

Original Research

The biological effect of cobalt chloride mimetic-hypoxia on nucleus pulposus cells and the comparability with physical hypoxia *in vitro*

Xiao-Xin Gao¹, Chen-Hao Liu¹, Zhi-Lei Hu¹, Hai-Yin Li¹, Xian Chang¹, Yue-Yang Li¹, Yu-Yao Zhang¹, Yu Zhai¹, Chang-Qing Li^{1,*}

¹Department of Orthopedics, Xinqiao Hospital, Army Military Medical University, 400037 Chongqing, China

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1. Abstract

Objective: Nucleus pulposus cells (NPCs) are cells extracted from the intervertebral disc and are important for research into intervertebral disc degeneration (IVDD). NPCs live in an avascular and relatively hypoxic environment. Cobalt chloride (CoCl_2) has been used in many cell studies to mimic hypoxia. The objective of this study was to explore the possibility of using CoCl_2 to induce mimetic-hypoxia for NPCs and the comparison with hypoxia (1% O_2) *in vitro*. **Materials and methods:** Rat nucleus pulposus cells of Passage 3–5 were used in this research. Cell viability, rate of cell apoptosis, ROS (reactive oxygen species) generation, cell migration, extracellular pH and extracellular matrix metabolism were determined to compare the influence of hypoxia (1% O_2) and CoCl_2 on NPCs. **Results:** We found that the effects of CoCl_2 on NPCs was dose-dependent. At the proper concentration, CoCl_2 could be used to elicit chemical hypoxia for nucleus pulposus cells *in vitro* and many biological effects, analogous to physical hypoxia (1% O_2), could be achieved such as enhanced cell viability, decreased apoptosis and activated extracellular matrix metabolism. On the other hand, CoCl_2 mimetic-hypoxia did not affect NPCs glycolysis and migration compared to physical hypoxia. In addition, high concentration of CoCl_2 ($>200 \mu\text{M}$) is harmful to NPCs with high rates of apoptosis and ECM (extracellular matrix) degradation. **Conclusions:** It is feasible and convenient to use CoCl_2 to induce chemical mimetic hypoxia for culturing NPCs on the premise of appropriate concentration. But in aspects of cell migration and glycolysis, CoCl_2 could not achieve similar results with physical hypoxia. This study may provide a convenient method and enlightenment to induce mimetic-hypoxia for researchers studying NPCs and IVDD.

2. Introduction

IVDD (Intervertebral disc degeneration) affects a large part of the world's population and is a major cause of lower back pain (LBP) and can result in permanent disability [1]. Nucleus pulposus cells (NPCs) are extracted from the intervertebral disc and are the most commonly used cell type to explore the underlying mechanism of IVDD. In addition, NPCs are indispensable in some promising therapies of IVDD like NPCs transplantation. The surrounding environment of NPCs is avascular [2–4] and hypoxia as low as 1% O_2 has been documented [5, 6]. Recent evidence has revealed that hypoxia plays a crucial role in maintaining the physiological functions of NPCs, including stable cell survival, coordinated metabolism and extracellular matrix synthesis [7–10]. It is logical to culture NPCs in environments of hypoxia to investigate the biological behavior of NPCs *in vitro* [11]. Low oxygen incubators are the most ideal apparatus to culture NPCs under physical hypoxia, however, a problem encountered by many researchers is access to a hy-

poxia incubator with downregulated oxygen levels, so this is not feasible for many laboratories [12]. Consequently, discovering novel and simple methods to mimic physical hypoxia in cultured NPCs is requisite and beneficial for the research and therapy of IVDD.

Previous studies have shown that some chemical compounds can be used to mimic physical hypoxia, such as divalent metals or iron chelators [13, 14]. One of the most frequently used chemical compounds is cobalt chloride (CoCl_2). CoCl_2 has been used for many cell lines as a hypoxia-mimetic compound, for it can stabilize hypoxia-inducible factors 1α (HIF1 α) under normoxic conditions based on the inhibition of PHDs by substitution of the Fe^{2+} [15–17]. The differences between CoCl_2 -generated hypoxia and physical hypoxia were demonstrated by Munoz-Sanchez *et al.* [12], however, to our knowledge, few studies to date have explored the possibility of using CoCl_2 to induce mimetic-hypoxia for NPCs *in vitro*. NPCs can maintain a certain level of HIF1 α expression under normoxic conditions and some researchers regard HIF1 α as one of the biomarkers of NPCs [18, 19]. Whether the biological behavior of NPCs under cobalt chloride hypoxia is comparable to physical hypoxia requires exploring. If so, CoCl_2 can be regarded as a simple and accessible compound to simulate a hypoxic environment for NPC experiments.

In these studies, we hypothesized that CoCl_2 mimetic-hypoxia would have analogous effects compared with physical hypoxia, and we aimed to verify the possibility to use CoCl_2 to induce mimetic-hypoxia for NPCs. We conducted *in-vitro* experiments to prove our hypothesis through detecting routine biological behaviors under hypoxia, such as cell survival, apoptosis, migration, glycolysis, ROS generation and extracellular matrix metabolism.

3. Materials and methods

3.1 Cell isolation and culture

NPCs were isolated from eight-week-old male Sprague-Dawley rats, which were brought from Animal Center of the Xinqiao Hospital. All experiments were approved by the Ethics Committee of the Army Medical University (Code: AMUWEC20211846). Rats were sacrificed after appropriate anesthesia. Nucleus pulposus tissue was separated from caudal vertebra and cut into small pieces. After digestion with 0.1% collagenase for 6 h, the suspension was centrifuged at 300 g for 5 min and then resuspended in DMEM-F12 (BI, Kibbutz Beit-Haemek, Israel) media with 10% fetal bovine serum (FBS, LONSA, Canelones, UY) and 100 U/mL penicillin-streptomycin. Afterwards, the partially digested tissue was cultured in an incubator containing 5% CO_2 , 20% O_2 with humidified atmosphere at 37 °C. After 1-week incubation, NPCs migrated from the partially digested tissue, then incubated in culture flasks in humidified atmosphere containing 5% CO_2 , 20% O_2 at 37 °C. Nonadherent cells were removed by

replacing the media after 48 h. At 90% confluence, NPCs were washed with phosphate-buffered saline (PBS) twice, and then passaged in a 1:2 ratio. Culture media was changed every 3 days. Cells used in this research were Passage 3–Passage 5.

3.2 Treatment with hypoxia condition and CoCl₂

NPCs in all groups were cultured in DMEM-F12 media with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin. CoCl₂ (Sigma, Saint Louis, MO, USA) was dissolved in DMEM-F12 for a stock concentration of 10 mmol/L and then diluted in culture media for required concentrations. For physical hypoxia, NPCs were cultured in an incubator with humidified atmosphere containing 1% O₂, 5% CO₂ at 37 °C. For mimetic-hypoxia, NPCs were incubated with different concentrations of CoCl₂ solution (50 μM, 100 μM, 200 μM, 300 μM, 400 μM) and cultured in an incubator with humidified atmosphere containing 20% O₂, 5% CO₂ at 37 °C.

