

ANTIOXIDANT EFFECTS ON HYPOXIA-INDUCED OXIDATIVE STRESS AND APOPTOSIS IN RAT ROTATOR CUFF FIBROBLASTS

R.J. Kim¹, S.H. An², J.Y. Gwark^{1,3} and H.B. Park^{1,3,*}

¹Gyeongsang Institute of Health Sciences, Gyeongsang National University, Jinju, Republic of Korea

²Graduate School of Medical Science, Gyeongsang National University, Jinju, Republic of Korea

³Department of Orthopaedic Surgery, Gyeongsang National University Changwon Hospital, Changwon, Republic of Korea

Abstract

Most cells, highly sensitive to oxygen levels, undergo apoptosis under hypoxia. Therefore, the involvement of hypoxia in rotator cuff tendon degeneration has been proposed. While previous studies have reported that hypoxia induces apoptosis in rotator cuff fibroblasts (RCFs), little research has investigated whether antioxidants have cytoprotective effects against RCF apoptosis. The present study aimed at determining whether the antioxidant N-acetylcysteine (NAC) exerted cytoprotective effects against hypoxia-induced RCF apoptosis. Third-passage rat RCFs were divided into normoxia, NAC, hypoxia and NAC-hypoxia groups. The hypoxia inducer was 1,000 $\mu\text{mol/L}$ cobalt chloride (CoCl_2); the antioxidant was 20 mmol/L NAC. Expressions of hypoxia-inducible factor-1 α (HIF-1 α) and heme oxygenase-1 (HO-1), cell viability, intracellular reactive oxygen species (ROS) production, apoptosis rates as well as expressions of cleaved caspase-3, cleaved poly ADP-ribose polymerase-1 (PARP-1), vascular endothelial growth factors- β (VEGF- β) and matrix metalloproteinase-2 (MMP-2) were evaluated. Expression of HIF-1 α and HO-1 was significantly higher in the hypoxia group than in the normoxia group ($p < 0.001$). Cell viability was significantly lower in the hypoxia group than in the normoxia group ($p < 0.001$). Intracellular ROS production, apoptosis rate and expressions of cleaved caspase-3, cleaved PARP-1, VEGF- β and MMP-2 were significantly higher in the hypoxia group than in the normoxia group ($p < 0.001$). All these responses were significantly attenuated by pre-treatment with NAC ($p \leq 0.001$). ROS were involved in hypoxic RCF apoptosis induced by CoCl_2 ; NAC, an ROS scavenger, inhibited hypoxia-induced RCF apoptosis by inhibiting ROS production.

Keywords: Hypoxia, reactive oxygen species, apoptosis, vascular endothelial growth factors- β , matrix metalloproteinase-2, N-acetylcysteine, rotator cuff tendinopathy.

***Address for correspondence:** Hyung Bin Park, Department of Orthopaedic Surgery, Gyeongsang National University School of Medicine and Gyeongsang National University Changwon Hospital, 11 Samjeongja-ro, Seongsan-gu, Changwon 51472, Republic of Korea.

Email: hbinpark83@gmail.com

Copyright policy: This article is distributed in accordance with Creative Commons Attribution Licence (<http://creativecommons.org/licenses/by-sa/4.0/>).

List of Abbreviations

AIF	apoptosis inducing factor	FACS	fluorescence-activated cell sorting
ANOVA	analysis of variance	FBS	foetal bovine serum
AP-1	activator protein 1	FITC	fluorescein isothiocyanate
BNip3	BCL2 interacting protein 3	GSH	glutathione
BSA	bovine serum albumin	HIF-1 α	hypoxia-inducible factor-1 α
CD	cluster of differentiation	HO-1	heme oxygenase-1
CO	carbon monoxide	IL	interleukin
CoCl_2	cobalt chloride	MMP	matrix metalloproteinase
DAPI	4',6-diamidino-2-phenylindole	MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DCF-DA	2',7'-dichlorofluorescein diacetate	NAC	N-acetylcysteine
DMEM	Dulbecco's modified Eagle medium	NRF	National Research Foundation of Korea
DMSO	dimethyl sulphoxide	PARP-1	poly ADP-ribose polymerase-1
EDTA	ethylenediaminetetraacetic acid	PBS	phosphate-buffered saline

PDH	pyruvate dehydrogenase
PRP	platelet-rich plasma
PVDF	polyvinylidene difluoride
RCFs	rotator cuff fibroblasts
RIPA	radioimmunoprecipitation assay buffer
ROS	reactive oxygen species
SD	standard deviation
SDS	sodium dodecyl sulphate
TBS-T	Tris-buffered saline-0.1 % Tween 20
VEGF- β	vascular endothelial growth factor- β

Introduction

Rotator cuff tendinopathy, or tendon degeneration, is one of the most common musculoskeletal diseases. Despite increased interest and research, the factors involved in the development of tendon degeneration are not yet completely understood (Via *et al.*, 2013; Yamamoto *et al.*, 2010). Hypovascularity has been proposed as a cause of rotator cuff tendon tear, but this remains controversial (Funakoshi *et al.*, 2010; Lohr and Uthoff, 1990; Moseley and Goldie, 1963; Nho *et al.*, 2008; Rathbun and Macnab, 1970). Excessive apoptosis is associated with tendinopathy and is present in degenerative tendons; it has been observed in rotator cuff tendons with impingement and full thickness tears (Lian *et al.*, 2007; Yamamoto *et al.*, 2007; Yuan *et al.*, 2002). The supraspinatus tendon, a gliding tendon, is the tendon most involved in rotator cuff disease. A characteristic of gliding tendons is the fibrocartilaginous character of the portion close to the insertion site, which is associated with hypoxia (Pufe *et al.*, 2005).

Oxygen homeostasis is essential for the body's constant use of energy. Hypoxia (0.1-1 % O₂), physioxia or physoxia (~ 1-13 %) and normoxia (~ 20 %) are terms used to define oxygen concentration in the cellular environment (Kumar and Choi, 2015). A condition of insufficient oxygen (hypoxia) or of excessive oxygen (hyperoxia) could be deleterious to cellular adaptation and survival. Hypoxia induces stress in organisms either through physiological conditions – such as cell differentiation, embryonic development, exercise, diving at great water depth, exposure to high altitude – or through pathological conditions – such as inflammation, solid-tumour formation, lung disease, neonatal hypoxia as well as cerebral and cardiac ischemia (Fuhrmann and Brune, 2017; Semenza, 2014). In musculoskeletal research, an hypoxic change is associated with tendinopathy in several tendons. Benson *et al.* (2010) found increased expression of HIF-1 α and of an HIF-1 α -regulated pro-apoptotic protein (BNip3), as well as increased rates of apoptosis in impingements and torn rotator cuff tendons. Based on that finding, the authors proposed that hypoxic changes to rotator cuff tendons contribute to the loss of cells through apoptosis and may be responsible for the development of lesions (Benson *et al.*, 2010). McBeath *et al.*

(2019) demonstrated that hypoxia drives tenocyte phenotypic changes, thereby providing a molecular insight into the development of human tendinosis that occurs with ageing. Millar *et al.* (2012) proposed hypoxic cell injury as a critical pathophysiological mechanism in early tendinopathy. Regarding protective strategies for tenocytes at risk of hypoxic death, pro-survival growth factors, insulin and PRP were proposed as potentially protecting tenocytes (Liang *et al.*, 2012). HIF-1, which is composed of HIF-1 α and HIF-1 β subunits, acts as a major regulator of oxygen homeostasis within cells (Semenza, 2003; Soni and Padwad, 2017; Yoon *et al.*, 2006; Ziello *et al.*, 2007). HIF-1 α is also known to be an important upregulator of VEGF, which stimulates endothelial cells and vessels to invade hypovascularised tissues (Gerber *et al.*, 1997). In cellular-level studies, cultured fibroblasts undergoing cyclical strain present increased HIF-1 α and VEGF (Petersen *et al.*, 2004; Pufe *et al.*, 2005). Some studies have found higher concentrations of VEGF in degenerative Achilles' tendons than in healthy tendon tissues (Petersen *et al.*, 2002; Pufe *et al.*, 2001). Although rotator cuff tendon degeneration has been attributed to hypovascularity, there is some evidence that neovascularisation is also a factor (Lohr and Uthoff, 1990). Lewis *et al.* (2009) described increased neovascularity in patients with rotator cuff tendinopathy. Lakemeier *et al.* (2010) reported increased HIF and VEGF expressions in torn rotator cuff tendons. They also found that HIF and VEGF expressions, as well as vessel density, significantly increase with the extent of tendon retraction.

CoCl₂, a chelator, is a well-known chemical hypoxic agent. Ever since Goldberg *et al.* (1988) established that CoCl₂ can mimic hypoxic conditions experimentally, CoCl₂ has been widely used as a hypoxia mimic in both *in vitro* and *in vivo* studies (Abdel-Rahman Mohamed *et al.*, 2019; Chen *et al.*, 2020; Nguyen *et al.*, 2020; Rana *et al.*, 2019; Yu *et al.*, 2019). The hypoxia simulated by CoCl₂ is similar to the hypoxic *in vivo* microenvironment in terms of signal transduction and transcription regulation depending on concentration and exposure time to CoCl₂ and in terms of cell susceptibility or resistance (Munoz-Sanchez and Chanez-Cardenas, 2019; Triantafyllou *et al.*, 2006).

Antioxidants have been reported to prevent several types of RCF death induced by excessive oxidative stress and excitotoxicity induced by glutamate (Kim *et al.*, 2019; Kim *et al.*, 2014; Nam *et al.*, 2016; Park *et al.*, 2010). NAC is a well-known antioxidative agent that protects multiple organs and cells against ischemic, oxidative and other stressors (Bartekova *et al.*, 2018; Colovic *et al.*, 2018; Heil *et al.*, 2018; Kim *et al.*, 2019; Kim *et al.*, 2017). NAC's chemical structure permits it to act as a glutathione precursor and substitute and it has been used in several clinical and *in vivo* studies (Lee *et al.*, 2013).

