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

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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 μmol/L cobalt chloride ($CoCl_2$); the antioxidant was 20 mmol/L NAC. Expressions of hypoxia-inducible factor-1α (HIF-1α) 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-1α 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.

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List of Abbreviations

AIF	apoptosis inducing factor
ANOVA	analysis of variance
AP-1	activator protein 1
BNip3	BCL2 interacting protein 3
BSA	bovine serum albumin
CD	cluster of differentiation
CO	carbon monoxide
$CoCl_2$	cobalt chloride
DAPI	4′,6-diamidino-2-phenylindole
DCF-DA	2′,7′-dichlorofluorescin diacetate
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulphoxide
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
GSH	glutathione
HIF-1α	hypoxia-inducible factor-1α
HO-1	heme oxygenase-1
IL	interleukin
MMP	matrix metalloproteinase
MTT	3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetylcysteine
NRF	National Research Foundation of Korea
PARP-1	poly ADP-ribose polymerase-1
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 Uhthoff, 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 % O_2), 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-1α and of an HIF-1α-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-1α and HIF-1β 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-1α 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-1α 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 Uhthoff, 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.

$CoCl_2$, a chelator, is a well-known chemical hypoxic agent. Ever since Goldberg *et al.* (1988) established that $CoCl_2$ can mimic hypoxic conditions experimentally, $CoCl_2$ 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 $CoCl_2$ is similar to the hypoxic *in vivo* microenvironment in terms of signal transduction and transcription regulation depending on concentration and exposure time to $CoCl_2$ 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 Uhthoff, 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 $CoCl_2$) and NAC-hypoxia groups. The exposure time to $CoCl_2$ 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 $CoCl_2$. The RCFs of both subsets were cultured on plates or dishes, incubated overnight and exposed to $CoCl_2$ or NAC and $CoCl_2$, 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 % CO_2 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 the12th passage was confirmed by PCR analyses (data not shown).

MTT assay

Cell viability was estimated by measuring the metabolism of MTT (M2128, Sigma-Aldrich). 2×10^4 RCFs were seeded in each well of a 24-well plate. Cells were maintained in an incubator at 5 % CO_2 and 37 °C for 24 h. Then, the study groups were exposed to $CoCl_2$ or to NAC and $CoCl_2$, 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. 1×10^5 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. 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. 1×10^5 RCFs were seeded in each well of a 6-well plate. After 24 h of incubation, the study groups were exposed to $CoCl_2$ or to NAC and $CoCl_2$, 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. 1×10^4 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 $CoCl_2$ or to NAC and $CoCl_2$, according to their study subset. After

medium removal, cells were stained for 30 min at room temperature using a prepared staining solution (2 μmol/L of acetoxymethyl ester of calcein and 4 μmol/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 μg/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 ×*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-1α (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 μmol/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 CXP 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 μmol/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 ± 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-1α and HO-1 expressions and of suppressive effects of NAC on those expressions