3.3 Detection of cell viability by Cell Counting Kit-8 (CCK-8)

To measure the cell viability of NPCs, a CCK-8 assay (CCK-8, Beyotime, Shanghai, China) was performed according to the manufacturer's instructions. CCK-8 is a colorimetric reaction-based assay that yields an orange formazan dye to an extent proportional with the cell number. The cell viability of NPCs was calculated by evaluating the absorbance at 450 nm on a spectrophotometer [20]. NPCs were seeded into 96-well plates (2000 cells/100 μL) and cultured in groups of normoxia, physical hypoxia (1% O₂) and CoCl₂ mimetic-hypoxia (50 μM, 100 μM, 200 μM, 300 μM, 400 μM). After 24 h, 48 h and 72 h, cell culture media was replaced with 110 μL CCK-8 solution (10% concentration) per well and incubated for 2.5 h, respectively. The OD value of each well was measured to obtain Ac (absorbance of control well), As (absorbance of the experimental well), Ab (absorbance of blank well) by a microplate reader at 450 nm (Spectra Max M2, Molecular Devices, Sunnyvale, CA, USA). Cell viability (%) = [(As-Ab) / (Ac-Ab)] × 100.

3.4 Detection of apoptosis rate by flow cytometry

Apoptosis was detected using the Annexin V-PE/7-AAD apoptosis detection kit (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. After treatments of normoxia, physical hypoxia (1% O₂) and CoCl₂ mimetic-hypoxia for 24 h, 48 h and 72 h, NPCs were collected and suspended in 1 × binding buffer at a concentration of 1 × 10⁶ cells/mL. 100 μL of this cell suspension (1 × 10⁵ cells) was then transferred to a 5 mL culture tube. After addition of 5 μL of Annexin V-PE and 5 μL of 7-AAD, the solution was gently mixed and incubated for 15 min at room temperature in the dark. Subsequently, 400 μL of 1 × binding buffer was added to each tube. Flow cytometry of NPCs was performed within 1 h,

with Annexin V positive cells representing the occurrence of apoptosis.

3.5 Migration assay of NPCs

Scratch tests were conducted to determine the effect of normoxia, physical hypoxia (1% O₂), and CoCl₂ mimetic-hypoxia on NPCs migration. A total of 3 × 10⁵ NPCs cells were seeded into 6-well plates and cultured overnight in media containing 10% FBS. A scratch wound was generated in the center of each well by a sterile 200 μL pipette tip. After washing twice with PBS, fresh serum-free culture media was added to the plates. Cells were then cultured in physical hypoxia (1% O₂) and CoCl₂ mimetic-hypoxia, respectively. Images were taken at 0 h, 12 h, 24 h using an inverted phase contrast microscope and cell migration area was quantified using ImageJ software (National Institutes of Health, New York, NY, USA).

3.6 Extracellular pH detection

Cells were cultured in 6-well plates and incubated for 24 h, 48 h, 72 h under normoxia (20% O₂), physical hypoxia (1% O₂) and CoCl₂ mimetic-hypoxia (50 μM, 100 μM, 200 μM, 300 μM, 400 μM). The conditioned cell media was collected and analysed with a pH meter (Sartorius, Goettingen, Germany). Data were collected and analysed to detect the extracellular pH change.

3.7 Reactive oxygen species (ROS) detection with flow cytometry

Intracellular accumulation of ROS was measured by DCFH-DA (Solarbio, Beijing, China) following the manufacturer's instructions. After physical hypoxia and CoCl₂ treatments for 6 h, cell samples were washed three times with serum-free culture media and stained with 10 μM of DCFH-DA in serum-free culture media for 20 min at 37 °C in the dark. The cells were washed three times with serum-free culture media to remove unbound probe outside of the cells. Cells were then collected in 5 mL polystyrene tubes and the mean fluorescence intensity of DCF was analyzed with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

3.8 Quantitative RT-PCR analysis

Following treatment of CoCl₂ (0 μM, 50 μM, 300 μM) and hypoxia (1% O₂), NPCs were washed twice with PBS and then treated with the RNAiso Plus (Takara, Tokyo, Japan) to extract total RNA from samples based on manufacturer's protocols. PrimeScript RT reagent kit (Takara, Tokyo, Japan) was used for RNA reverse transcription to synthesize cDNA. SYBR Premix Ex Taq II Kit (Takara, Tokyo, Japan) was used for amplification and detecting the relative mRNA expression of target genes with the Real-Time PCR System (Cobas z 480, Basel, Switzerland). The primers for QRT-PCR are presented in Table 1. *β-actin* was used to normalize target gene mRNA and we utilized formula $2^{-\Delta\Delta C_t}$ to measure the relative mRNA expression.

Table 1. The primers for QRT-PCR.

	RNA sequence (5'-3')
Sox9-Forward	GCACATCAAGACGGAGCAACT
Sox9-Reverse	TTCTGGTGGTCGGTGTAGTCAT
Glut1-Forward	ATCCACCACACTCACCACACT
Glut1-Reverse	CCATAAGCACGGCAGACACAA
Col2a1-Forward	GCAGCAAGAGCAAGGAGAAGAA
Col2a1-Reverse	CAGTGGACAGTAGACGGAGGAA
Mmp1-Forward	TGCCGTTTGTGAGGAAGAGAC
Mmp1-Reverse	CTGCGTTGAACTGATTGGTGAA
Timp1-Forward	TGGCATCCTCTTGTGCTATCA
Timp1-Reverse	AACGCTGGTATAAGGTGGTCTC
Acan-Forward	TGGCCTGCCTGACTTTAGTG
Acan-Reverse	CCTGAACCACTGACGCTGAT
Hif1 α -Forward	TTGATGTGGACAGCGATATGGT
Hif1 α -Reverse	GGCAGTGACAGTGATGGTAGG
β -actin-Forward	CTGTGTGGATTGGTGGCTCT
β -actin-Reverse	CAGCTCAGTAACAGTCCGCC

Sox9, SRY-box transcription factor 9; *Glut1*, glucose transporter 1; *Col2a1*, collagen type II alpha 1 chain; *Mmp1*, matrix metalloproteinase 1; *Timp1*, tissue inhibitor of metalloproteinase 1; *Acan*, aggrecan; *Hif1 α* , hypoxia inducible factor 1 subunit alpha.