Most cells are highly sensitive to oxygen levels and undergo apoptosis following periods of hypoxia. Hypoxia has been proposed as a possible causative

factor in rotator cuff tendon degeneration. Previous studies have reported that hypoxia induces apoptosis in RCFs and have suggested that hypoxia is a cause of rotator cuff tendon degeneration (Benson *et al.*, 2010; Lakemeier *et al.*, 2010; Lohr and Uthoff, 1990). However, there has been little research investigating whether antioxidants have cytoprotective effects against hypoxia-induced apoptosis of RCFs. Therefore, this study's purpose was to determine whether NAC, an antioxidant, exerted cytoprotective effects against hypoxia-induced RCF apoptosis.

Materials and Methods

Study design

The study comprised two subsets. The first subset's study groups were divided into normoxia and hypoxia groups. The RCFs of the first subset's hypoxia groups were treated with various concentrations of CoCl₂ (232696, Sigma-Aldrich): 25, 50, 100, 500 and 1,000 μmol/L. The second subset's study groups were divided into normoxia, NAC (A9165, Sigma-Aldrich), hypoxia (1,000 μmol/L CoCl₂) and NAC-hypoxia groups. The exposure time to CoCl₂ was 24 h for all experiments, with the exception of 4 h for the HIF-1α expression experiments and 1 min for the ROS formation experiments. The NAC-hypoxia group was exposed to 20 mmol/L NAC for 1 h, before exposure to CoCl₂. The RCFs of both subsets were cultured on plates or dishes, incubated overnight and exposed to CoCl₂ or NAC and CoCl₂, depending on their study subset. These study groups were evaluated for cell viability by MTT assay, phase contrast microscope, crystal violet staining, live and dead assay, DAPI staining and annexin V/PI double staining. They were also evaluated for intracellular ROS and expression levels of HIF-1α, HO-1, cleaved caspase-3, cleaved PARP-1, VEGF-β and MMP-2. The study was approved by the Institutional Review Board of the Gyeongsang National University (IRB: GNU-170918-R0043).

Cell culture

The study used primary cultured fibroblasts originating from intact rat supraspinatus tendons harvested from 6-week-old male Sprague-Dawley rats (*n* = 6). The rat supraspinatus tendon tissues were washed twice with PBS (Lonza), then minced into small pieces with a sterile scalpel. Next, tissues were placed on three 6-well culture plates (Corning) containing DMEM (Lonza) supplemented with 20 % FBS (Thermo Fisher Scientific) and 1 % antibiotic-antimycotic (Thermo Fisher Scientific). Plates (Corning) were incubated in a humidified 5 % CO₂ atmosphere at 37 °C. After 2 weeks, cells had reached 90 % confluence. Then, cells were trypsinised (12605010, TrypLE™ Express, Thermo Fisher Scientific) for 5 min, centrifuged at 15,928 ×g for 3 min and expanded for a second passage. Next, cells were harvested using TrypLE™ Express and

cryopreserved. These cryopreserved third passage cells were later thawed and used for the study experiments. Cultured cells, which were characterised by flow cytometry, expressed fibroblast markers such as AS02 and 5B5. However, they were negative for both CD68, a macrophage marker, and CD45, a leukocyte marker. The expression of scleraxis, a specific marker for tendon cells, was confirmed in the cultured cells (Park *et al.*, 2010). The high expression of tenomodulin in the studied cells was confirmed by flow cytometry analysis (data not shown). Scleraxis gene expression up to the 12th passage was confirmed by PCR analyses (data not shown).

MTT assay

Cell viability was estimated by measuring the metabolism of MTT (M2128, Sigma-Aldrich). 2 × 10⁴ RCFs were seeded in each well of a 24-well plate. Cells were maintained in an incubator at 5 % CO₂ and 37 °C for 24 h. Then, the study groups were exposed to CoCl₂ or to NAC and CoCl₂, according to their study subset. Briefly, a 500 μL MTT solution (0.5 mg/mL in free medium) was added to each well of the 24-well plate. Then, the plate was incubated for 2 h. Next, the cell supernatant was removed and 500 μL DMSO (D8418, Merck KGaA) was added to each well of the plate. Absorbance of the plate was measured at 570 nm, using a microplate reader (ECLIPSE Ti2-U, Nikon). Cell viability was expressed as a percentage of live cells, compared with the control set at 100 %.

Phase contrast microscope analyses

The cell viability analysis was performed as follows. 1 × 10⁵ RCFs were seeded in each well of a 6-well plate. After 24 h incubation, the study groups were exposed to CoCl₂ or to NAC and CoCl₂, according to their study subset. The cell morphology of the fibroblasts was observed (40× magnification) using an inverted phase contrast microscope (VERSA Max, Molecular Devices, San Jose, CA, USA).

Crystal violet assay

Cell viability analysis using the crystal violet assay was performed as follows. 1 × 10⁵ RCFs were seeded in each well of a 6-well plate. After 24 h of incubation, the study groups were exposed to CoCl₂ or to NAC and CoCl₂, according to their study subset. Cells were stained for 2 h at room temperature using a 0.1 % crystal violet solution (V5265, Sigma-Aldrich). Then, they were washed with PBS and analysed using a scanner (PowerLook, 2100XL, UMAX, Dallas, TX, USA).

Live and dead assay

The live and dead assay was performed using the LIVE/DEAD™ Viability/Cytotoxicity kit (L3224, Thermo Fisher Scientific) as follows. 1 × 10⁴ RCFs were seeded in each of the confocal image dishes (SPL Life Sciences, Pocheon, Korea). After 24 h of incubation, the study groups were exposed to CoCl₂ or to NAC and CoCl₂, according to their study subset. After

medium removal, cells were stained for 30 min at room temperature using a prepared staining solution (2 $\mu\text{mol/L}$ of acetoxymethyl ester of calcein and 4 $\mu\text{mol/L}$ of ethidium homodimer-1). Live and dead cells were analysed using a laser-scanning confocal imaging system (Olympus IX70, Olympus).

DAPI staining

DAPI staining for evaluation of DNA fragmentation was performed as follows. 1×10^5 RCFs were seeded in each well of a 6-well plate. After 24 h incubation, the study groups were exposed to CoCl_2 or to NAC and CoCl_2 , according to their study subset. Cells were fixed with methanol for 5 min at -20°C and washed with cold PBS. Cells were kept in 1 % Triton X-100 (1610407, Biorad) for 10 min at room temperature and, then, washed with PBS. Next, cells were stained with DAPI staining solution (1 $\mu\text{g/mL}$, D9542, Sigma-Aldrich) for 5 min at 37°C and washed with PBS. Then, cells were imaged using a fluorescence microscope (Olympus IX70, Olympus).

Annexin V/PI double staining

1×10^5 RCFs were seeded in each well of a 6-well plate. After 24 h incubation, the study groups were exposed to CoCl_2 or to NAC and CoCl_2 , according to their study subset. RCFs were harvested after trypsinisation, then centrifuged and collected. Those cells were washed with PBS, then stained using an FITC Annexin V/PI kit (556547, BD Biosciences), according to the manufacturer's instructions. Cell viability was determined by flow cytometry (Cytomics FC500, Beckman Coulter) as follows: live cells were labelled with neither stain, early apoptotic cells were labelled only with annexin V, necrotic cells were labelled only with annexin PI, apoptotic cells were labelled with both annexin V and PI.

Western blot assay

3×10^5 RCFs were seeded in a 60 mm culture dish. After exposure to CoCl_2 or to NAC and CoCl_2 according to the study subset, cells were washed with cold PBS and total cell lysates were prepared by scraping using 100 μL of RIPA buffer (89900, Thermo Fisher Scientific). Then, the digested cells were sonicated and centrifuged for 20 min at 4°C and $159,280 \times g$ to remove insoluble debris. Samples were resolved on a 10 % SDS-polyacrylamide gel and electrophoretically transferred onto a PVDF membrane using the wet transfer technique. After blocking for 1 h with 5 % skim milk in a TBS-T buffer solution (IBS-BT008, iNtRon, Seongnam, Korea), the membrane was incubated with primary antibodies against HIF-1 α (1 : 10,000, A300-286A, BETHYL Laboratories, Montgomery, TX, USA), HO-1, cleaved caspase-3, cleaved PARP-1 (1 : 1,000, 43966, 9662, 9542, Cell Signaling Technology), VEGF- β , MMP-2 (1 : 1,000, sc-80442, sc-10736, Santa Cruz) and β -actin (1 : 10,000, MA1-744, Thermo Fisher Scientific) in TBS-T buffer containing 5 % BSA (A3294, Sigma-Aldrich). Specific antibody binding was detected

by horseradish peroxidase-conjugated secondary antibodies (anti-rabbit and anti-mouse, 1 : 5,000; 1460, 31430, Thermo Fisher Scientific) and visualised using an enhanced chemiluminescence detection reagent (W1001, Promega).

Measurement of intracellular ROS

1×10^5 RCFs were seeded in each well of a 6-well cell culture plate, incubated overnight and exposed to CoCl_2 or to NAC and CoCl_2 , depending on their study subset. Cells were stained for 15 min at 37°C with 5 $\mu\text{mol/L}$ DCF-DA (D6883, Sigma-Aldrich) in serum-free medium; then, they were washed with PBS three times and removed from the plate using trypsin-EDTA (12605010, Thermo Fisher Scientific). Next, cells were again suspended in PBS and intracellular ROS production was measured by FACS (Cytomics FC500, Beckman). The fluorescence intensity of the cells was determined by flow cytometry with an excitation wavelength of 480 nm and an emission wavelength of 525 nm. Data were analysed using the XCP software (Beckman).

