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Original Article

Cobalt Chloride Alters Mitochondrial Function of *In Vitro* Cultured Cardiomyocytes in a Dose-dependent Manner

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Abstract: This study was carried out to evaluate the effect of Cobalt chloride ($CoCl_2$) on cardiac mitochondrial function in an *in vitro* model. In the study, H9C2 cardiomyocytes were cultured in a medium containing different concentrations of $CoCl_2$. Cell viability, cardiolipin content, mitochondrial function, and mitochondrial oxidative stress were assessed by using Cell Counting Kit-8 and suitable fluorescence kits. The obtained data show that $CoCl_2$ (200÷400 µM) induced cell death and decreased mitochondrial function of H9C2 cardiomyocytes. Particularly, $CoCl_2$ at the dose of 300 µM significantly altered the values of mitochondrial membrane potential, H_2O_2 and O_2^- to 63.79±2.15%, 145.81±5.83% and 143.10±3.07% (of 100% control), respectively. Altogether, $CoCl_2$ strongly induced cardiomyocyte death via altering mitochondrial function in a dose-dependent manner.

Keywords: H9C2, mitochondria, cell counting kit-8.

1. Introduction

Oxygen (O_2)-deficient environment plays a very important role in the pathological conditions, including myocardial ischemia [1]. Cells respond to the O_2-deficiency conditions by activating factor hypoxia inducing factor 1α (HIF1α), a transcription factor regulating the expression of genes involved in cell survival, metabolism, and migration [2]. Under normal conditions, HIF1α is hydroxylated, ubiquitinated, and degraded in the proteasome; however, under hypoxic conditions, the activity of hydroxylase enzymes is inhibited and HIF1α becomes stable [3]. Previous research shows that Cobalt chloride ($CoCl_2$) has the ability to induce O_2 deficiency by inhibiting hydroxylase activity and sustaining HIF1α [1]; therefore, this compound has been widely used for establishing *in vitro* models of ischemic heart disease. Mitochondria are intracellular respiratory organelles and strongly affected by oxygen deprivation. Also, abnormal mitochondrial function might exacerbate the progression of ischemic heart disease. To understand the influence of $CoCl_2$ on mitochondria, this study was carried out to assess the alteration of cardiac mitochondria under different treatments of $CoCl_2$.

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2. Methodology

2.1. Cell Culture

H9C2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (PS) at 37 °C, 5% CO_2. Culture medium was changed every 2-3 days.

2.2. $CoCl_2$ Treatment

H9C2 cells were further grown in a 96-well black plate, glass bottom at a density of 10^4 cells/well at 37 °C, 5% CO_2 for 24 h. The cells were then subjected to $CoCl_2$ (100÷400 µM) for 24 h. Then, the medium containing $CoCl_2$ was removed, the cells were continued to be grown for 24 h in new media containing DMEM supplemented with 10% FBS and 1% PS at 37 °C, 5% CO_2. In the normal control group, the H9C2 cells were continuously cultured in normal media (DMEM, 10% FBS, 1% PS, 37 °C, 5% CO_2) for 48 h.

At the end of the experiment, the values of cell viability, mitochondrial membrane potential, cardiolipin content and H_2O_2 and O_2^- levels were determined indirectly through the analysis of Cell Counting Kit-8 (CCK-8), fluorescent indicators. Experiments were performed in triplicate.

2.3. Cell Viability Assay

After the above $CoCl_2$ treatments, cell viability was assessed by using CCK-8 as previously described [4]. For each group, H9C2 cells were further incubated for 1-4 h with CCK-8. The absorbance value indicating cell viability was measured at 450 nm using a microplate reader. The number of alive cells in each well was expressed as a value relative to the normal control. Experiments were repeated in triplicate.

2.4. Measurement of Mitochondrial Cardiolipin and Mitochondrial Membrane Potential

H9C2 cells were seeded at a density of 10^4 cells/well in 96-well black, glass bottom plates (CAT. 33196, SPL) and subjected to $CoCl_2$ treatments. After treatment, the cells were stained with either 0.1 µM NAO (ex/em: 495/519 nm, Invitrogen, USA) or 1 µM TMRE (ex/em: 535/570 nm) for 30 min at room temperature. The cells were washed twice with Phosphate Buffered Saline (PBS) before measuring fluorescence intensity using a microplate reader [5]. The NAO or TMRE intensity in each well was expressed as a percentage value relative to the normal control. Experiments were repeated 3 times.

2.5. Measurement of Oxidative Stress

After being treated to different conditions, H9C2 cells were stained with either 5 µM CM-H_2DCFDA (ex/em: 485/525 nm) or 1 µM Mitosox Red (ex/em: 510/580 nm) at 37 °C for 30 min at room temperature to detect changes in H_2O_2 or O_2^- levels. After being washed twice with PBS 1X, the different fluorescence intensities of the dyes were measured using a microplate reader. The total intensity in each well was expressed as a percentage value relative to the normal control. Experiments were performed in triplicate.