3.9 Western blot analysis

NPCs were incubated under normoxia, with selected concentrations of CoCl₂ (50 μ M, 300 μ M) and physical hypoxia for 24 h, respectively. Total protein was extracted by RIPA buffer (Beyotime Biotechnology, Shanghai, China) containing 1% PMSF (Beyotime Biotechnology, Shanghai, China) and then centrifuged at 12,000 g at 4 °C for 5 min to discard the cell debris. Protein concentrations were determined by standard bicinchoninic acid (BCA; Beyotime Biotechnology, Shanghai, China) method. A total of 40 μ g of protein was loaded into each well and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to polyvinylidene fluoride membranes (PVDF; Merck Millipore, Darmstadt, Germany). Membranes were blocked by 5% nonfat milk in TBST, incubated with specific antibodies overnight at 4 °C, and then washed with TBST three times. The membranes were then incubated with horseradish peroxidase-labeled secondary antibody (dilution 1:5000) for 1.5 h and visualized using an enhanced chemiluminescence substrate (Bio-Rad, Hercules, CA, USA) and Bio-Rad Chemidoc (Hercules, CA, USA). The relative level of target protein to β -ACTIN was calculated by using the ImageJ software. The antibodies used were as follows: β -ACTIN (Abcam, Cambridge, UK, 1:1000 dilution), COL2/ACAN/SOX9/MMP1/TIMP1/BAX/BCL2 (Proteintech, Wuhan, China, 1:500 dilution), P53 (Proteintech, Wuhan, China, 1:200 dilution), HIF1 α /GLUT1 (Abcam, Cambridge, UK, 1:1000 dilution).

3.10 Statistical analysis

Data are presented as mean \pm standard deviation (SD) for at least three independent experiments. SPSS 23.0 software (SPSS Inc. IL, New York, NY, USA) was used to conduct statistical analysis. Multiple comparison of data among the groups were determined by a one-way ANOVA followed by the least significant difference test (Fisher test) and statistical significance was evaluated by an unpaired Student's test for comparisons between two means. Differences were considered statistically significant when $p < 0.05$.

4. Results

4.1 CoCl₂ -mimetic and physical hypoxia effects on cell viability

A dose response test of CoCl₂ was performed to explore the effects of various concentrations on cell viability. When CoCl₂ concentration was no more than 100 μ M, no significant difference in cell viability was found in NPCs with normoxia (20% O₂) or physical hypoxia (1% O₂) at 24 h, 48 h and 72 h, respectively. However, when the concentration of CoCl₂ increased (over 200 μ M), the cell viability of NPCs under CoCl₂ were significantly inhibited compared with NPCs in normoxia or physical hypoxia at 24 h, 48 h and 72 h, respectively ($p < 0.05$) (Fig. 1).

4.2 CoCl₂ -mimetic and physical hypoxia effects on cell apoptosis

Flow cytometry results demonstrated that there were no significant differences between the apoptosis rates of NPCs under CoCl₂ (50 μ M and 100 μ M), 1% O₂ and normoxia ($p > 0.05$). When the concentration of CoCl₂ was greater than 200 μ M, cell apoptosis rates increased (Fig. 2A,B).

4.3 CoCl₂ -mimetic and physical hypoxia effects on cell migration

The scratch test was conducted to explore the effect of physical hypoxia, and CoCl₂-mimetic hypoxia on NPCs migration. 1% O₂ stimulated hypoxia elicited stronger NPC migration than CoCl₂-mimetic hypoxia and normoxia, as shown by the NPCs healing area/wounded area at 0 h, 12 h and 24 h after scratch injury ($p < 0.05$). The migration speed of NPCs under CoCl₂ was negatively correlated with the concentration of CoCl₂ ($p < 0.05$) (Fig. 3A,B).

4.4 CoCl₂ -mimetic and physical hypoxia effects on extracellular pH

The pH of culture media was determined at 24 h, 48 h and 72 h after normoxia, physical hypoxia, and CoCl₂-mimetic hypoxia. In each group, extracellular pH was time-dependently downregulated. The pH of culture media under 1% O₂ were lower than those under normoxia and CoCl₂ treatment at 24 h, 48 h and 72 h ($p < 0.05$) (Fig. 3C,D).

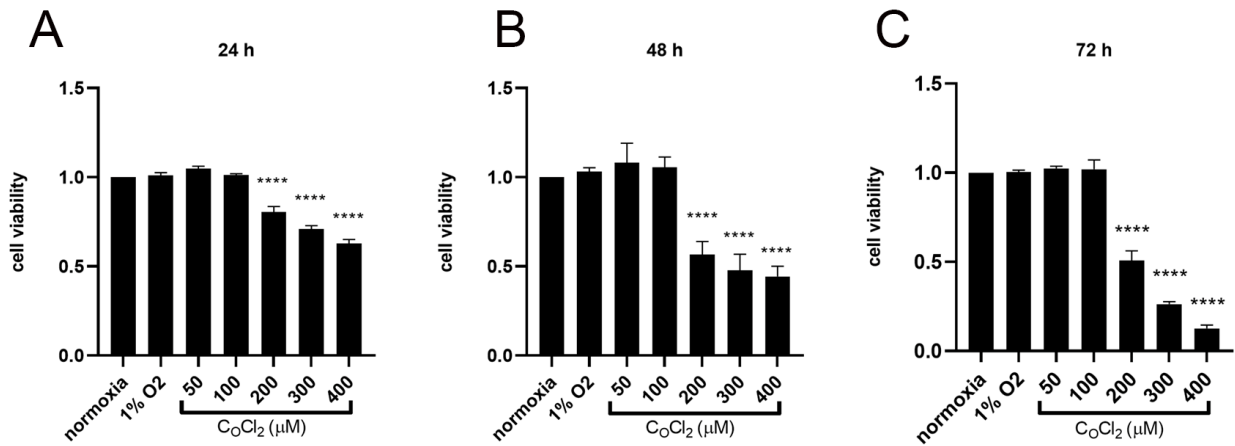


Fig. 1. The cell viability of NPCs measured by CCK-8 assay in normoxia (20% O₂), physical hypoxia (1% O₂), and CoCl₂-mimetic hypoxia. Data are expressed as mean \pm SD from three independent experiments (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 vs. normoxia; No statistical significance/ns).

4.5 CoCl₂ -mimetic and physical hypoxia effects on ROS generation

The intracellular ROS level was detected by flow cytometry. Compared with normoxia, NPCs under physical hypoxia and CoCl₂ mimetic-hypoxia exhibited upregulated ROS generation. ROS fluorescence intensity was dose-dependent under CoCl₂ culture (Fig. 4A,B).

4.6 Quantitative RT-PCR analysis

As 50 μ M CoCl₂ appeared to have similar cell viability compared with 1% O₂, while 300 μ M induced cell apoptosis of NPCs, we analyzed the mRNA expression of several biomarkers of NPCs under normoxia, physical hypoxia and CoCl₂ mimetic-hypoxia at low concentration (50 μ M) and high concentration (300 μ M). Compared with normoxia, both 1% O₂ and CoCl₂ groups upregulated the transcription of *Hif1 α* . The elevation of *Hif1 α* of CoCl₂ groups was concentration-dependent. *Acan*, *Col2a1*, *Sox9*, *Mmp1* and *Timp1* had the same tendency with *Hif1 α* while *Glut1* was upregulated most in the physical hypoxia group. The ratio of *Mmp1/Timp1* was upregulated in CoCl₂ (300 μ M) while it was downregulated in physical hypoxia (Fig. 5A).