The intracellular ROS production of each study subset was assessed qualitatively using a confocal microscope, as follows. 1×10^4 RCFs were seeded on the cover glass of each well of a 24-well cell culture plate. After 24 h incubation, the study groups were exposed to CoCl_2 or to NAC and CoCl_2 , according to their study subset. Cells were incubated with a 5 $\mu\text{mol/L}$ DCF-DA solution for 15 min at 37°C and washed with PBS. After the addition of serum-free medium, each cover glass was moved to a confocal dish. The intracellular ROS production was analysed using a laser-scanning confocal imaging system (Olympus IX70, Olympus).

Statistical analysis

Each experiment was performed at least three times and the results were presented as the mean of the total number of trials performed to obtain more objective data. All values were expressed as mean \pm SD. All statistical analyses were performed by one-way ANOVA, followed by Tukey's *post-hoc* test. Differences with a probability of less than 0.05 were considered statistically significant. All statistical analyses were performed by SPSS 17.0 for Windows (SPSS).

Results

Analyses of CoCl_2 -induced HIF-1 α and HO-1 expressions and of suppressive effects of NAC on those expressions

Western blot analyses showed that the expression of HIF-1 α in all CoCl_2 study groups was significantly higher than in the normoxia group ($p < 0.001$) (Fig. 1a,b). The expression of HO-1 in the 100, 500 and 1,000 $\mu\text{mol/L}$ CoCl_2 study groups was significantly higher than in the normoxia group ($p \leq 0.020$) (Fig. 1c,d).

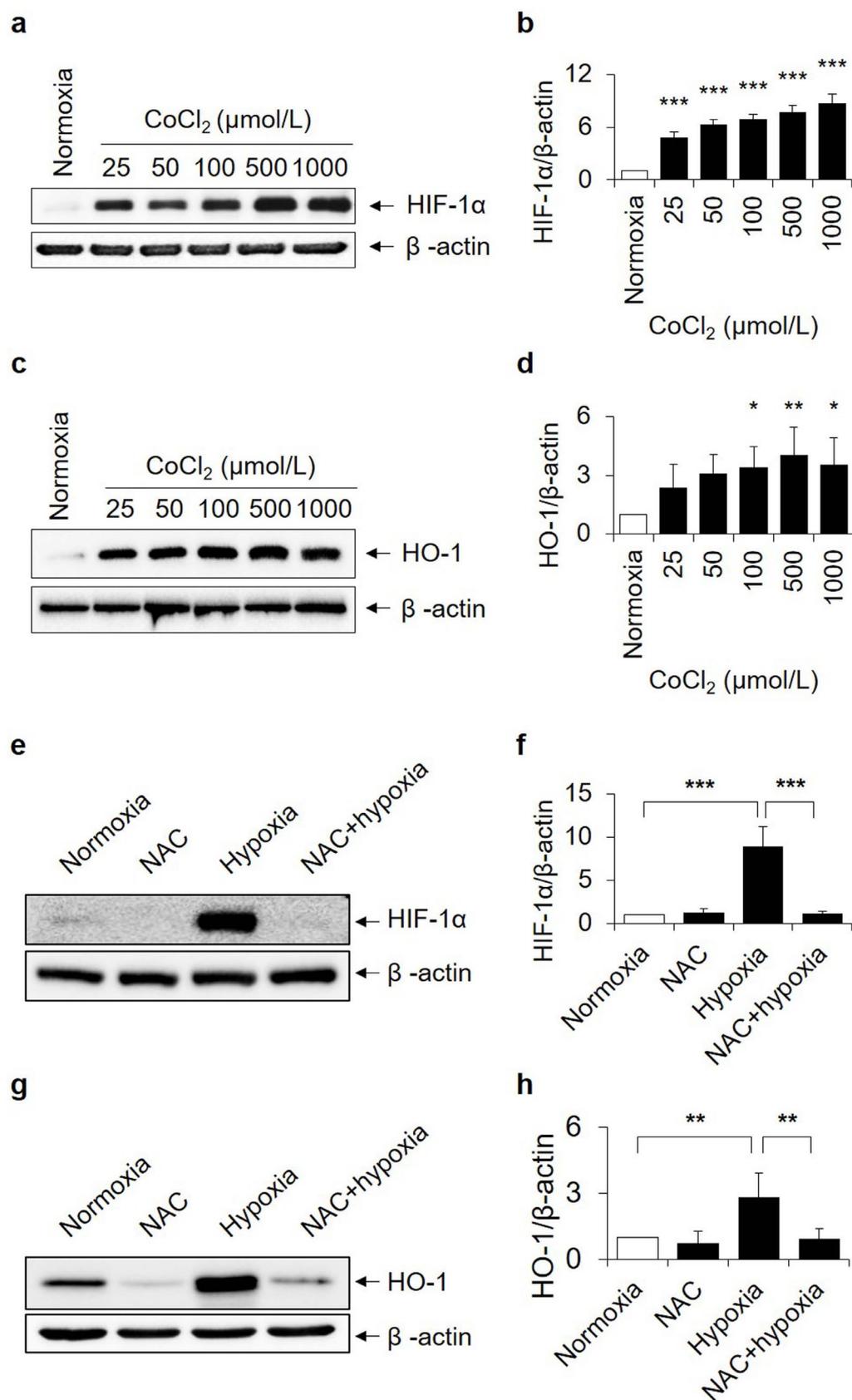


Fig. 1. Analyses of CoCl₂-induced HIF-1α and HO-1 expressions and of suppressive effects of NAC on those expressions. (a,b) HIF-1α expression in all CoCl₂ study groups was significantly higher than in the normoxia group ($p < 0.001$). (c,d) HO-1 expression was significantly higher in the 100, 500 and 1,000 μmol/L CoCl₂ study groups than in the normoxia group ($p \leq 0.020$). (e,f) HIF-1α expression in the NAC-hypoxia group was significantly lower than in the hypoxia group ($p < 0.001$). (g,h) HO-1 expression in the NAC-hypoxia group was significantly lower than in the hypoxia group ($p = 0.002$). (b,d,f,h) Mean \pm SD, $n = 5$, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

The assessment of NAC's effects against hypoxia indicated that the increase in HIF-1 α expression was significantly lower in the NAC-hypoxia group than in the hypoxia group ($p < 0.001$) (Fig. 1e,f); the increase in HO-1 expression was significantly attenuated in the NAC-hypoxia group ($p = 0.002$) (Fig. 1g,h).

Analyses of CoCl₂-induced cytotoxicity and of suppressive effects of NAC on that cytotoxicity

The cytotoxicity analyses showed that cell viability decreased in a concentration-dependent manner ($p < 0.045$) (Fig. 2a). The morphological analyses using a phase contrast microscope (Fig. 2b) and crystal violet staining (Fig. 2c) showed that the cell populations decreased with exposure to increasing concentrations of CoCl₂. The live and dead assays showed that live cells (green) decreased and dead cells (red) increased after exposure to increasing concentrations of CoCl₂ (Fig. 2d).

The assessment of the cell-protective effects of NAC against hypoxia indicated that cell viability was significantly higher in the NAC-hypoxia group than in the hypoxia group ($p < 0.001$) (Fig. 2e). The live and dead assay found markedly more live cells

and markedly fewer dead cells in the NAC-hypoxia group than in the hypoxia group (Fig. 2f).

Analyses of CoCl₂-induced apoptosis and of NAC's suppressive effects on that apoptosis

FACS analyses using annexin V/PI double staining indicated that apoptosis rates were significantly higher in the 1,000 $\mu\text{mol/L}$ CoCl₂ group than in the normoxia group and the other CoCl₂ study groups ($p = 0.001$) (Fig. 3a,b). DAPI staining showed that the cell population with DNA fragmentation, a morphological characteristic of apoptotic cells, was greater in the hypoxia group than in the normoxia group (Fig. 3c). Expressions of cleaved caspase-3 and of cleaved PARP-1 significantly increased in 1,000 $\mu\text{mol/L}$ CoCl₂, as compared with the normoxia group ($p < 0.001$) (Fig. 3d-f). The assessment of NAC's effects against apoptosis induced by hypoxia indicated that the NAC-hypoxia group's apoptosis rate was significantly lower than the hypoxia group's rate ($p < 0.001$) (Fig. 3g,h). The expression of cleaved caspase-3 and cleaved PARP-1 was also significantly lower in the NAC-hypoxia group than in the hypoxia group ($p < 0.001$) (Fig. 3i-k).