2.6. Cell Imaging

Normal images of H9C2 cells were captured by using an Inverted Microscope with Optical view 7.0. For fluorescence images, the cells were grown in confocal dishes and dyed with the mitochondrial indicators. The images were captured using an ApoTome Fluorescence Microscope (ApoTome 2) and reconstructed from individual tiles (X:6, Y:9) using ZEN Blue 2.5 software (Carl Zeiss).

2.7. Statistical Analysis

Data are presented as means ± standard deviation (SD) by using Excel 2016, Origin 8.5 software. Differences between the two groups were evaluated by ANOVA and Tukey test; a p-value ≤ 0.05 was considered significant.

3. Results and Discussion

3.1. $CoCl_2$ Significantly Decreased the H9C2 Cell Viability

In this study, the effects of $CoCl_2$ on cell viability of H9C2 cardiomyocytes were

evaluated by observing the changes in cell morphology (Figure 1) and the percentage of alive cells under different conditions (Figure 2). H9C2 cells were grown in medium containing $CoCl_2$ for 24 h to simulate oxygen deprivation (hypoxic condition) and then were grown in fresh medium (without $CoCl_2$) for the next 24 h to simulate reoxygenation conditions. The H9C2 cell images are presented in Figure 1. In $CoCl_2$-subjected cell groups, the number of dead cells, detaching from the surface of the well bottom and floating in the medium, suggesting the decline in H9C2 cell viability as previously mentioned [6]. This phenomenon was clearly observed and gradually increased when the concentration of $CoCl_2$ increased from 100 µM to 400 µM. Treatment of $CoCl_2$ at dose of 200 µM to 400 µM significantly induced H9C2 death, so the number of remaining H9C2 cells adhering to the bottom was quite small. Also, the number of dead cells suspended in the culture medium was large (Figure 1E, dots/balls shape). Meanwhile, in the control group, the number of adherent cells was the highest, the number of cells covering the surface area and the cell image was clear (Figure 1A).

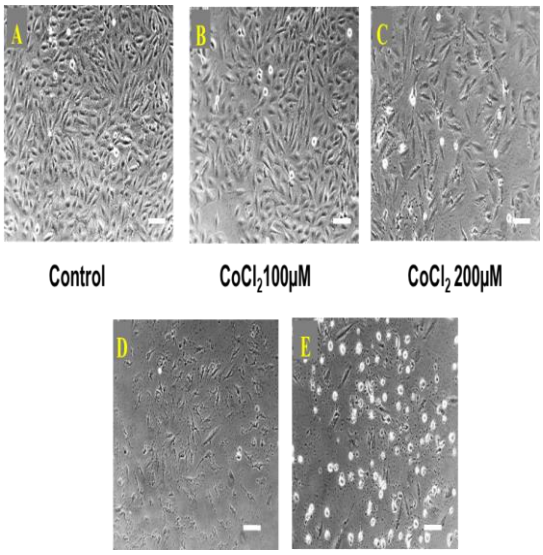

Figure 1. H9C2 cell images under different conditions. A) Cells were cultured in normal condition; B-E) Cells were subjected to $CoCl_2$ (100-400 µM); Magnification: 5X, scale bar: 5µm.

The morphology results were quite similar to the quantified data of cell viability using CCK-8 (Figure 2).

The data in Figure 2 show that the survival rate of the H9C2 cell groups supplemented with $CoCl_2$ decreased compared with the control. This ratio gradually decreased as the concentration of $CoCl_2$ gradually increased from 100 µM to 400 µM. The survival rate of H9C2 cells treated with $CoCl_2$ at a dose of 100 µM was insignificant compared to normal control. The cell viability was significantly decreased in the test group supplemented with 200÷400 µM $CoCl_2$. Particularly, under treatment condition with $CoCl_2$ at dose of 400 µM, the percentage of alive cells was only 37.12±0.17 (% of control).

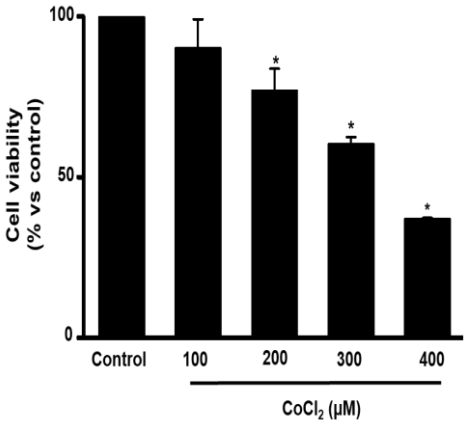

Figure 2. Cell viability of H9C2 cells.
Control: cells were cultured in normal condition,
$CoCl_2$: cells were subjected to $CoCl_2$ (100÷400 µM);
* $p<0.05$ vs Control.

This can be explained by the accumulation of Co^{2+} in the cell and displacing the Fe^{2+} ion in the center and inactivating the hydroxylase enzyme [7], thus helping to stabilize HIF1α and the amount of this molecule in cells increased [8]. HIF1α activity increases oxygen-sensitizing genes [9], helping cells respond to hypoxia. On the other hand, an increase in the number of dead cells can also attribute to the fact that $CoCl_2$ increases the production of oxidative free radicals, decreases the mitochondrial membrane potential, stimulates induced programmed cell

death, thereby causing cell damage and death [10]. Besides, the data also show that $CoCl_2$ affected H9C2 cells at a concentration of 200 µM or higher ($p<0.05$). The obtained results are consistent with previous research results [11, 12] on the use of $CoCl_2$ concentration.