4.7 Western blot analysis

The protein levels of several biomarkers of NPCs were analysed under normoxia, physical hypoxia and CoCl₂-mimetic-hypoxia at low concentration (50 μ M) and high concentration (300 μ M). Compared with normoxia (20% O₂), proteins such as HIF1 α , GLUT1, SOX9, ACAN, COL2, MMP1 and TIMP1 had similar increased levels under physical hypoxia and CoCl₂-mimetic-hypoxia (Fig. 5B,C). Under 1% O₂ hypoxia, the level of GLUT1 was upregulated more than that under CoCl₂-mimetic-hypoxia. The BAX/BCL2 ratio was upregulated in CoCl₂ compared with normoxia or physical hypoxia (p < 0.05). P53 was de-

tected in CoCl₂ treated groups and the trend was coincident with HIF1 α expression, while it was expressed at low level under normoxia or physical hypoxia (Fig. 6A,B).

5. Discussion

The intervertebral disc is the largest avascular structure of the human body [2]. In our study, the cell type studied was NPCs which have adapted to the avascular and hypoxic environment of the intervertebral disc. Therefore, culturing NPCs in an analogous hypoxic condition may be beneficial for basic medical research and development of potential clinical therapies for IVVD [21]. However, a problem exists for some researchers that do not have access to a hypoxia incubator to maintain low oxygen during cell studies. Employing chemical compounds is a feasible choice to induce mimetic-hypoxia. To this end, CoCl₂ has been used in many cell lines to induce mimetic hypoxia because it can stabilize HIF1 α under normoxia. Based on the inhibition of PHDs by substitution of the Fe²⁺, high levels of HIF1 α could be detected in the presence of CoCl₂. But according to some researchers, differences between CoCl₂ and physical hypoxia still exist and might be specific for different cell lines [12]. Therefore, we did this research to explore the possibility to use CoCl₂ for NPCs as mimetic-hypoxia and compare the similarities and differences between mimetic-hypoxia and physical hypoxia. Though NPCs could maintain HIF1 α under normoxia, it was found that both CoCl₂ mimetic-hypoxia and physical hypoxia upregulated the mRNA and protein level of HIF1 α in this study. Additionally, some of the biological manifestations had the same trend under physical hypoxia and mimetic hypoxia, such as ECM metabolism, cell viability, ROS generation and apoptosis. However, in aspects of cell migration and glycolysis, CoCl₂ could not achieve similar results as physical hypoxia, which should be noted.

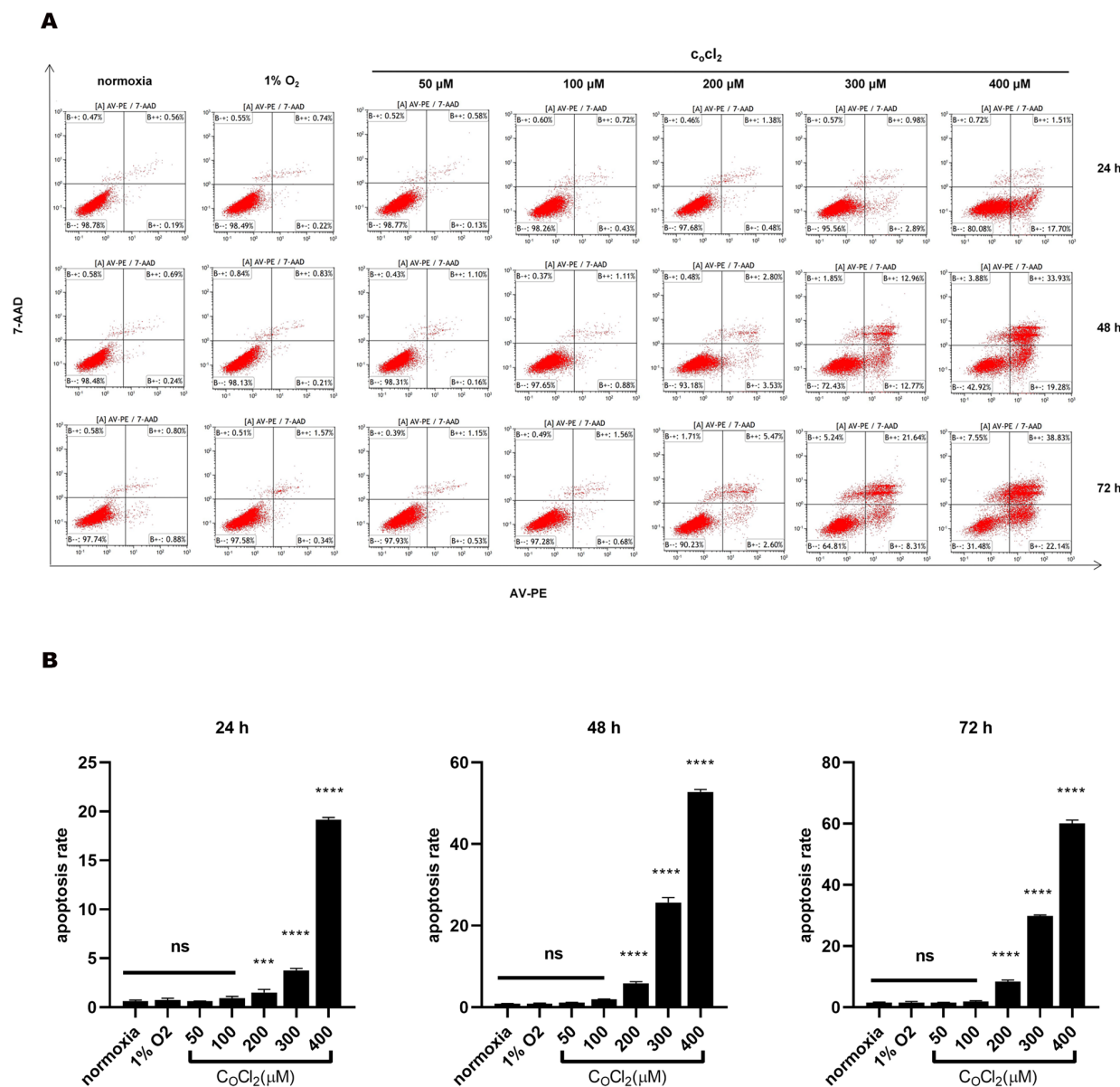


Fig. 2. The apoptosis rates of NPCs measured by flow cytometry assay in normoxia (20% O₂), physical hypoxia (1% O₂), and CoCl₂-mimetic hypoxia. (A) Cell apoptosis detected by flow cytometry analysis after Annexin V-PE/7-AAD double-staining. Apoptotic rate was represented as a percentage of total cell populations. The proportion of dead cells (annexin V+/7AAD+), live cells (annexin V-/7AAD-), early apoptotic cells (annexin V+/7AAD-) and late apoptotic cells (annexin V+/PI+) was measured for comparison; (B) Histograms showing the apoptosis rate (the sum of early and late apoptotic cells) of NPCs treated for 24 h, 48 h and 72 h in normoxia, physical hypoxia, and CoCl₂-mimetic hypoxia. The values are expressed as mean \pm SD from three independent experiments (*** p < 0.001, **** p < 0.0001 vs. normoxia; No statistical significance/ns).