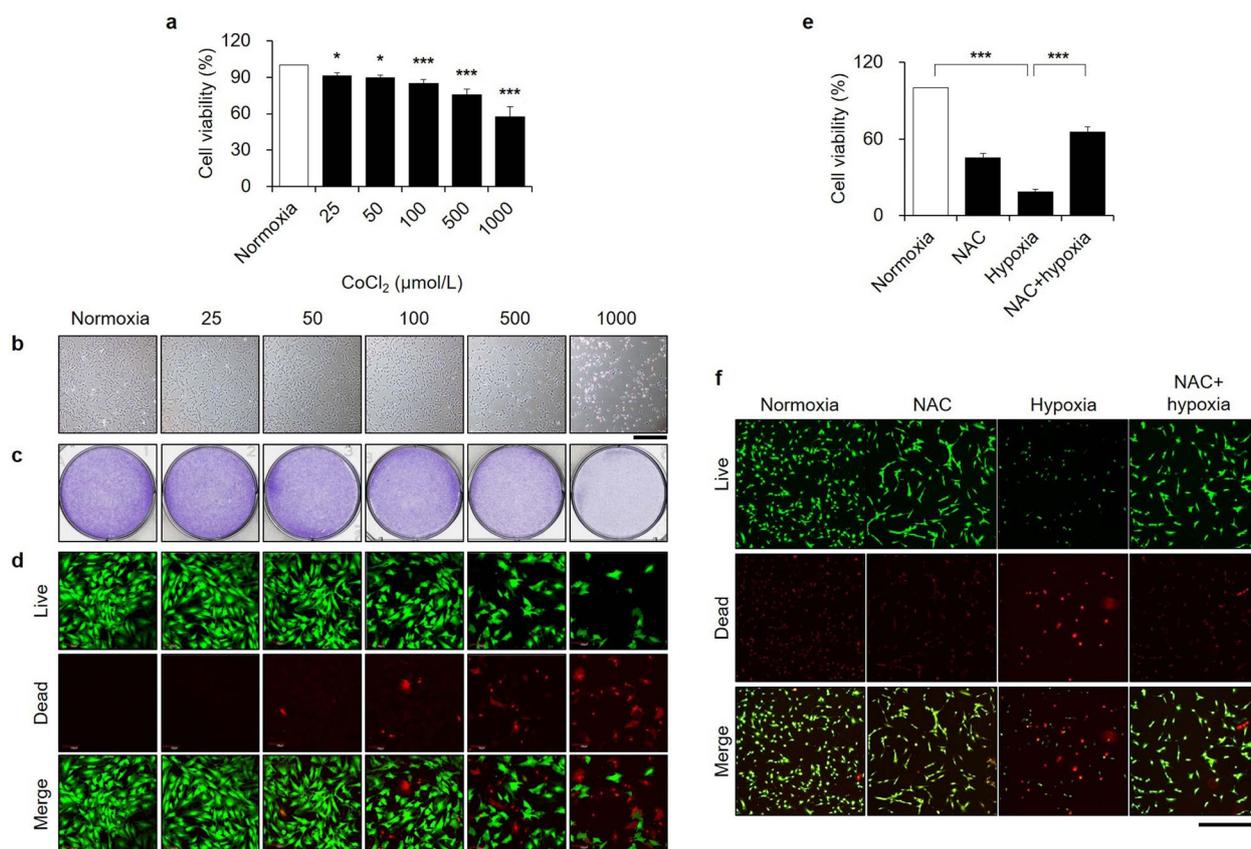


Fig. 2. Analyses of CoCl₂-induced cytotoxicity and of suppressive effects of NAC on that cytotoxicity. (a) Cell viability when cells were exposed to CoCl₂ decreased in a concentration-dependent manner ($p < 0.001$). The morphological analyses were performed by (b) a phase contrast microscope, (c) crystal violet staining and (d) live and dead assay. Results showed that the live-cell populations were decreased by exposure to CoCl₂ in a dose-dependent manner. (e) Cell viability in the NAC-hypoxia group was significantly higher than in the hypoxia group ($p < 0.001$). (f) Live and dead assay indicated that the rate of live cells was markedly higher and the rate of dead cells was markedly lower in the NAC-hypoxia group than in the hypoxia group. (a,e) Mean \pm SD, $n = 5$, * $p < 0.05$, *** $p < 0.001$. (b) Scale bar: 300 μm . (d,f) Scale bar: 500 μm .

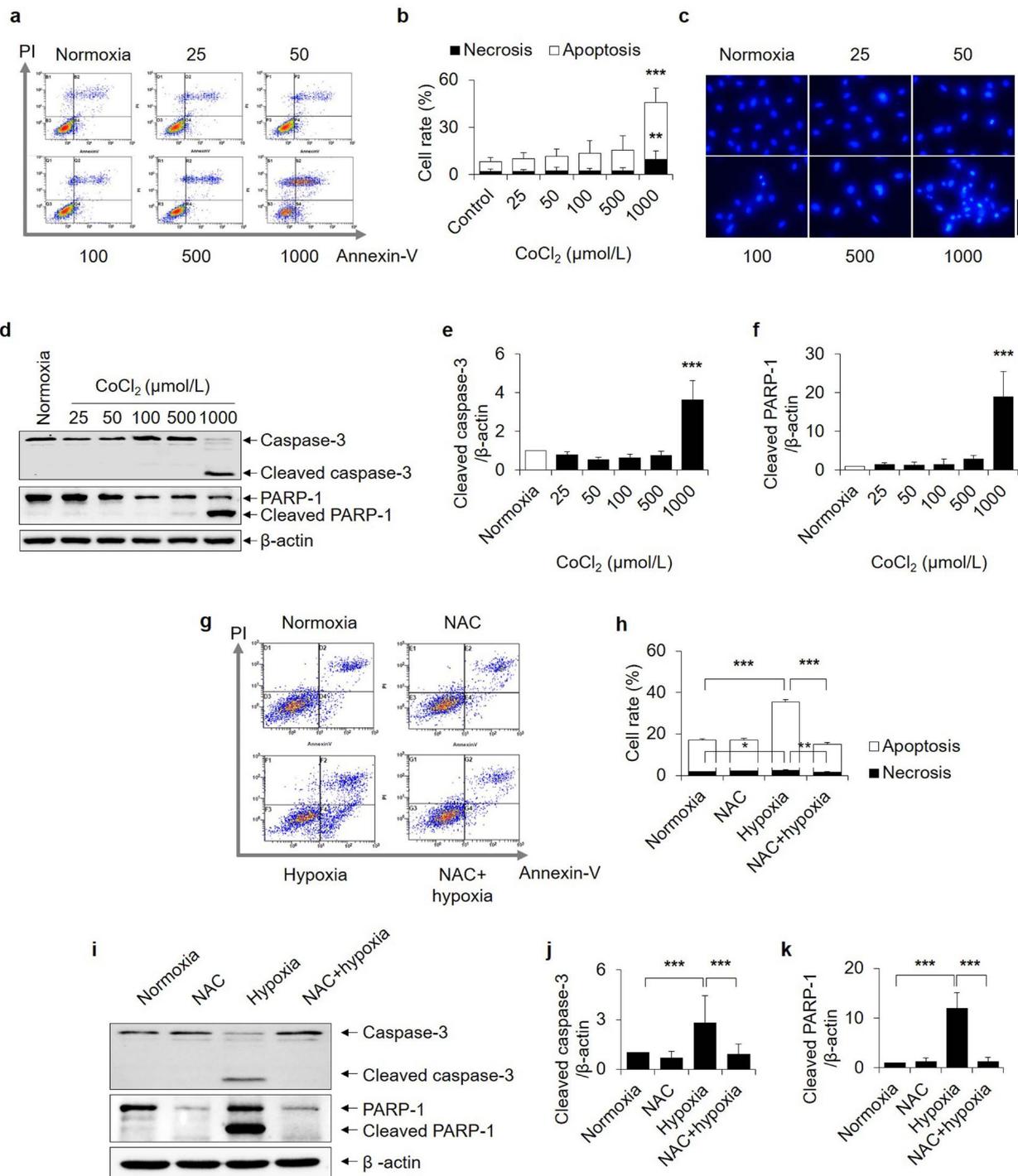


Fig. 3. Analyses of CoCl₂-induced apoptosis and of suppressive effects of NAC on that apoptosis. (a,b) According to the FACS analyses using annexin V/PI staining, the apoptosis rate was significantly higher in the 1,000 μmol/L CoCl₂ group than in the normoxia group ($p < 0.001$). (c) DAPI staining showed that cells with DNA fragmentation were more numerous in the 1,000 μmol/L CoCl₂ group than in the normoxia group. (d-f) Expression of cleaved caspase-3 ($p < 0.001$) and cleaved PARP-1 ($p < 0.001$) in the 1,000 μmol/L CoCl₂ group was significantly higher than in the normoxia group. (g,h) Apoptosis rate in the NAC-hypoxia group was significantly lower than in the hypoxia group ($p < 0.001$). (i,j) Western blot analyses showed that the cleaved caspase-3 expression in the NAC-hypoxia group was significantly lower than in the hypoxia group ($p < 0.001$). (i,k) Expressions of cleaved PARP-1 was significantly lower in the NAC-hypoxia group than in the hypoxia group ($p < 0.001$). (b,e,f,h,j,k) Mean \pm SD, $n = 5$, $*** p < 0.001$. (c) Scale bar: 250 μm.

Analyses of CoCl₂-induced intracellular ROS production and of NAC's suppressive effects on that production

FACS analysis indicated that the levels of intracellular ROS production were higher in all the CoCl₂ study groups than in the normoxia group (Fig. 4a,b). In particular, the levels of intracellular ROS were significantly higher in the 500 μmol/L group ($p = 0.027$) and in the 1,000 μmol/L group ($p < 0.001$) than in the normoxia group. According to the confocal microscope analysis, the levels of intracellular ROS production were markedly higher in all the studied hypoxia groups than in the normoxia group (Fig. 4c). The assessment of NAC's effects against the intracellular ROS production induced by hypoxia indicated that such ROS production was significantly lower in the NAC-hypoxia group than in the hypoxia group ($p < 0.001$) (Fig. 4d,e). The confocal microscope analyses confirmed that intracellular ROS production was markedly lower in the NAC-hypoxia group than in the hypoxia group (Fig. 4f).

Analyses of CoCl₂-induced VEGF-β and MMP-2 expressions and of NAC's suppressive effects on those expressions

Expression of VEGF-β was significantly higher in the 1,000 μmol/L CoCl₂ study group than in the normoxia

group ($p < 0.001$) (Fig. 5a,b). The expression of MMP-2 was significantly higher in the 500 and 1,000 μmol/L CoCl₂ study groups than in the normoxia group ($p < 0.001$) (Fig. 5c,d). The assessment of inhibitory NAC effects against expression of VEGF-β and MMP-2 induced by hypoxia indicated that there was significantly lower expression of VEGF-β ($p < 0.001$) (Fig. 5e,f) and MMP-2 ($p = 0.001$) (Fig. 5g,h) in the NAC-hypoxia groups than in the hypoxia groups.