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

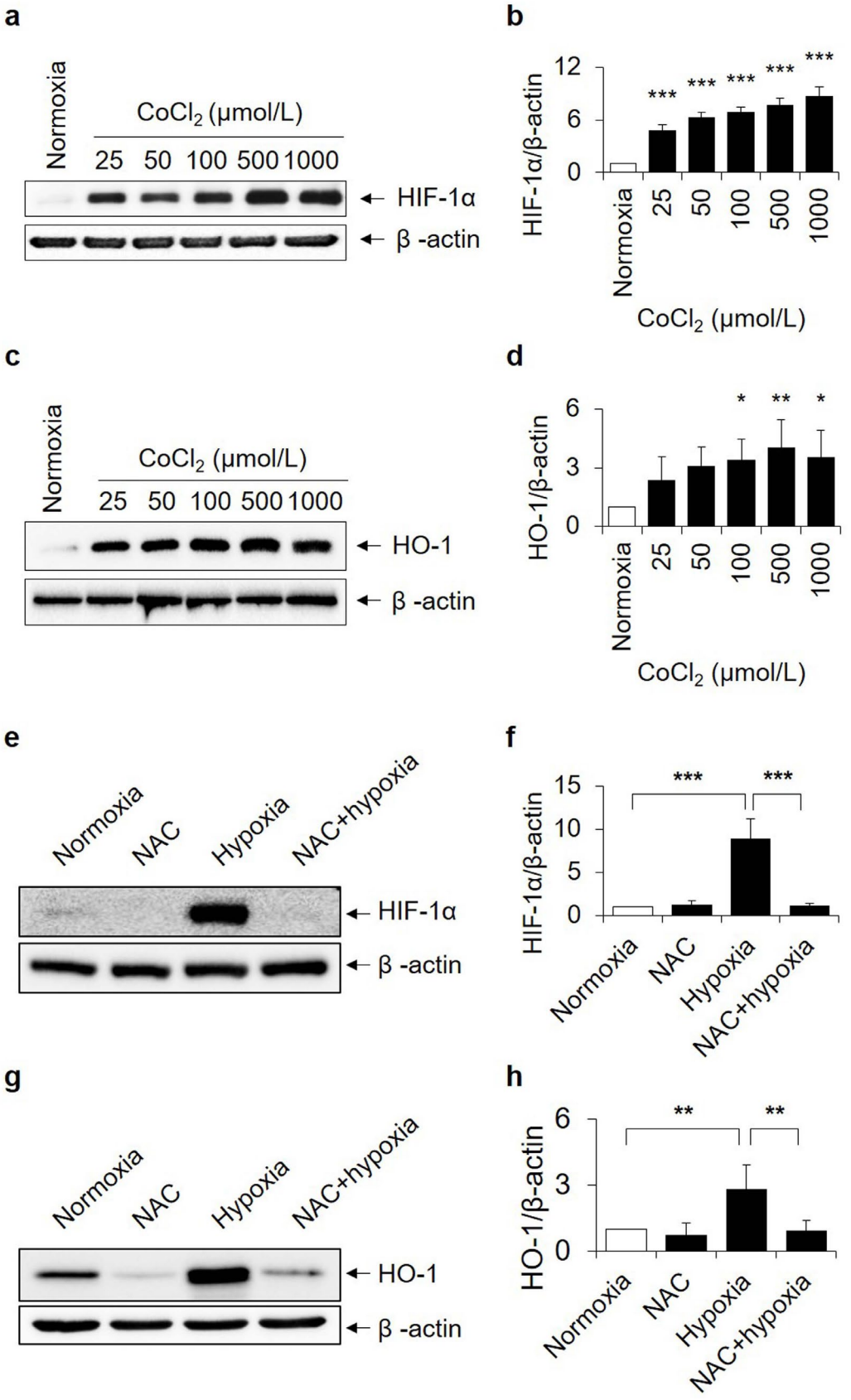

Fig. 1. Analyses of $CoCl_2$-induced HIF-1α and HO-1 expressions and of suppressive effects of NAC on those expressions. (**a**,**b**) HIF-1α expression in all $CoCl_2$ study groups was significantly higher than in the normoxia group ($p < 0.001$). (**c**,**d**) HO-1 expression was significantly higher in the 100, 500 and 1,000 μmol/L $CoCl_2$ 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 ± 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-1α expression was significantly lower in the NAC-hypoxia group than in the hypoxia group ($p < 0.001$) (Fig. 1**e**,**f**); the increase in HO-1 expression was significantly attenuated in the NAC-hypoxia group ($p = 0.002$) (Fig. 1**g**,**h**).

Analyses of $CoCl_2$-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. 2**a**). The morphological analyses using a phase contrast microscope (Fig. 2**b**) and crystal violet staining (Fig. 2**c**) showed that the cell populations decreased with exposure to increasing concentrations of $CoCl_2$. The live and dead assays showed that live cells (green) decreased and dead cells (red) increased after exposure to increasing concentrations of $CoCl_2$ (Fig. 2**d**).