5.1 ECM metabolism and cell phenotype

SOX9 is considered as one of the biomarkers for NPC [22–24]. In our study, physical hypoxia and CoCl₂-mimetic hypoxia all upregulated the level of SOX9. This result is consistent with previous studies [6] and that the upregulation of SOX9 under hypoxia may be via HIF1 α pathway [25].

Nucleus pulposus tissue contain abundant ECM including collagen II (COL2) and aggrecan (ACAN) that

are responsible for maintaining the mechanical load of IVD [26, 27]. Over the years, many studies have shown that hypoxia could induce various cell types to enhance COL2 and ACAN expression via HIF1 α , including NPCs [10, 28–32]. Matrix metalloproteinases (MMPs) are endopeptidases of the ECM that have the ability to degrade almost all known components of the ECM in IVDs. Among these, MMP1 is a collagenase that is precisely regulated by its endogenous protein inhibitors, the tissue inhibitors of metallopro-

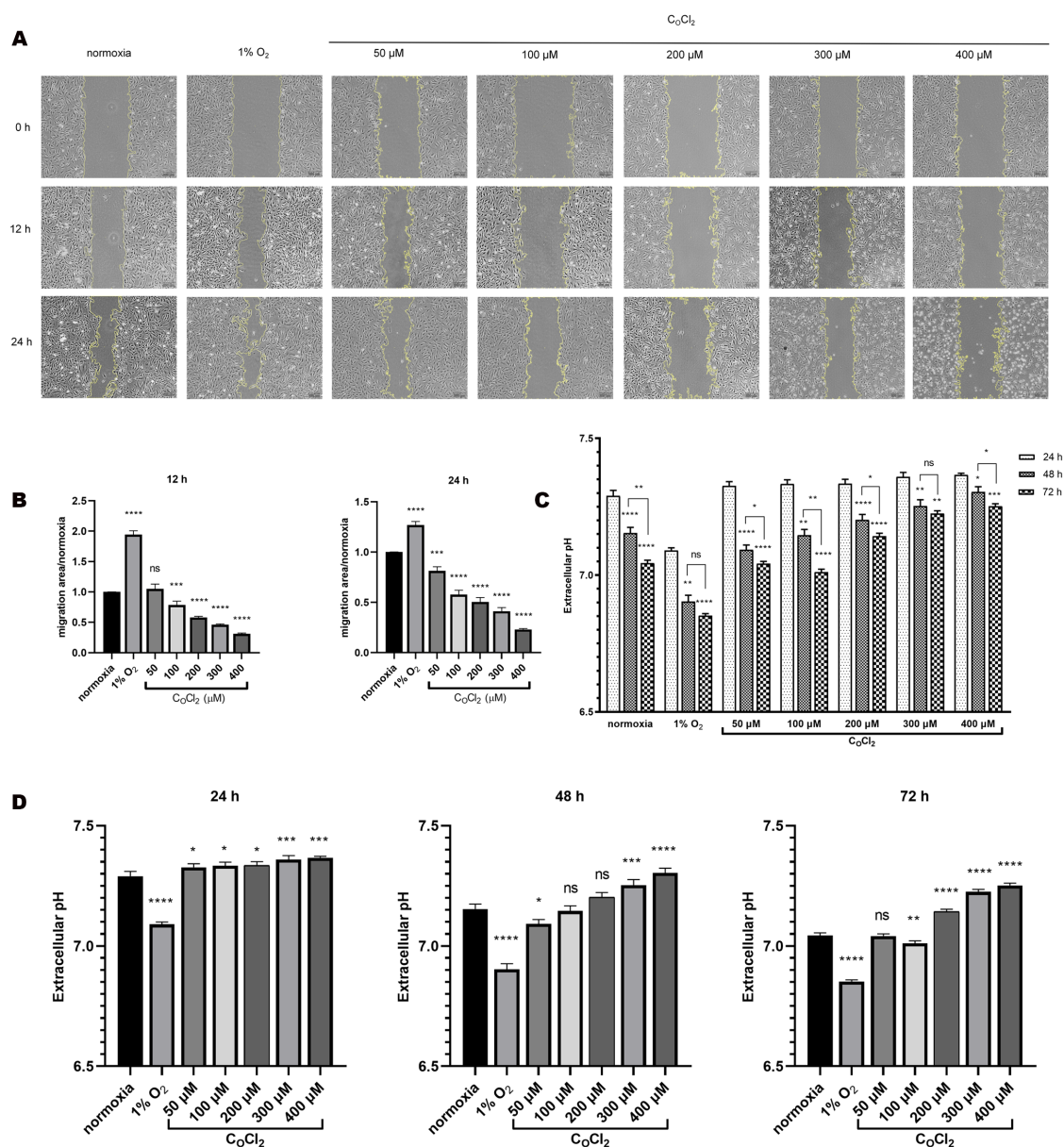


Fig. 3. Effect of physical hypoxia, and CoCl₂-mimetic hypoxia on NPC migration and extracellular pH. (A) Images illustrating NPCs migration detected by scratch test at 0 h, 12 h, and 24 h; (B) Histogram showing the migration ratio of NPCs treated with normoxia, physical hypoxia, and CoCl₂-mimetic hypoxia for 12 h and 24 h; (C) Extracellular pH of NPCs culture media collected at 24 h, 48 h, and 72 h under normoxia, physical hypoxia, and CoCl₂-mimetic hypoxia; (D) Extracellular pH detected by pH meter at 24 h, 36 h and 72 h, respectively. pH of 1% O₂ group was of the lowest at 24 h, 48 h and 72 h. Data are expressed as mean ± SD from three independent experiments (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001 vs. normoxia; No statistical significance/ns).

teinas 1 (TIMP1) [33, 34]. In our study, both physical hypoxia and CoCl₂-mimetic-hypoxia upregulated the levels of COL2 and ACAN compared with normoxia. In addition, both groups upregulated MMP1 and upregulated the level of TIMP1, which is consistent with previous studies [35, 36]. The level of MMP1 was coincident with the increased expression of HIF1α. In the group treated with CoCl₂ at 300 μM concentration, the MMP1/TIMP1 ratio was higher than normoxia, 50 μM CoCl₂, and physical hy-

poxia, which may be attributed to the high level of HIF1α as has been previously reported [21].