Discussion

The supraspinatus is the tendon most frequently involved in rotator cuff tendinopathy and tear, which have been shown to be associated with hypoxia (Pufe *et al.*, 2005). Hypoxic changes to the rotator cuff tendon have been proposed as contributors to the development of a rotator cuff tendon tear, through apoptosis (Benson *et al.*, 2010). This study was designed to induce hypoxia in RCFs using CoCl₂ and then to reduce the hypoxic effect by treatment with NAC, an antioxidant. The notable finding of the present study was that, after the induced hypoxia resulted in increases in intracellular ROS production, RCF apoptosis and VEGF-β and MMP-2 expressions, those increases were reduced by treatment with NAC.

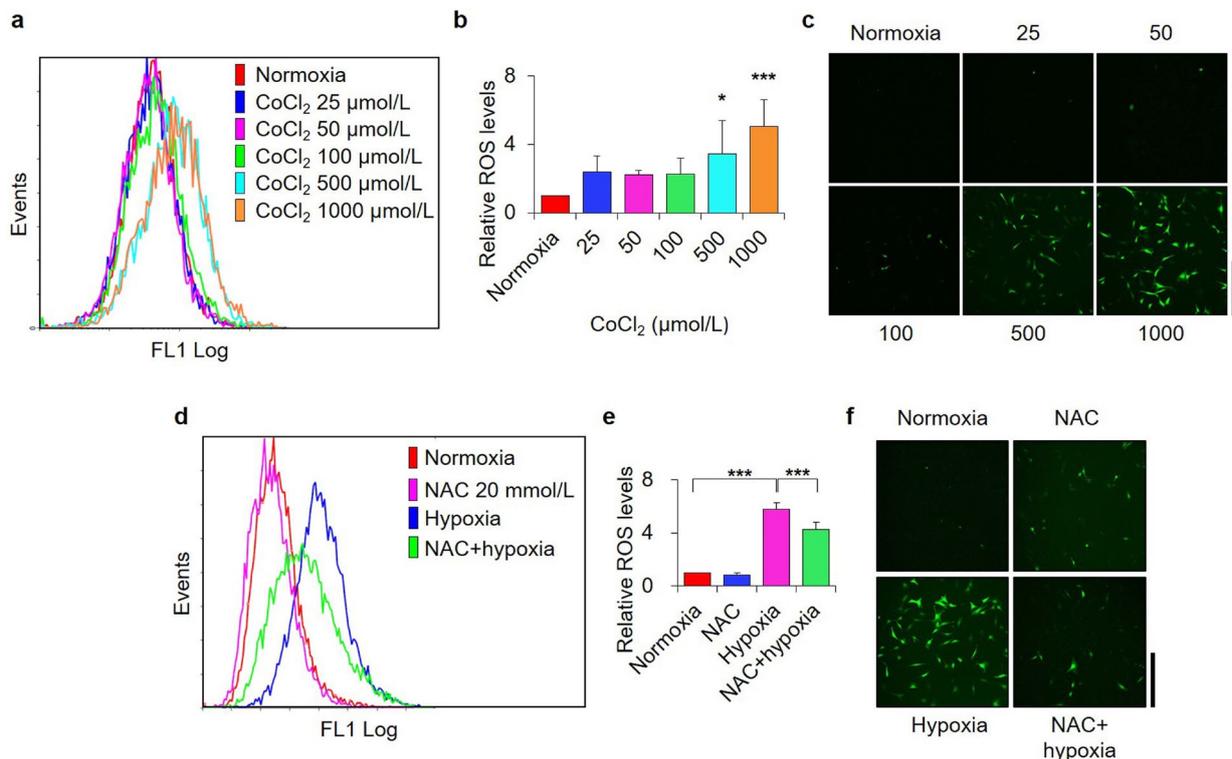


Fig. 4. Analyses of CoCl₂-induced intracellular ROS production and of suppressive effects of NAC on that production. (a,b) FACS analyses indicated that the levels of intracellular ROS production were significantly higher in the 500 μmol/L ($p = 0.027$) and 1,000 μmol/L ($p < 0.001$) CoCl₂ study groups than in the normoxia group. (c) Confocal microscope analyses confirmed that the levels of intracellular ROS production in the 500 μmol/L and 1,000 μmol/L CoCl₂ groups were markedly higher than in the normoxia group. (d,e) The levels of intracellular ROS production in the NAC-hypoxia group were significantly lower than those in the hypoxia group ($p < 0.001$). (f) The confocal microscope analyses indicated that the levels of intracellular ROS production in the NAC-hypoxia group were markedly lower than those in the hypoxia group. (b,e) Mean \pm SD, $n = 5$, * $p < 0.05$, *** $p < 0.001$. (c,f) Scale bar: 500 μm.

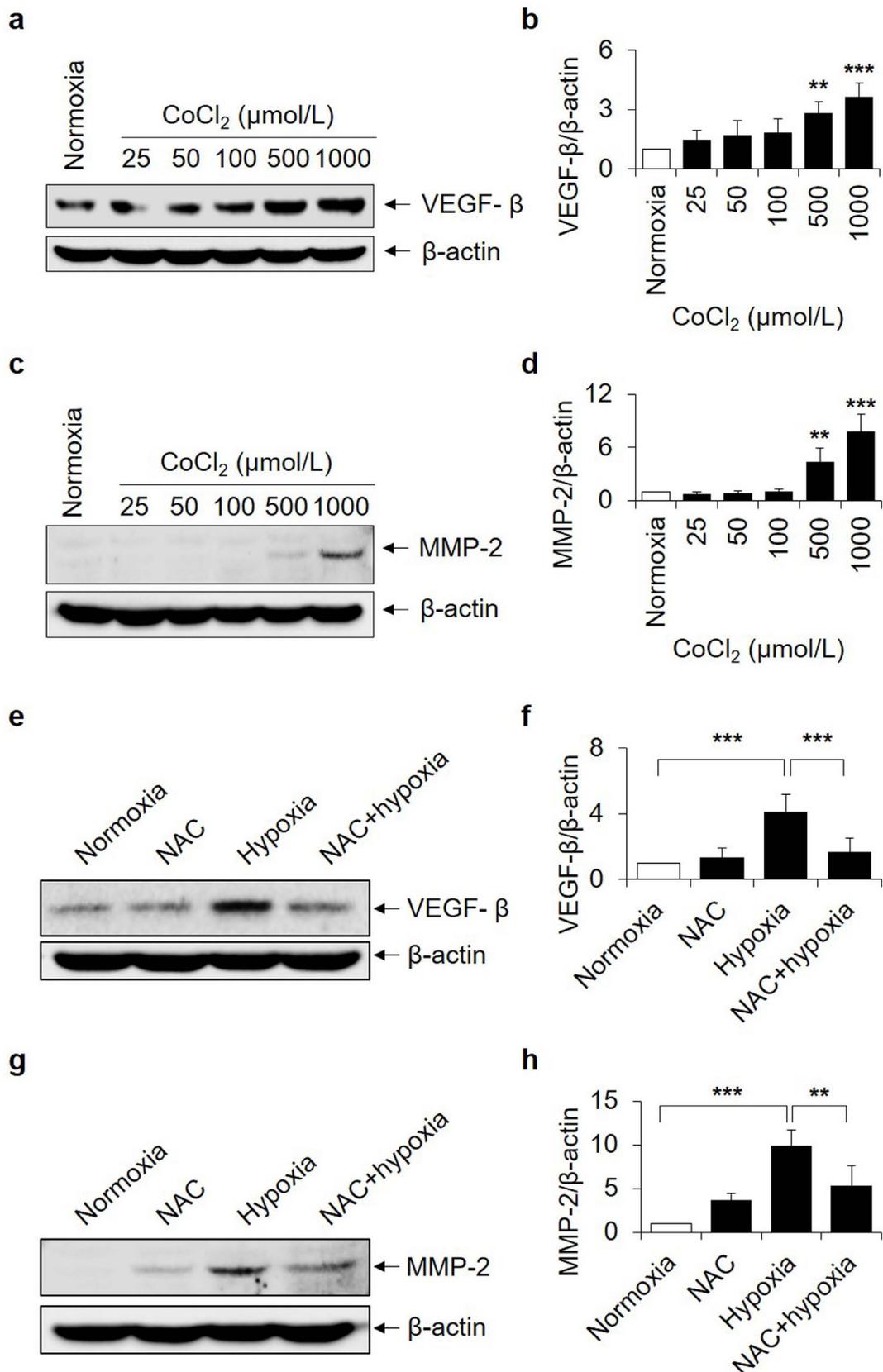


Fig. 5. Analyses of CoCl₂-induced VEGF-β and MMP-2 expressions and of suppressive effects of NAC on those expressions. (a,b) VEGF-β expression was significantly higher in the 500 and 1,000 μmol/L CoCl₂ groups than in the normoxia group ($p < 0.05$). (c,d) MMP-2 expression was significantly higher in the 500 and 1,000 μmol/L CoCl₂ groups than in the normoxia group ($p < 0.05$). (e,f) VEGF-β expression in the NAC-hypoxia group were significantly lower than in the hypoxia group ($p < 0.001$). (g,h) MMP-2 expression in the NAC-hypoxia group was significantly lower than in the hypoxia group ($p = 0.001$). (b,d,f,h) Mean \pm SD, $n = 5$, ** $p < 0.05$, *** $p < 0.001$.