3.2. $CoCl_2$ Highly Attenuated Mitochondrial Cardiolipin Contents in H9C2 Cells

Effects of $CoCl_2$ on mitochondrial cardiolipin were tested by the NAO fluorescence kit. The obtained results were shown in Figure 3.

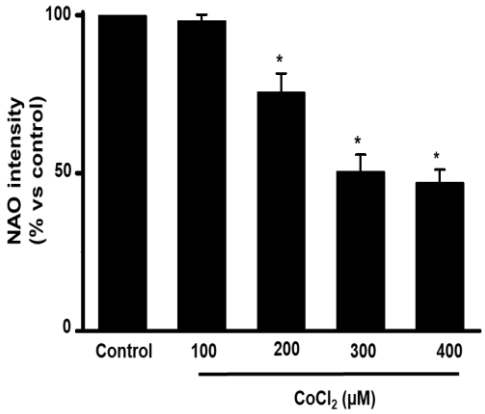

Figure 3. NAO intensity in H9C2 cells.
Control: cells were cultured in normal condition, $CoCl_2$: cells were subjected to $CoCl_2$ (100-400 µM); * $p<0.05$ vs Control.

Cardiolipin is a characteristic phospholipid of the inner mitochondrial membrane and is essential for many mitochondrial functions such as respiratory chain activity and energy metabolism [13]. The decrease in the number and structure of cardiolipin leads to mitochondrial dysfunction and is closely associated with a large number of diseases [14]. The results in Figure 3 show that the group of cells cultured in 100÷400 µM $CoCl_2$ medium had lower cardiolipin content than the control. However, these significant differences were seen in $CoCl_2$ treatment groups at dose of 200 µM (75.56±5.97%), 300 µM (50.47±5.31%) and 400 µM (46.9±4.12%) compared to those in normal control.

3.3. $CoCl_2$ Strongly Altered Cardiac Mitochondrial Function

As mitochondrial membrane potential is an important index to assess mitochondrial function, the changes of this index might lead to cell death. Also, the integrity of mitochondrial membrane structure and function was further shown in examining of H_2O_2 and O_2^- productions. The obtained results are shown in Figure 4 and 5.

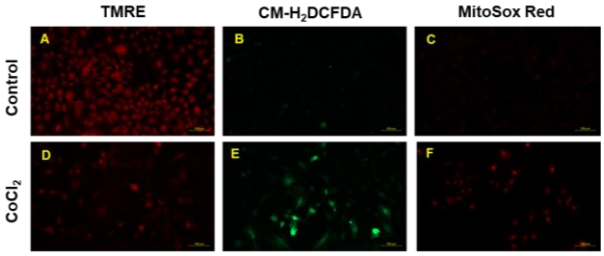

Figure 4. The images of H9C2 cells stained mitochondrial dyes.
Control: cells were cultured in normal; $CoCl_2$: cells were subjected to $CoCl_2$ 300 µM; A&D, B&E, C&F: cells were stained with TMRE, CM-H_2DCFDA and MitoSox Red dye, respectively. The images were taken by Apoptome 2; Magnification: 20X; scale bar: 100 µm.

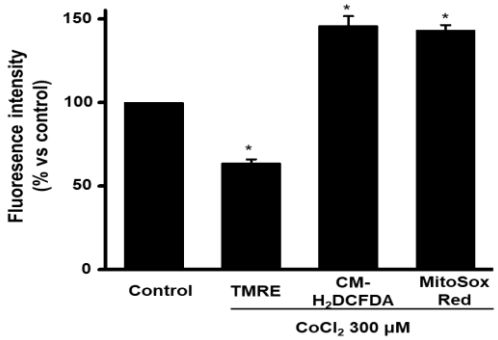

Figure 5. Mitochondrial function in $CoCl_2$-exposed H9C2 cells.
Control: cells were cultured in normal condition; TMRE, CM-H_2DCFDA, MitoSox Red: intensity of TMRE, CM-H_2DCFDA and MitoSox Red of H9C2 cells under $CoCl_2$ 300 µM condition.
* $p<0.05$ vs Control.

Figure 4 and Figure 5 show that supplementation of $CoCl_2$ 300 µM to culture media significantly decreased mitochondrial

membrane potential to 63.79±2.15% of the control. Also, $CoCl_2$ strongly elevated H_2O_2 and O_2^- productions to 145.81±5.83% and 143.10±3.07%, respectively. This is consistent with previous study, H_2O_2 and O_2^- mitochondrial decreased membrane integrity under ischemia injuries [14]. The data in this study proved that $CoCl_2$ markedly altered mitochondrial structure and function and consequently, cell death in a dose-dependent manner.

4. Conclusion

Taken together, this study demonstrates that $CoCl_2$ strongly induced H9C2 cardiomyocyte death via altering mitochondrial function in a dose-dependent manner.

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