5.2 Cell viability

It has been confirmed that hypoxia could slightly promote cell viability for NPCs via HIF1α [7, 37]. But few studies have focused on the impact of CoCl₂ on NPCs cell viability. He *et al.* [38] performed CCK-8 tests and found that 10 μM to 100 μM CoCl₂ was safe in mimetic-hypoxia

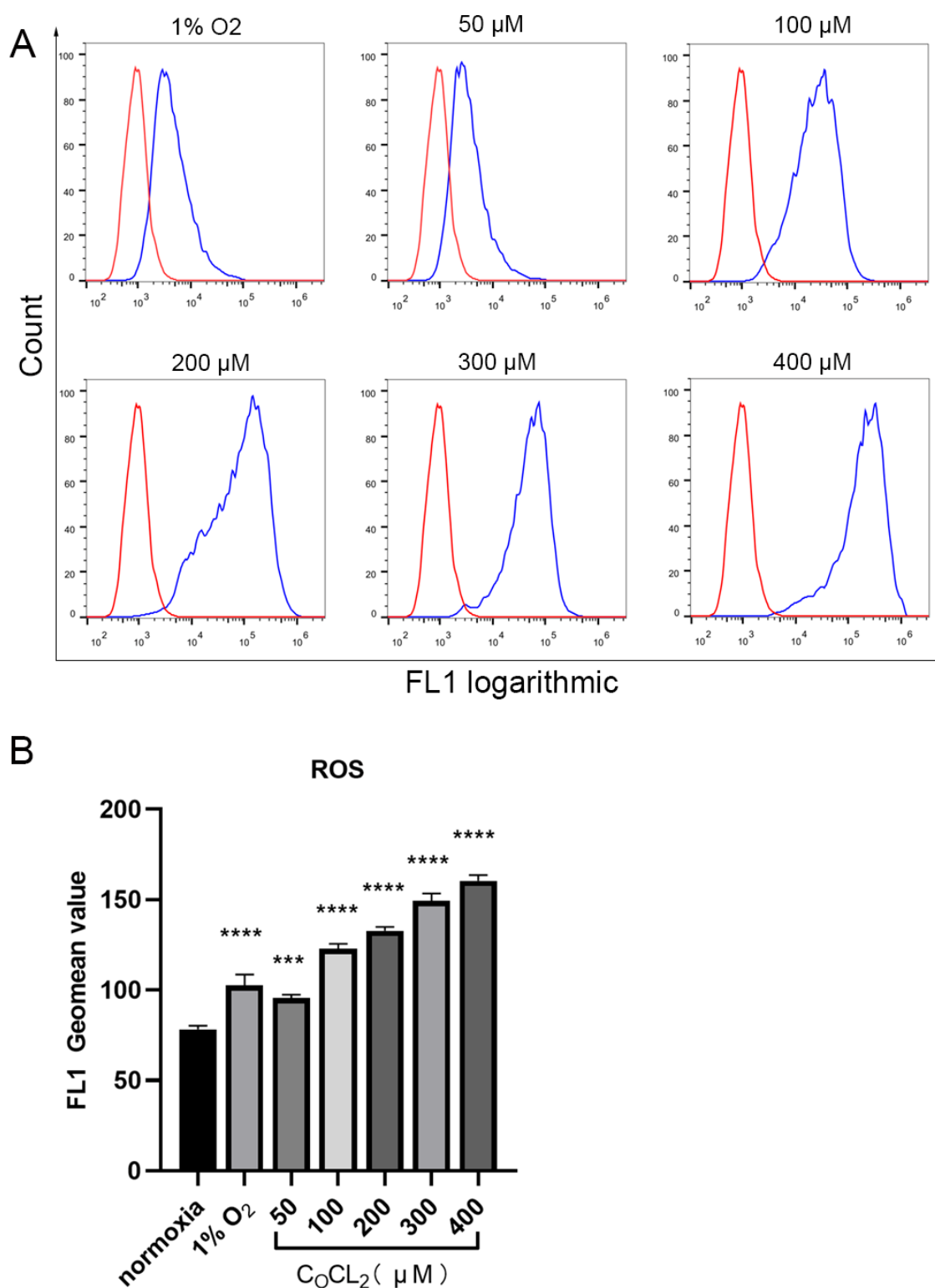


Fig. 4. The intracellular ROS of NPCs detected by flow cytometry after DCFH-DA staining. (A) ROS detected by flow cytometry after treated with 1% O₂ and CoCl₂ (50 μ M, 100 μ M, 200 μ M, 300 μ M, 400 μ M) for 6 h and DCFH-DA staining for 20 min (Red histogram represents group of normoxia); (B) Histogram showing the ROS inside NPCs treated with 1% O₂ and CoCl₂ for 6 h compared with normoxia. Data are expressed as mean \pm SD from three independent experiments (*** p < 0.001, **** p < 0.0001).

