                        | 15.7048                          |
| 22.13     | 99.4396                       | 24.5691                          | 196.5238                        | 15.6075                          |
| 22.17     | 99.3865                       | 24.5704                          | 196.5021                        | 15.608                           |
FOXO, a highly conserved transcriptional regulatory protein, is a downstream protein of the PI3K/Akt and AMPK/14-3-3 signaling pathways [39, 40]. The PI3K/Akt/FOXO signaling pathway is involved in the regulation of various physiological processes such as proliferation, apoptosis, and insulin resistance. The PI3K/Akt pathway can...
be activated by several of these stimuli, and ROS is one of the most important factors. Adipogenic differentiation can be mediated through activation of the PI3K/Akt pathway by oxidative stress in primary rat osteoblasts [41]. Stitt et al. found that the IGF1/PI3K/Akt pathway plays an important role in preventing the expression of ubiquitin ligases by inhibiting FOXO transcription factors [42]. AMPK is an important protein kinase in eukaryotic cells. The energy regulation of AMPK can maintain normal ATP levels in cardiomyocytes [43]. AMPK regulates the utilization of energy throughout the body, and it is considered the energy regulator [44]. FOXO is an important downstream molecule of AMPK involved in the signal transduction and regulation of various cellular biological processes such as inhibiting cell proliferation and promoting apoptosis [45, 46]. IGF1 stimulation can decrease the level of AMPK phosphorylation in F1 and F3/4 granulosa cells [47]. Hinchy proved that the ROS released from mitochondria can activate AMPK indirectly [48]. Furthermore, FOXO has been proven to be required in endothelial but not myocardial cell lineages during cardiovascular development [49]. Evans-Anderson et al. demonstrated that a FOXO transcription factor is a negative regulator of cardiomyocyte proliferation during heart development [50]. In our present study, we found that IGF1 depletion inhibited the expression of IRS1, PI3K, and Akt and upregulated both the mRNA and protein expression of AMPK and FOXO; meanwhile, the expression of JNK significantly increased in the IGF1 suppression group, which is important upstream of IRS1. All of these results suggest that IGF1 knockdown can activate FOXO through inhibiting the expression of IRS1. Combined with the results of ROS, we concluded that IGF1 suppression triggers ROS release to activate FOXO, which further inhibits myocardial development.

Mitochondria, as an energy converter in animal cells, are a main site for intracellular oxidative phosphorylation and ATP formation. Pawlikowska et al. showed that mitochondria are essential organelles for insulin-mediated muscle formation and that insulin stimulates mitochondria and promotes mitochondrial function [51]. Insulin-like growth factor-binding proteins (IGFBPs) are important parts of the IGF family; IGFBPs cannot bind with insulin but rather form a complex with IGFs. They can regulate the normal growth of embryonic and postnatal organs, which is also a very important tool for IGF1 transport and storage. The IGF1-IGFBP complex will decompose and release IGF1, which will further combine with IGF1R in the cell membrane under the catalysis of IR [52]. IGF1 is protected from decomposition by its high affinity with IGFBP, prolonging the half-life of IGF1 in the body’s circulation cells. IGFBPs avoid the body’s negative effects for insulin overdose by reducing the concentration of free IGF1 in the blood. In addition, studies have shown that IGFBP can help IGF1 recognize target cell and regulate the activity of IGF1 [53]. The IGF1-IGFBP complex can cause a cascade of various phosphorylation processes in the cell, ultimately leading to the entry of the glucose transporter molecules (GLUT1, 3, and 8) into the cell membrane and increasing the rate of glucose transport in the cell. Glucose transport is mainly dependent on GLUT, and it plays an important role in regulating glucose transport and maintaining cardiac energy balance [54]. IGF1RS/IRS signaling regulates cellular energy metabolism through the PI3K/Akt signaling pathway, and its downstream genes have been

Figure 8: Morphological observation was performed in cardiomyocytes transfected with Opti-MEM (C group, a) and siRNA (KD group, b) for 24 h. Cardiomyocytes were stained by H.E., and the results are shown in (c) and (d). H.E. staining for myocardial tissues in the N group (e) and the Si group (f). C indicates the control group; KD indicates the knockdown group in vitro; N indicates the normal group; Si indicates the knockdown group in vivo.
found [55]. FOXO plays an important role in mediating the effects of insulin and growth factors on diverse physiological functions [56]. In the present study, we demonstrated that IGF1 knockdown significantly decreased the expression of GLUT3 and IGFBP, indicating that IGF1 suppression blocks energy metabolism. To further verify these results, we also detected the ATP content and oxygen consumption rate in two different groups; the results revealed that IGF1 knockdown can significantly impede the ability of using oxygen for cardiomyocytes, thereby reducing the production of ATP. Considering our results showing FOXO activation by IGF1 knockdown, we conclude that FOXO can inhibit myocardial development by interfering with myocardial energy metabolism.