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. 2**e**). 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. 2**f**).

Analyses of $CoCl_2$-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 µmol/L $CoCl_2$ group than in the normoxia group and the other $CoCl_2$ 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. 3**c**). Expressions of cleaved caspase-3 and of cleaved PARP-1 significantly increased in 1,000 µmol/L $CoCl_2$, as compared with the normoxia group ($p < 0.001$) (Fig. 3**d**-**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. 3**g**,**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. 3**i**-**k**).

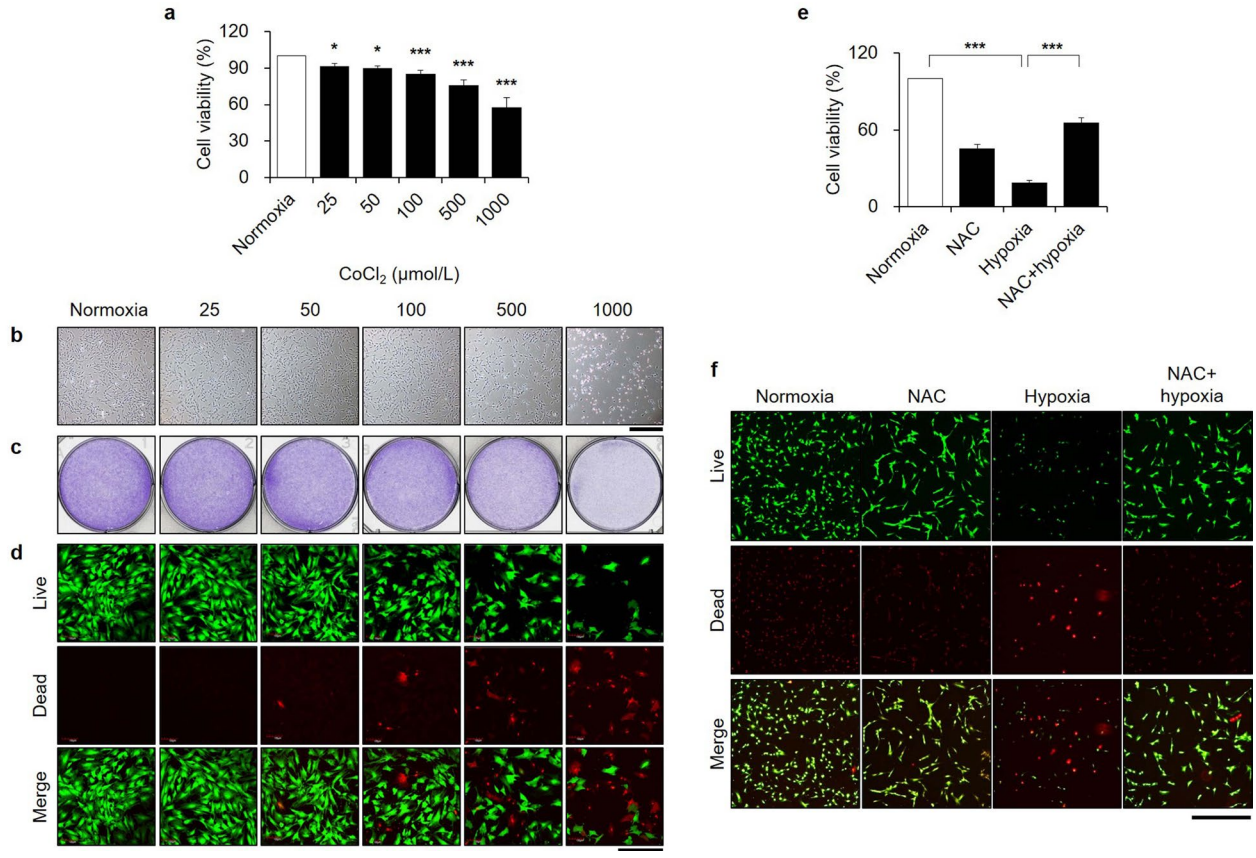

Fig. 2. Analyses of $CoCl_2$-induced cytotoxicity and of suppressive effects of NAC on that cytotoxicity. (**a**) Cell viability when cells were exposed to $CoCl_2$ decreased in a concentration-dependent manner ($p < 0.001$). The morphological analyses were performed by (**b**) a phase contrast microscope, (**c**) crystal violet staining and (**d**) live and dead assay. Results showed that the live-cell populations were decreased by exposure to $CoCl_2$ 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 ± SD, $n = 5$, * $p < 0.05$, *** $p < 0.001$. (**b**) Scale bar: 300 µm. (**d**,**f**) Scale bar: 500 µm.