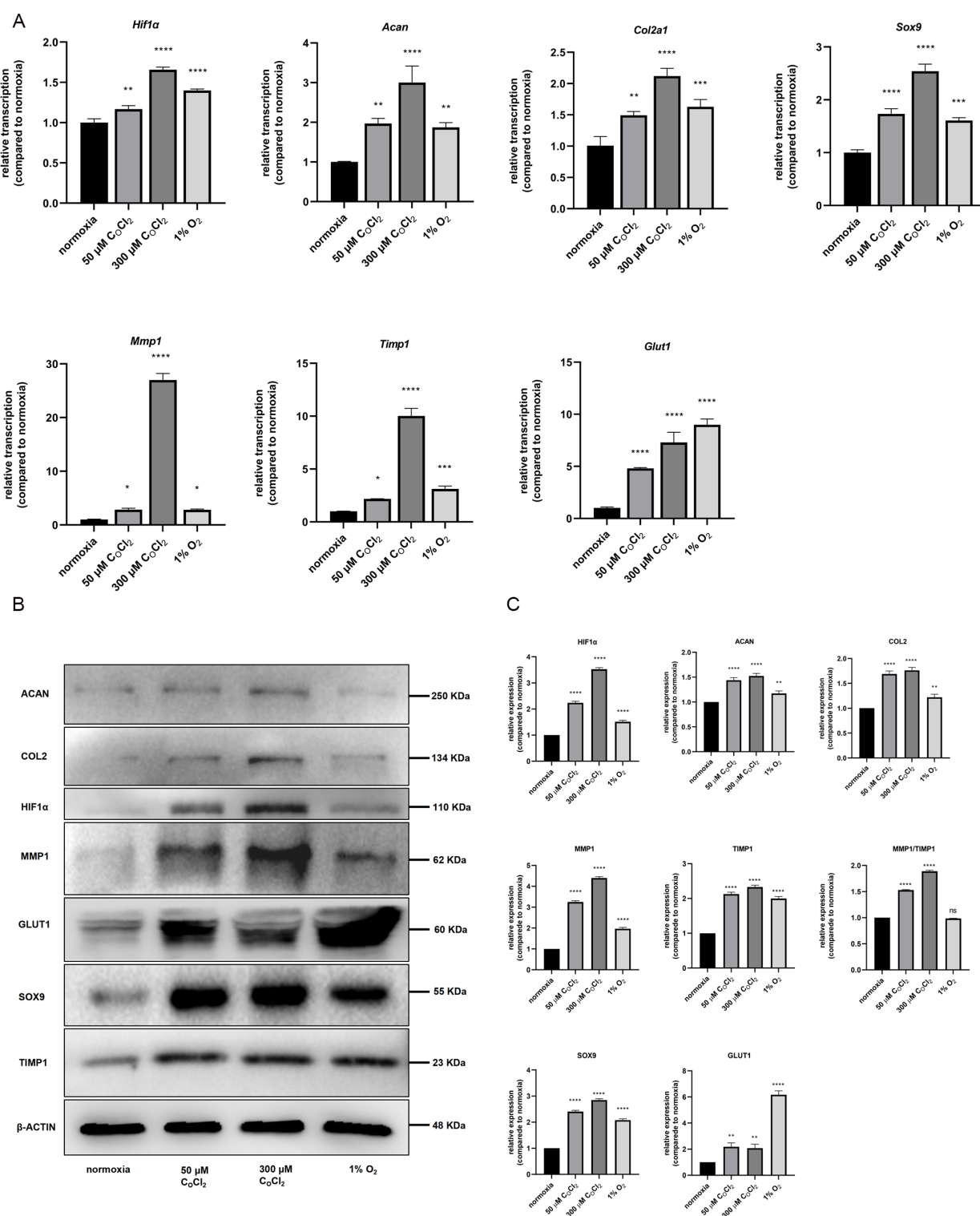


Fig. 5. The relative mRNA and protein expression of *Hif1α*, *Glut1*, *Acan*, *Col2a1*, *Sox9*, *Mmp1* and *Timp1* of NPCs under normoxia, physical hypoxia, and CoCl_2 -mimetic hypoxia. (A) The relative mRNA expressions of target genes with QRT-PCR. β -actin was used to normalize target gene mRNA and formula $2^{-\Delta\Delta C_t}$ were utilized to measure the relative mRNA expression compared to normoxia. Data are expressed as mean \pm SD from three independent experiments; (B) The Western blot bands of target protein HIF1 α , GLUT1, ACAN, COL2A1, SOX9, MMP1 and TIMP1; (C) Histogram exhibiting the relative protein level compared to normoxia group. Data are expressed as mean \pm SD from three independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; No statistical significance/ns).

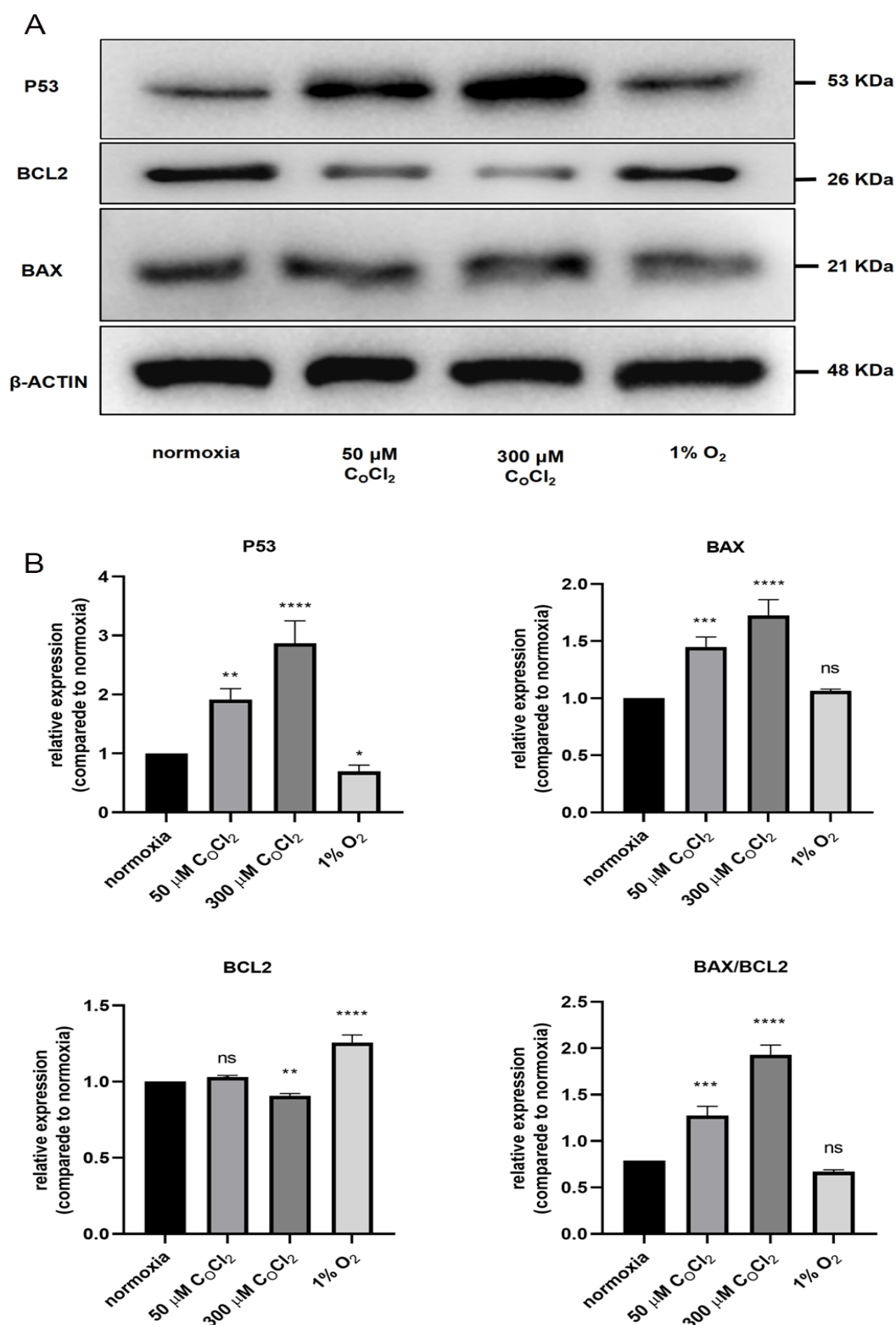


Fig. 6. Relative expression of protein P53, BAX, BCL2 of NPCs under normoxia, physical hypoxia, and CoCl_2 -mimetic hypoxia. (A) The Western blot bands of target protein P53, BAX, BCL2; (B) Histogram exhibiting for statistical analysis of the relative protein expression compared to normoxia group. Data are expressed as mean \pm SD from three independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; No statistical significance/ns).

for the *in vitro* study of NPCs. Jiang *et al.* [39] treated NPCs with 200 μM CoCl_2 for mimetic hypoxia. In our study, both physical hypoxia and low concentration CoCl_2 (50 μM , 100 μM) slightly enhanced the cell viability detected by CCK-8 assay but these differences were not statistically significant. Nevertheless, high concentrations of CoCl_2 (>200 μM) appeared to inhibit cell viability. Thus, we conclude that as a chemical compound, CoCl_2 has some toxic effects on NPCs viability, but the effects may be minimal at low concentrations. Once the critical concentration of 200 μM is exceeded, CoCl_2 will be detrimental to the NPCs.