Several CoCl_2 hypoxia-inducing models have been reported in studies using various biomolecular markers (Borcar *et al.*, 2013; Huang *et al.*, 2014; Lendahl *et al.*, 2009). 1 h exposure to 500 $\mu\text{mol/L}$ CoCl_2 or 6 h exposure to 1 % oxygen increases IL-8 expression in human endothelial cells (Kim *et al.*, 2006). 4 h exposure either to 150 $\mu\text{mol/L}$ CoCl_2 or to 1 % oxygen has the effect on HeLa cells of inducing HIF-1 α (Triantafyllou *et al.*, 2006). 24 h exposure to 25–1,000 $\mu\text{mol/L}$ CoCl_2 or to 1 % oxygen causes decreased PDH phosphorylation in HepG2 cells (Borcar *et al.*, 2013). CoCl_2 treatment has been reported to up-regulate HIF-1 transcription factor and HO-1 in many cell types (Amersi *et al.*, 1999; Azenshtein *et al.*, 2005; Taketani *et al.*, 1988; Wang and Semenza, 1995). The present study confirmed previous studies' findings according to which CoCl_2 increases expressions of the hypoxic markers HIF-1 α and HO-1 (Amersi *et al.*, 1999; Azenshtein *et al.*, 2005; Taketani *et al.*, 1988; Wang and Semenza, 1995). Moreover, it found that expression of HIF-1 α was induced by relatively high concentrations of CoCl_2 and particularly by 500 $\mu\text{mol/L}$ and 1,000 $\mu\text{mol/L}$ CoCl_2 . ROS production, rates of apoptosis as well as expressions of caspase-3, PARP-1, VEGF and MMP2 were also significantly increased by 1,000 $\mu\text{mol/L}$ CoCl_2 . Several studies have reported increases in HIF-1 α expression induced by different concentrations of CoCl_2 , exposure times and cell types, respectively: 500 $\mu\text{mol/L}$, 4h, human endothelial cells (Kim *et al.*, 2006); 100–,000 $\mu\text{mol/L}$, 12 h, HepG2 cells (Liu *et al.*, 2015); 600 $\mu\text{mol/L}$, 12 h, cardiac myocytes (Mao *et al.*, 2013); 400 $\mu\text{mol/L}$, 48 h, human periodontal ligament cells (Song *et al.*, 2012); 100 $\mu\text{mol/L}$, 24 h, mesenchymal stem cells (Yoo *et al.*, 2016). Except for Kim *et al.* (2006) study, which had a similar exposure time and concentration, the present study involved a shorter exposure time (4 h) than the other studies (12–48 h) and might, therefore, have required 500 $\mu\text{mol/L}$ and 1,000 $\mu\text{mol/L}$ of CoCl_2 (relatively high concentrations) to induce hypoxia. Additionally, because CoCl_2 concentrations and exposure times were determined according to the types of experimental cells and evaluated biomolecules used for the hypoxia models (Munoz-Sanchez and Chanez-Cardenas, 2019; Triantafyllou *et al.*, 2006), direct comparisons among those models are difficult, at least regarding their CoCl_2 concentrations.

ROS released during hypoxia act as signalling agents that trigger diverse functional responses, including activation of gene expression through the stabilisation of the transcription factor HIF-1 α (Guzy and Schumacker, 2006). CoCl_2 -induced hypoxia has been reported to increase the level of ROS in PC12 cells (Kotake-Nara and Saida, 2006; Zou *et al.*, 2001). The present study confirmed that CoCl_2 -induced hypoxia increased ROS production. A previous study reported that CoCl_2 -induced hypoxia promoted apoptosis in PC12 cells, through ROS production accompanied by AP-1 activation (Zou *et al.*, 2001). CoCl_2 -induced hypoxia was reported to increase apoptosis, through mitochondrial dysfunction, in a

study using human periodontal ligament cells (Song *et al.*, 2012). In the present study, CoCl_2 -induced hypoxia increased apoptosis by increasing ROS production and expressions of caspase-3 and PARP-1. Caspase-3 is a recognised common executioner of both the intrinsic and extrinsic apoptosis pathways (McIlwain *et al.*, 2013); PARP-1 also plays a role in the main pathways of apoptosis by stimulating the release of AIF (Tewari *et al.*, 1995). The present study supported previous studies' finding (McIlwain *et al.*, 2013; Tewari *et al.*, 1995) according to which CoCl_2 -induced hypoxia promotes apoptosis.

In the present study, CoCl_2 -induced hypoxia increased expressions of VEGF- β and MMP-2 in RCFs. ROS are recognised as important mediators and modulators of the synthesis and activity of VEGF, a major angiogenic molecule (Huang and Nan, 2019; Kim and Byzova, 2014). HO-1 has been reported to be involved in angiogenesis through initiation of VEGF expression (Dulak *et al.*, 2008b). Genetic overexpression of HO-1 has been shown to enhance VEGF synthesis (Dulak *et al.*, 2008a). The present study supported the findings of previous studies in which hypoxia sequentially induced ROS, HO-1 and VEGF (Dulak *et al.*, 2008a; Sadaghianloo *et al.*, 2017). MMPs play roles in collagenolysis and elastolysis during periods of development, wound healing and major inflammatory disease (Antonicelli *et al.*, 2007; Fields, 2013). MMP-2 has been reported as expressed and activated during the healing process of acute supraspinatus tendon tear where it play a role in remodelling (Choi *et al.*, 2002). Collagen types I and III are the main collagens present in the tendon matrix. MMP-2 degrades collagen types I, II and III (Aimes and Quigley, 1995; Kannus, 2000; Kontinen *et al.*, 1998; Patterson *et al.*, 2001; Riley, 2004). This suggests that MMP-2 may act as an executioner during matrix degradation. The present study's results suggested that hypoxia induced degradation of the rotator cuff tendon matrix through the increasing expression of MMP-2.

NAC is a well-known antioxidant that protects multiple organs and cells against ischemic, oxidative and other stressors (Bartekova *et al.*, 2018; Colovic *et al.*, 2018; Heil *et al.*, 2018; Kim *et al.*, 2019; Kim *et al.*, 2017). In the present study, NAC attenuated CoCl_2 -induced HIF-1 α , HO-1 and apoptosis by the reductions in ROS production and expression of cleaved caspase-3 and cleaved PARP-1. NAC has been reported as an inhibitor of HIF-1 α and of HO-1, both of which are induced by hypoxia (Gao *et al.*, 2007; Ryter *et al.*, 2000). CoCl_2 -induced hypoxia has been reported to increase the level of ROS in PC12 cells; the antioxidant NAC has been reported to inhibit that response (Kotake-Nara and Saida, 2006; Zou *et al.*, 2001). Some studies have reported that the inhibition of HIF-1 α and HO-1 expression is induced by the scavenging of ROS by pre-treatment with NAC (Greer *et al.*, 2012; He *et al.*, 2018; Kim *et al.*, 2002). NAC has been reported to inhibit apoptosis induced by hypoxia in various cells: hepatocytes, PC12 cells,

human periodontal ligament cells, hippocampal cells and mesenchymal stem cells (Bernard *et al.*, 2018; Heil *et al.*, 2018; Jayalakshmi *et al.*, 2005; Lan *et al.*, 2011; Song *et al.*, 2012). Those previous studies support the present study findings, which suggested that NAC had the potential to reduce hypoxia-induced rotator cuff tendon degeneration, as antioxidants act upon the oxidative stress (Kim *et al.*, 2014; Park *et al.*, 2010) or upon a neurotransmitter (Kim *et al.*, 2019) involved in that degeneration. The present study's findings also suggested that the CoCl₂-induced hypoxia model could be valuable in the search for therapeutic targets involved in hypoxia-induced tendon degeneration.

The study had several limitations. Because it focused on apoptotic cell death, which is a major type of cell death related to rotator cuff tendon degeneration or tear, it did not investigate necrotic cell death and its related mechanism, although necroptosis is inducible by CoCl₂ in the form of programmed necrotic cell death (Gong *et al.*, 2017; Rovetta *et al.*, 2013). Moreover, the study did not address whether overuse activity could induce hypoxia in an animal model or whether NAC, an antioxidant, could prevent hypoxia-induced cell death in an animal overuse model. Therefore, further studies should examine these issues.

Conclusion

ROS were involved in hypoxic RCF apoptosis induced by CoCl₂; NAC, an ROS scavenger, inhibited hypoxia-induced RCF apoptosis by inhibiting ROS production.

Acknowledgement

This research was supported by the Basic Science Research Program, through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2021R1A2C1008931).