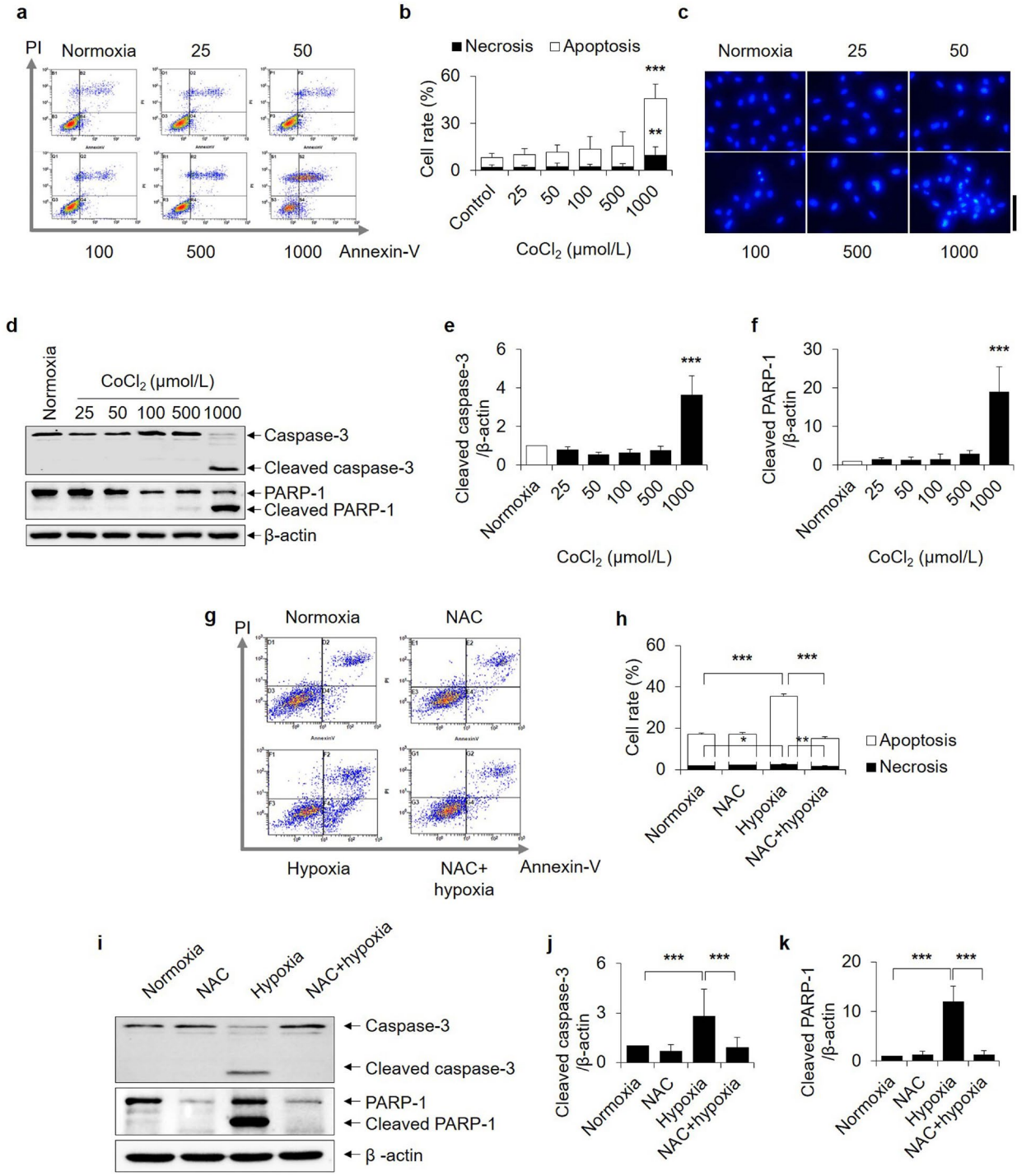

Fig. 3. Analyses of $CoCl_2$-induced apoptosis and of suppressive effects of NAC on that apoptosis. (**a**,**b**) According to the FACS analyses using annexin V/PI staining, the apoptosis rate was significantly higher in the 1,000 μmol/L $CoCl_2$ group than in the normoxia group ($p < 0.001$). (**c**) DAPI staining showed that cells with DNA fragmentation were more numerous in the 1,000 μmol/L $CoCl_2$ 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 $CoCl_2$ 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 ± SD, $n = 5$, *** $p < 0.001$. (**c**) Scale bar: 250 μm.

Analyses of $CoCl_2$-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 $CoCl_2$ study groups than in the normoxia group (Fig. 4**a**,**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. 4**c**). 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. 4**d**,**e**). The confocal microscope analyses confirmed that intracellular ROS production was markedly lower in the NAC-hypoxia group than in the hypoxia group (Fig. 4**f**).

Analyses of $CoCl_2$-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 $CoCl_2$ study group than in the normoxia group ($p < 0.001$) (Fig. 5**a**,**b**). The expression of MMP-2 was significantly higher in the 500 and 1,000 μmol/L $CoCl_2$ study groups than in the normoxia group ($p < 0.001$) (Fig. 5**c**,**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. 5**e**,**f**) and MMP-2 ($p = 0.001$) (Fig. 5**g**,**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 $CoCl_2$ 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.