5.3 Cell migration

Cell migration is a fundamental biological process involved in tissue homeostasis and is an important part of cell transplantation. It has a complex mechanism which is still not clear and may be related to the MAPK signaling pathway, phosphatidylinositol signaling pathway and cytokine–cytokine receptor pathway [40, 41]. Many studies have shown that cells migrate faster under hypoxia than normoxia, including mesenchymal stem cells and carcinoma cells [42–44]. Magdaleno *et al.* [43] compared the migration of renal carcinoma cells under hypoxia and CoCl_2 and found that physical hypoxia promoted migration but CoCl_2 failed to achieve similar effect. They concluded that HIF1 α independent mechanisms modulate the divergent outcomes in assembly of fibronectin, which is a core matrix protein that assembles to promote cell migration. Heirani-Tabasi *et al.* [41] compared the effects of three hypoxia-mimicking agents on migration-related signaling pathways in mesenchymal stem cells and found that CoCl_2 failed to promote cell migration and the mechanism of which may be partly related to the MAPK signaling pathway through IL8/CXCR2 axis or similar mechanisms. In addition, some studies found that CoCl_2 inhibited cell migration compared with normoxia [2, 45]. In our study, it was obvious that NPCs migrated faster under physical hypoxia compared with normoxia, while NPCs migrated slower in all groups under CoCl_2 mimetic-hypoxia. Our data indicated that stabilization of HIF1 α under mimetic-hypoxia is not sufficient to enhance cell migration and it requires the synergistic contribution of some other signaling pathways driven by physical hypoxia to affect this phenotype, the detailed mechanism of which still needs further research.

5.4 Glycolysis

Under hypoxia, energy metabolism is switched from oxidative phosphorylation to glycolysis by upregulating the expression of glycolytic enzymes and glucose transporters [46]. Studies have shown that NPCs keep HIF1 α under normoxia and generate energy through anaerobic glycolysis [19, 47]. Under hypoxia, NPCs could express more GLUTs to facilitate glucose transport [48]. In our study, both physical hypoxia and CoCl_2 mimetic-hypoxia upregu-

lated the expression of GLUT1, but interestingly the physical hypoxia group expressed the highest GLUT1 and down-regulated the extracellular pH the most. This may indicate that physical hypoxia promoted glycolysis in NPCs compared with normoxia but CoCl_2 did not. This result concurs with a recent research report by Zhigalova *et al.* [49]. They performed RNA-seq experiments to explore transcriptomes of human Caki-1 cells under real hypoxia and CoCl_2 treatment and found that glycolysis was not controlled by HIF1 α , indicating that CoCl_2 failed to affect some of the essential downstream consequences of hypoxia, particularly the glycolysis/gluconeogenesis pathway. There may be some underlying mechanisms which trigger the downstream events of NPCs glycolysis in hypoxia apart from HIF1 α .

5.5 ROS generation

As our results have shown, both physical hypoxia and CoCl_2 mimetic-hypoxia upregulated the ROS level of NPCs. For the CoCl_2 groups, ROS generation had a positive correlation with concentration (≤ 400 μM), which is consistent with previous research that demonstrated that CoCl_2 could stimulate cells to generate more ROS and thus cause a negative impact on cell survival [50]. Though the result may be similar, the mechanism could be different. In research of Hep3B cells and wild-type Hep3B cells, using either hypoxia (1.5% O_2) or CoCl_2 incubation, it was found that physical hypoxia activates ROS generation through a mitochondria-dependent signaling pathway, while CoCl_2 stimulating ROS generation via a mitochondria-independent mechanism [51]. The precise details of this mechanism of ROS generation under physical hypoxia and CoCl_2 for NPCs still needs more in-depth study.

5.6 Apoptosis

As is shown in our study, the apoptosis rates of NPCs under both physical hypoxia and CoCl_2 mimetic-hypoxia (<200 μM) had no obvious difference compared with normoxia. But when the concentration of CoCl_2 was larger than 200 μM (including 300 μM , 400 μM), the apoptosis rates rose significantly. This is in line with the findings of Bae *et al.* [52] that enhanced hypoxia by further increasing CoCl_2 concentrations can promote cell apoptosis. We found that both the ratio of BAX/BCL2 and P53 expression have a positive correlation with the concentration of CoCl_2 , but they were all downregulated under physical hypoxia. Previous studies reported that CoCl_2 induces cell apoptosis via different pathways. For example, CoCl_2 -induced HIF1 α expression correlated with apoptosis and may be related to the PI3K/Akt pathway [53]. Other researchers revealed that apoptosis associated with oxidative stress and DNA damage [54–57], may involve P53. Rana *et al.* [58] found that P53 in breast cancer cells is HIF1 α -dependent and overexpression of HIF1 α -dependent BAX ultimately

leads to apoptosis. They speculated that hypoxia affects the P53-dependent pathway in a HIF1 α -dependent manner, thereby targeting the genes involved in P53 pathway which alters the expression of pro-apoptotic genes. Additionally, Lee *et al.* [59] reported that CoCl₂ induced apoptosis, through both mitochondria and death receptor-mediated pathways, is regulated by the BCL2 family in mES cells. In our study, NPCs under hypoxia down-regulated P53 but CoCl₂ up-regulated it. According to Zhang *et al.* [60], hypoxia appears to regulate P53 and is related to the severity of hypoxia, resulting in the increase or decrease of P53 levels and activities in cells. We thus speculated that the way that high concentrations of CoCl₂ induces apoptosis in NPCs is through excessive HIF1 α , P53, superfluous ROS or cell toxicity of CO²⁺. The mechanisms of these processes need to be further explored in future studies.

5.7 Limitations

There are some shortcomings in our experiments. Firstly, we just compared the similarity and difference of some primary phenotypes between physical hypoxia and CoCl₂ mimetic-hypoxia but didn't investigate further underlying mechanisms. Secondly, the hypoxia incubator we used could only be set at 1% O₂ as hypoxia, and the effects of different O₂ concentrations on NPCs were not examined.

Generally, this research has presented an experimental study of the CoCl₂ for mimetic-hypoxia environment for culturing NPCs *in vitro*. This may bring convenience and enlightenment for other researchers studying NPCs and IVVD.

6. Conclusions

Our results indicate that mimetic-hypoxia by CoCl₂ can achieve some similar effects with physical hypoxia, which induces the accumulation of HIF1 α protein and altering the expression of hypoxia-associated genes involved in ECM generation, ROS generation and apoptosis. For similar research studies, it is feasible and convenient to use CoCl₂ to induce chemical mimetic hypoxia for culturing NPCs on the premise of appropriate concentration. But in aspects of cell migration and glycolysis, CoCl₂ could not achieve similar results with physical hypoxia. In addition, excessive CoCl₂ can generate negative effects, such as high apoptosis rate and ECM degradation, which should be considered.

7. Author contributions

X-XG and C-QL conceived and designed the experiments; X-XG and Z-LH performed the experiments; X-XG and C-HL revised the article and conducted the experiments complementation; H-YL and XC analyzed the data; Y-YL, and Y-YZ edited the figures, YZ polished the language. X-XG wrote this article. C-QL reviewed and edited the article.

8. Ethics approval and consent to participate

Ethics approval were obtained with the informed consent of all participants. The Ethics Committee of the Army Medical University approved this research. Code: AMUWEC20211846.

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11. Conflict of interest

The authors declare no conflict of interest.

12. References

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Send correspondence to: Chang-Qing Li, Department of Orthopedics, Xinqiao Hospital, Army Military Medical University, 400037 Chongqing, China, E-mail: changqli1970@126.com