References

- Abdel-Rahman Mohamed A, M M Metwally M, Khalil SR, Salem GA, Ali HA (2019) Moringa oleifera extract attenuates the CoCl₂ induced hypoxia of rat's brain: expression pattern of HIF-1 α , NF- κ B, MAO and EPO. *Biomed Pharmacother* **109**: 1688-1697.
- Aimes RT, Quigley JP (1995) Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyses the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments. *J Biol Chem* **270**: 5872-5876.
- Amersi F, Buelow R, Kato H, et al. (1999) Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. *J Clin Invest* **104**: 1631-1639.
- Antonicelli F, Bellon G, DeBelle L, Hornebeck W (2007) Elastin-elastases and inflamm-aging. *Curr Top Dev Biol* **79**: 99-155.
- Azenshtein E, Meshel T, Shina S, Barak N, Keydar I, Ben-Baruch A (2005) The angiogenic factors CXCL8 and VEGF in breast cancer: regulation by an array of pro-malignancy factors. *Cancer Lett* **217**: 73-86.
- Bartekova M, Barancik M, Ferenczyova K, Dhalla NS (2018) Beneficial effects of N-acetylcysteine and N-mercaptopyrionylglycine on ischemia reperfusion injury in the heart. *Curr Med Chem* **25**: 355-366.
- Benson RT, McDonnell SM, Knowles HJ, Rees JL, Carr AJ, Hulley PA (2010) Tendinopathy and tears of the rotator cuff are associated with hypoxia and apoptosis. *J Bone Joint Surg Br* **92**: 448-453.
- Bernard O, Jeny F, Uzunhan Y, Dondi E, Terfous R, Label R, Sutton A, Larghero J, Vanneaux V, Nunes H, Boncoeur E, Planes C, Dard N (2018) Mesenchymal stem cells reduce hypoxia-induced apoptosis in alveolar epithelial cells by modulating HIF and ROS hypoxic signaling. *Am J Physiol Lung Cell Mol Physiol* **314**: L360-L371.
- Borcar A, Menze MA, Toner M, Hand SC (2013) Metabolic preconditioning of mammalian cells: mimetic agents for hypoxia lack fidelity in promoting phosphorylation of pyruvate dehydrogenase. *Cell Tissue Res* **351**: 99-106.
- Chen S, Xu H, Hu F, Wang T (2020) Identification of key players involved in CoCl₂ hypoxia induced pulmonary artery hypertension *in vitro*. *Front Genet* **11**: 232. DOI: 10.3389/fgene.2020.00232.
- Choi HR, Kondo S, Hirose K, Ishiguro N, Hasegawa Y, Iwata H (2002) Expression and enzymatic activity of MMP-2 during healing process of the acute supraspinatus tendon tear in rabbits. *J Orthop Res* **20**: 927-933.
- Colovic MB, Vasic VM, Djuric DM, Krstic DZ (2018) Sulphur-containing amino acids: protective role against free radicals and heavy metals. *Curr Med Chem* **25**: 324-335.
- Dulak J, Deshane J, Jozkowicz A, Agarwal A (2008a) Heme oxygenase-1 and carbon monoxide in vascular pathobiology: focus on angiogenesis. *Circulation* **117**: 231-241.
- Dulak J, Loboda A, Jozkowicz A (2008b) Effect of heme oxygenase-1 on vascular function and disease. *Curr Opin Lipidol* **19**: 505-512.
- Fields GB (2013) Interstitial collagen catabolism. *J Biol Chem* **288**: 8785-8793.
- Fuhrmann DC, Brune B (2017) Mitochondrial composition and function under the control of hypoxia. *Redox Biol* **12**: 208-215.
- Funakoshi T, Iwasaki N, Kamishima T, Nishida M, Ito Y, Kondo M, Minami A (2010) *In vivo* visualisation of vascular patterns of rotator cuff tears using contrast-enhanced ultrasound. *Am J Sports Med* **38**: 2464-2471.
- Gao P, Zhang H, Dinavahi R, Li F, Xiang Y, Raman V, Bhujwalla ZM, Felsner DW, Cheng L, Pevsner J, Lee LA, Semenza GL, Dang CV (2007) HIF-dependent

antitumorigenic effect of antioxidants *in vivo*. *Cancer Cell* **12**: 230-238.

Gerber HP, Condorelli F, Park J, Ferrara N (1997) Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. *J Biol Chem* **272**: 23659-23667.

Goldberg MA, Dunning SP, Bunn HF (1988) Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. *Science* **242**: 1412-1415.

Gong YN, Guy C, Crawford JC, Green DR (2017) Biological events and molecular signaling following MLKL activation during necroptosis. *Cell Cycle* **16**: 1748-1760.

Greer SN, Metcalf JL, Wang Y, Ohh M (2012) The updated biology of hypoxia-inducible factor. *EMBO J* **31**: 2448-2460.

Guzy RD, Schumacker PT (2006) Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. *Exp Physiol* **91**: 807-819.

He C, Zhang W, Li S, Ruan W, Xu J, Xiao F (2018) Edaravone improves septic cardiac function by inducing an HIF-1 α /HO-1 pathway. *Oxid Med Cell Longev* **2018**: 5216383. DOI: 10.1155/2018/5216383.

Heil J, Schultze D, Schemmer P, Bruns H (2018) N-acetylcysteine protects hepatocytes from hypoxia-related cell injury. *Clin Exp Hepatol* **4**: 260-266.

Huang BW, Miyazawa M, Tsuji Y (2014) Distinct regulatory mechanisms of the human ferritin gene by hypoxia and hypoxia mimetic cobalt chloride at the transcriptional and post-transcriptional levels. *Cell Signal* **26**: 2702-2709.

Huang YJ, Nan GX (2019) Oxidative stress-induced angiogenesis. *J Clin Neurosci* **63**: 13-16.

Jayalakshmi K, Sairam M, Singh SB, Sharma SK, Ilavazhagan G, Banerjee PK (2005) Neuroprotective effect of N-acetyl cysteine on hypoxia-induced oxidative stress in primary hippocampal culture. *Brain Res* **1046**: 97-104.

Kannus P (2000) Structure of the tendon connective tissue. *Scand J Med Sci Sports* **10**: 312-320.

Kim HH, Lee SE, Chung WJ, Choi Y, Kwack K, Kim SW, Kim MS, Park H, Lee ZH (2002) Stabilisation of hypoxia-inducible factor-1 α is involved in the hypoxic stimuli-induced expression of vascular endothelial growth factor in osteoblastic cells. *Cytokine* **17**: 14-27.

Kim KS, Rajagopal V, Gonsalves C, Johnson C, Kalra VK (2006) A novel role of hypoxia-inducible factor in cobalt chloride- and hypoxia-mediated expression of IL-8 chemokine in human endothelial cells. *J Immunol* **177**: 7211-7224.

Kim RJ, Hah YS, Gwark JY, Park HB (2019) N-acetylcysteine reduces glutamate-induced cytotoxicity to fibroblasts of rat supraspinatus tendons. *Connect Tissue Res* **60**: 431-443.

Kim RJ, Hah YS, Sung CM, Kang JR, Park HB (2014) Do antioxidants inhibit oxidative-stress-

induced autophagy of tenofibroblasts? *J Orthop Res* **32**: 937-943.

Kim RJ, Kang JR, Hah YS, Park HB (2017) N-acetyl cysteine protects cells from chondrocyte death induced by local anesthetics. *J Orthop Res* **35**: 297-303.

Kim YW, Byzova TV (2014) Oxidative stress in angiogenesis and vascular disease. *Blood* **123**: 625-631.

Kontinen YT, Ceponis A, Takagi M, Ainola M, Sorsa T, Sutinen M, Salo T, Ma J, Santavirta S, Seiki M (1998) New collagenolytic enzymes/cascade identified at the pannus-hard tissue junction in rheumatoid arthritis: destruction from above. *Matrix Biol* **17**: 585-601.

Kotake-Nara E, Saida K (2006) Endothelin-2/ vasoactive intestinal contractor: regulation of expression *via* reactive oxygen species induced by CoCl₂ and biological activities including neurite outgrowth in PC12 cells. *ScientificWorldJournal* **6**: 176-186.

Kumar H, Choi DK (2015) Hypoxia inducible factor pathway and physiological adaptation: a cell survival pathway? *Mediators Inflamm* **2015**: 584758. DOI: 10.1155/2015/584758.

Lakemeier S, Reichelt JJ, Patzer T, Fuchs-Winkelmann S, Paletta JR, Schofer MD (2010) The association between retraction of the torn rotator cuff and increasing expression of hypoxia inducible factor 1 α and vascular endothelial growth factor expression: an immunohistological study. *BMC Musculoskelet Disord* **11**: 230. DOI: 10.1186/1471-2474-11-230.

Lan A, Liao X, Mo L, Yang C, Yang Z, Wang X, Hu F, Chen P, Feng J, Zheng D, Xiao L (2011) Hydrogen sulfide protects against chemical hypoxia-induced injury by inhibiting ROS-activated ERK1/2 and p38MAPK signaling pathways in PC12 cells. *PLoS One* **6**: e25921. DOI: 10.1371/journal.pone.0025921.

Lee HJ, Kim YS, Ok JH, Song HJ (2013) Apoptosis occurs throughout the diseased rotator cuff. *Am J Sports Med* **41**: 2249-2255.

Lendahl U, Lee KL, Yang H, Poellinger L (2009) Generating specificity and diversity in the transcriptional response to hypoxia. *Nat Rev Genet* **10**: 821-832.

Lewis JS, Raza SA, Pilcher J, Heron C, Poloniecki JD (2009) The prevalence of neovascularity in patients clinically diagnosed with rotator cuff tendinopathy. *BMC Musculoskelet Disord* **10**: 163. DOI: 10.1186/1471-2474-10-163.

Lian O, Scott A, Engebretsen L, Bahr R, Duronio V, Khan K (2007) Excessive apoptosis in patellar tendinopathy in athletes. *Am J Sports Med* **35**: 605-611.

Liang M, Cornell HR, Zargar Baboldashti N, Thompson MS, Carr AJ, Hulley PA (2012) Regulation of hypoxia-induced cell death in human tenocytes. *Adv Orthop* **2012**: 984950. DOI: 10.1155/2012/984950.

Liu Q, Xu Z, Mao S, Chen W, Zeng R, Zhou S, Liu J (2015) Effect of hypoxia on hypoxia inducible factor-1 α , insulin-like growth factor I and vascular

endothelial growth factor expression in hepatocellular carcinoma HepG2 cells. *Oncol Lett* **9**: 1142-1148.

Lohr JF, Uthoff HK (1990) The microvascular pattern of the supraspinatus tendon. *Clin Orthop Relat Res*: 35-38.

Mao X, Wang T, Liu Y, Irwin MG, Ou JS, Liao XL, Gao X, Xu Y, Ng KF, Vanhoutte PM, Xia Z (2013) N-acetylcysteine and allopurinol confer synergy in attenuating myocardial ischemia injury *via* restoring HIF-1 α /HO-1 signaling in diabetic rats. *PLoS One* **8**: e68949. DOI: 10.1371/journal.pone.0068949.