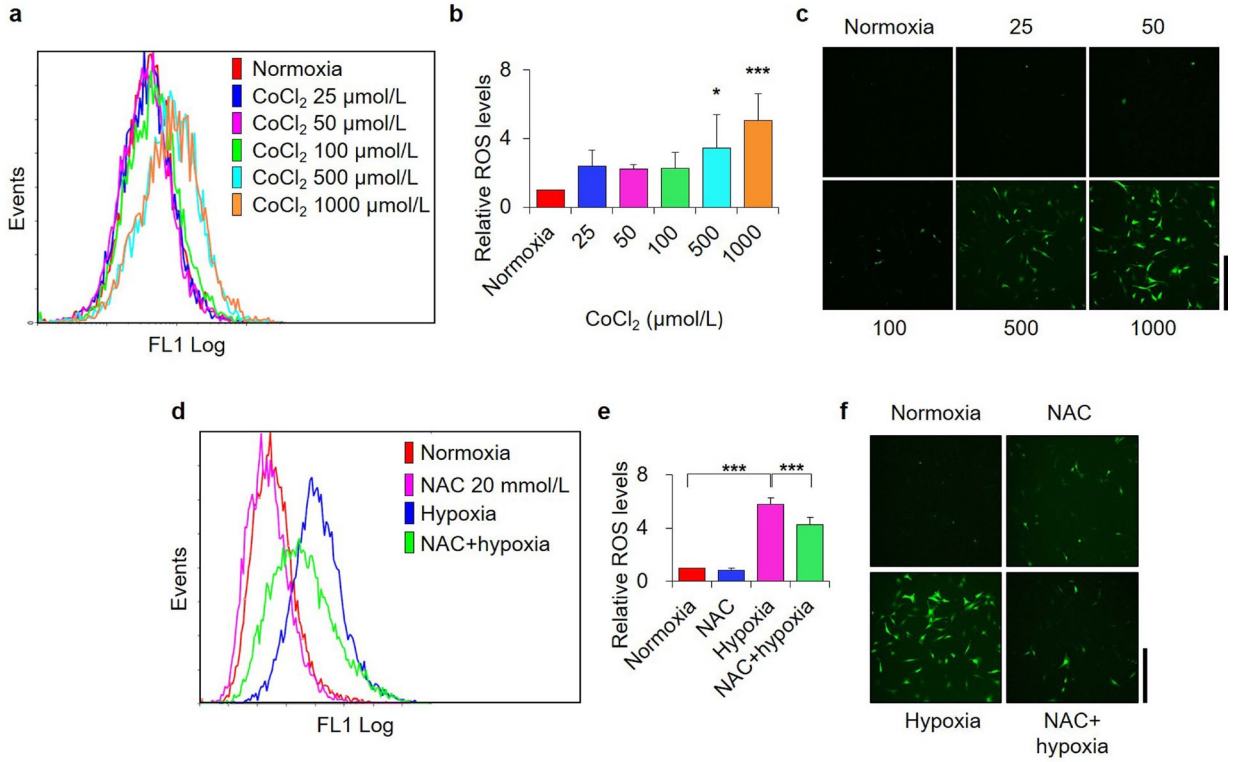

Fig. 4. Analyses of $CoCl_2$-induced intracellular ROS production and of suppressive effects of NAC on that production. (**a**,**b**) FACS analyses indicated that the levels of intracellular ROS production were significantly higher in the 500 μmol/L ($p = 0.027$) and 1,000 μmol/L ($p < 0.001$) $CoCl_2$ 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 $CoCl_2$ 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 ± SD, $n = 5$, * $p < 0.05$, *** $p < 0.001$. (**c**,**f**) Scale bar: 500 μm.