Myogenic regulatory factors (MRFs) are a class of muscle-specific regulators that determine the function of cell-directed differentiation. MRFs are essential for muscle development in vertebrates [56]. MRFs include MYF5, MyoD, MyoG, and MRF4; the function of MRFs is transforming mesenchymal stem cells into myoblasts, which will further activate and maintain the differentiation state of myocytes. Each member of the MRF family plays different roles at different stages. MyoD and MYF5 play an important role in the early proliferative phase of myocytes [57]. MYF5 is responsible for myoblast proliferation, and MyoD regulates the differentiation process for myoblasts. MyoG and MRF4 can drive terminal differentiation. The proliferation process of myoblasts is normal after knocking out MyoG, but the subsequent differentiation process is significantly inhibited [58]. Mesp1 and Mesp2 are key genes in regulating cardiac differentiation. Mesp1 can directly activate other genes in the cardiac core by binding to other gene promoters in cardiomyocytes. Mesp1 and Mesp2 reduction can impede heart development [59]. GATA4 and Nkx2.5 are transcription factors involved in heart development, and overexpression of GATA4 can accelerate myocardial differentiation [60]. Early embryo development requires the production and expenditure of large amounts of cellular energy for cell growth. Insulin can regulate skeletal muscle cell differentiation by mediating the activation of MAPK and PKB phosphorylation [61]. Montarras et al. demonstrated that insulin or IGF is necessary for cell differentiation [62]. Cellular energy metabolism that contains fatty acid is involved in fetal heart development [63]. In the present study, we found that IGF1 knockdown can significantly decrease the expression of MyoD, Mesp1, MYF5, MYF6, GATA4, GATA6, and Nkx2.5, indicating that IGF1 suppression can block myocardial development. We suggest that reducing energy metabolism inhibits the expression of myocardial development to factors through comprehensive results on energy metabolism.

In summary, we conclude that IGF1 knockdown hinders myocardial development through the energy metabolism dysfunction caused by ROS-dependent FOXO activation in the chicken heart. Our results indicate a novel hypothesis for IGF1 in the development of cardiomyocytes.

**Abbreviations**

IGF1: Insulin-like growth factor 1  
IGFs: Insulin-like growth factors  
IGF1R: Insulin-like growth factor 1 receptor  
IGF2: Insulin-like growth factor 2  
GH: Growth hormone  
BMP: Bone morphogenic protein  
FGF4: Fibroblast growth factor 4  
IR: Insulin receptor  
IGFR: Insulin-like growth factor receptor  
IRR: Insulin receptor-related receptor  
TK: Tyrosine kinase  
IRS: Insulin receptor substrate  
PI3K: Phosphatidylinositol 3-kinase  
VEGF: Vascular endothelial growth factor  
ROS: Reactive oxygen species  
H₂O₂: Hydrogen peroxide  
GSH: Glutathione  
GSH-Px: Glutathione peroxidase  
CAT: Catalase  
MDA: Malondialdehyde  
iNOS: Induced nitric oxide synthase  
SOD: Superoxide dismutase  
T-AOC: Total antioxidant capability  
IGFBP1: Insulin-like growth factor-binding protein 1  
IGFBP2: Insulin-like growth factor-binding protein 2  
IGFBP3: Insulin-like growth factor-binding protein 3  
IGFBP4: Insulin-like growth factor-binding protein 4  
IGFBP5: Insulin-like growth factor-binding protein 5  
IGFBP7: Insulin-like growth factor-binding protein 7  
GLUT1: Glucose transporters 1  
GLUT3: Glucose transporters 3  
GLUT8: Glucose transporters 8  
Akt: Threonine-protein kinase  
P-Akt: Phosphorylation-threonine-protein kinase  
FOXO: Forkhead box protein  
P-FOXO: Phosphorylation-forkhead box protein  
JNK: c-Jun N-terminal kinase  
P-JNK: Phosphorylation-c-Jun N-terminal kinase  
GATA4: GATA-binding protein 4  
GATA6: GATA-binding protein 6  
Nkx2.5: NK2 homeobox 5  
AMPK: AMP-activated protein kinase  
MyoG: Myogenin  
Mesp1: Cardiac transcription factor mesoderm posterior 1  
MYF5: Myogenic factor 5  
MYF6: Myogenic factor 6  
O₂⁻: Superoxide anion  
GSSH: Oxidized glutathione  
ATP: Adenosine triphosphate  
IGFBPs: Insulin-like growth factor-binding proteins  
MRFs: Myogenic regulatory factors  
MAPK: Mitogen-activated protein kinase  
Nrf2: Nuclear factor erythroid 2-related factor 2  
PBS: Phosphate-buffered solution  
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

**Data Availability**

The data used to support the findings of this study are available from the corresponding author upon request.
Conflicts of Interest
The authors declare that there are no conflicts of interest.

Authors’ Contributions
All authors have read the manuscript and agreed to submit it in its current form for consideration for publication in the Journal.

Acknowledgments
This study was supported by the National Natural Science Foundation of China (31872531), Earmarked Fund for China Agriculture Research System (No. CARS 35-04), Funding for State Key Laboratory of Animal Nutrition (2004DA125184F1725), Merit-based Funding for Returned Oversea Student of Heilongjiang Province (2018QD0005), Heilongjiang Postdoctoral Fund “Academic backbone” Project of Northeast Agricultural University (17XG11).

Supplementary Materials
The graphical abstract of the entire manuscript. (Supplementary Materials)

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