McBeath R, Edwards RW, O'Hara BJ, Maltenfort MG, Parks SM, Steplewski A, Osterman AL, Shapiro IM (2019) Tendinosis develops from age- and oxygen tension-dependent modulation of Rac1 activity. *Aging Cell* **18**: e12934. DOI: 10.1111/ace1.12934.

McIlwain DR, Berger T, Mak TW (2013) Caspase functions in cell death and disease. *Cold Spring Harb Perspect Biol* **5**: a008656. DOI: 10.1101/cshperspect.a008656.

Millar NL, Reilly JH, Kerr SC, Campbell AL, Little KJ, Leach WJ, Rooney BP, Murrell GA, McInnes IB (2012) Hypoxia: a critical regulator of early human tendinopathy. *Ann Rheum Dis* **71**: 302-310.

Moseley HF, Goldie I (1963) The arterial pattern of the rotator cuff of the shoulder. *J Bone Joint Surg Br* **45**: 780-789.

Munoz-Sanchez J, Chanez-Cardenas ME (2019) The use of cobalt chloride as a chemical hypoxia model. *J Appl Toxicol* **39**: 556-570.

Nam DC, Hah YS, Nam JB, Kim RJ, Park HB (2016) Cytoprotective mechanism of cyanidin and delphinidin against oxidative stress-induced tenofibroblast death. *Biomol Ther (Seoul)* **24**: 426-432.

Nguyen VT, Canciani B, Cirillo F, Anastasia L, Peretti GM, Mangiavini L (2020) Effect of chemically induced hypoxia on osteogenic and angiogenic differentiation of bone marrow mesenchymal stem cells and human umbilical vein endothelial cells in direct coculture. *Cells* **9**: 757. DOI: 10.3390/cells9030757.

Nho SJ, Yadav H, Shindle MK, Macgillivray JD (2008) Rotator cuff degeneration: etiology and pathogenesis. *Am J Sports Med* **36**: 987-993.

Park HB, Hah YS, Yang JW, Nam JB, Cho SH, Jeong ST (2010) Antiapoptotic effects of anthocyanins on rotator cuff tenofibroblasts. *J Orthop Res* **28**: 1162-1169.

Patterson ML, Atkinson SJ, Knauper V, Murphy G (2001) Specific collagenolysis by gelatinase A, MMP-2, is determined by the hemopexin domain and not the fibronectin-like domain. *FEBS Lett* **503**: 158-162.

Petersen W, Pufe T, Kurz B, Mentlein R, Tillmann B (2002) Angiogenesis in fetal tendon development: spatial and temporal expression of the angiogenic peptide vascular endothelial cell growth factor. *Anat Embryol (Berl)* **205**: 263-270.

Petersen W, Varoga D, Zantop T, Hassenpflug J, Mentlein R, Pufe T (2004) Cyclic strain influences the expression of the vascular endothelial growth factor

(VEGF) and the hypoxia inducible factor 1 alpha (HIF-1alpha) in tendon fibroblasts. *J Orthop Res* **22**: 847-853.

Pufe T, Petersen W, Tillmann B, Mentlein R (2001) The angiogenic peptide vascular endothelial growth factor is expressed in foetal and ruptured tendons. *Virchows Arch* **439**: 579-585.

Pufe T, Petersen WJ, Mentlein R, Tillmann BN (2005) The role of vasculature and angiogenesis for the pathogenesis of degenerative tendons disease. *Scand J Med Sci Sports* **15**: 211-222.

Rana NK, Singh P, Koch B (2019) CoCl₂ simulated hypoxia induce cell proliferation and alter the expression pattern of hypoxia associated genes involved in angiogenesis and apoptosis. *Biol Res* **52**: 12. DOI: 10.1186/s40659-019-0221-z.

Rathbun JB, Macnab I (1970) The microvascular pattern of the rotator cuff. *J Bone Joint Surg Br* **52**: 540-553.

Riley G (2004) The pathogenesis of tendinopathy. A molecular perspective. *Rheumatology (Oxford)* **43**: 131-142.

Rovetta F, Stacchiotti A, Faggi F, Catalani S, Apostoli P, Fanzani A, Aleo MF (2013) Cobalt triggers necrotic cell death and atrophy in skeletal C2C12 myotubes. *Toxicol Appl Pharmacol* **271**: 196-205.

Ryter SW, Si M, Lai CC, Su CY (2000) Regulation of endothelial heme oxygenase activity during hypoxia is dependent on chelatable iron. *Am J Physiol Heart Circ Physiol* **279**: H2889-2897.

Sadaghianloo N, Yamamoto K, Bai H, Tsuneki M, Protack CD, Hall MR, Declémy S, Hassen-Khodja R, Madri J, Dardik A (2017) Increased oxidative stress and hypoxia inducible factor-1 expression during arteriovenous fistula maturation. *Ann Vasc Surg* **41**: 225-234.

Semenza GL (2003) Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* **3**: 721-732.

Semenza GL (2014) Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. *Annu Rev Pathol* **9**: 47-71.

Song ZC, Zhou W, Shu R, Ni J (2012) Hypoxia induces apoptosis and autophagic cell death in human periodontal ligament cells through HIF-1 α pathway. *Cell Prolif* **45**: 239-248.

Soni S, Padwad YS (2017) HIF-1 in cancer therapy: two decade long story of a transcription factor. *Acta Oncol* **56**: 503-515.

Taketani S, Kohno H, Yoshinaga T, Tokunaga R (1988) Induction of heme oxygenase in rat hepatoma cells by exposure to heavy metals and hyperthermia. *Biochem Int* **17**: 665-672.

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* **81**: 801-809.

Triantafyllou A, Liakos P, Tsakalof A, Georgatsou E, Simos G, Bonanou S (2006) Cobalt induces hypoxia-

inducible factor-1alpha (HIF-1alpha) in HeLa cells by an iron-independent, but ROS-, PI-3K- and MAPK-dependent mechanism. *Free Radic Res* **40**: 847-856.

Via AG, De Cupis M, Spoliti M, Oliva F (2013) Clinical and biological aspects of rotator cuff tears. *Muscles Ligaments Tendons J* **3**: 70-79.

Wang GL, Semenza GL (1995) Purification and characterization of hypoxia-inducible factor 1. *J Biol Chem* **270**: 1230-1237.

Yamamoto A, Takagishi K, Osawa T, Yanagawa T, Nakajima D, Shitara H, Kobayashi T (2010) Prevalence and risk factors of a rotator cuff tear in the general population. *J Shoulder Elbow Surg* **19**: 116-120.

Yamamoto N, Itoi E, Tuoheti Y, Seki N, Abe H, Minagawa H, Shimada Y, Okada K (2007) Glenohumeral joint motion after medial shift of the attachment site of the supraspinatus tendon: a cadaveric study. *J Shoulder Elbow Surg* **16**: 373-378.

Yoo HI, Moon YH, Kim MS (2016) Effects of CoCl₂ on multi-lineage differentiation of C3H/10T1/2 mesenchymal stem cells. *Korean J Physiol Pharmacol* **20**: 53-62.

Yoon D, Pastore YD, Divoky V, Liu E, Mlodnicka AE, Rainey K, Ponka P, Semenza GL, Schumacher A, Prchal JT (2006) Hypoxia-inducible factor-1 deficiency results in dysregulated erythropoiesis signaling and iron homeostasis in mouse development. *J Biol Chem* **281**: 25703-25711.

Yu X, Wan Q, Ye X, Cheng Y, Pathak JL, Li Z (2019) Cellular hypoxia promotes osteogenic differentiation of mesenchymal stem cells and bone defect healing *via* STAT3 signaling. *Cell Mol Biol Lett* **24**: 64. DOI: 10.1186/s11658-019-0191-8.

Yuan J, Murrell GA, Wei AQ, Wang MX (2002) Apoptosis in rotator cuff tendonopathy. *J Orthop Res* **20**: 1372-1379.

Ziello JE, Jovin IS, Huang Y (2007) Hypoxia-inducible factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia. *Yale J Biol Med* **80**: 51-60.

Zou W, Yan M, Xu W, Huo H, Sun L, Zheng Z, Liu X (2001) Cobalt chloride induces PC12 cells apoptosis

through reactive oxygen species and accompanied by AP-1 activation. *J Neurosci Res* **64**: 646-653.

Discussion with Reviewers

Reviewer 1: Can the authors comment on oxygen availability in healthy tendons? What would be an *in vivo*-like oxygen tension for tendon cells? Please discuss with regard to the results of the present study.

Authors: The physiological oxygen tension for the tenocyte microenvironment is not determined completely. According to the present study and previous relevant studies, hypoxia (0.1-1 %) induces tenocyte apoptosis, phenotype change and calcification. A study (reference?) of mesenchymal stem cells reported that a physioxic culture condition (1-7 %) slows down cell cycle progression and differentiation. It is probable that normoxia (13-20 %) must be maintained in order to preserve optimal physiological function of the tendon cells. Further research is needed to determine the optimal oxygen tension in normal tendon cells, according to their specific phase in the cell cycle.

Reviewer 2: You conclude that NAC, as a ROS scavenger, inhibited hypoxia-induced RCF apoptosis. What further research is needed to develop this knowledge into a therapeutic approach? How can NAC be used in the treatment of tendinopathies?

Authors: These are very important questions. The next step for continuation of this research would be to determine whether NAC has the ability to reduce hypoxia-related changes in an overuse or hypoxia animal model, with hypoxia-mimicking agents. We are considering the possibility of intravenous NAC injection for patients who have rotator cuff tendinopathy.

Editor's note: The Scientific Editor responsible for this paper was Denitsa Docheva.