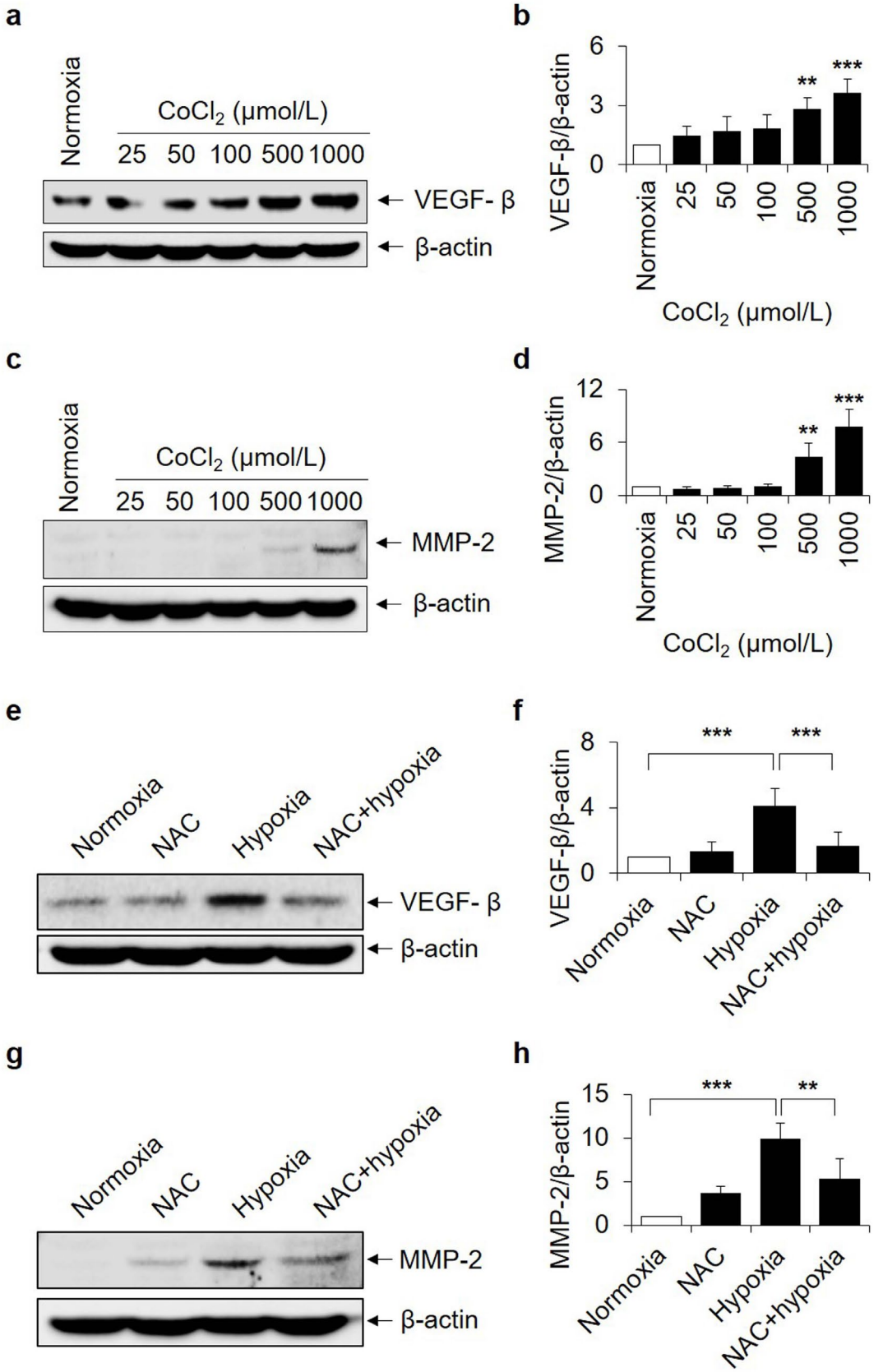

Fig. 5. Analyses of $CoCl_2$-induced VEGF-β and MMP-2 expressions and of suppressive effects of NAC on those expressions. (**a**,**b**) VEGF-β expression was significantly higher in the 500 and 1,000 μmol/L $CoCl_2$ groups than in the normoxia group ($p < 0.05$). (**c**,**d**) MMP-2 expression was significantly higher in the 500 and 1,000 μmol/L $CoCl_2$ 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 ± 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 μmol/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 μmol/L $CoCl_2$ or to 1 % oxygen has the effect on HeLa cells of inducing HIF-1α (Triantafyllou *et al.*, 2006). 24 h exposure to 25-1,000 μmol/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-1α 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-1α was induced by relatively high concentrations of $CoCl_2$ and particularly by 500 μmol/L and 1,000 μmol/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 μmol/L $CoCl_2$. Several studies have reported increases in HIF-1α expression induced by different concentrations of $CoCl_2$, exposure times and cell types, respectively: 500 μmol/L, 4h, human endothelial cells (Kim *et al.*, 2006); 100-,000 μmol/L, 12 h, HepG2 cells (Liu *et al.*, 2015); 600 μmol/L, 12 h, cardiac myocytes (Mao *et al.*, 2013); 400 μmol/L, 48 h, human periodontal ligament cells (Song *et al.*, 2012); 100 μmol/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 μmol/L and 1,000 μmol/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-1α (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; Konttinen *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-1α, 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-1α 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-1α 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 $CoCl_2$-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 $CoCl_2$, 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 $CoCl_2$; NAC, an ROS scavenger, inhibited hypoxia-induced RCF apoptosis by inhibiting ROS production.

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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.