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 (CoCl₂); 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 ≤ 0.001). ROS were involved in hypoxic RCF apoptosis induced by CoCl₂; 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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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 % O₂), physioxia or physiosis (~ 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 neovascularisation 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 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 CoCl$_2$ (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 10% 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 xg 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 x 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 x 10$^4$ 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 (40x 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 x 10$^4$ 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 x 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.
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 CoCl₂ or to NAC and CoCl₂, 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 CoCl₂ or to NAC and CoCl₂, 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 CoCl₂ or to NAC and CoCl₂, 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 × 10⁵ RCFs were seeded in each well of a 6-well cell culture plate, incubated overnight and exposed to CoCl₂ or to NAC and CoCl₂, 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 × 10⁵ 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 CoCl₂ or to NAC and CoCl₂, 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 CoCl₂-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 CoCl₂ 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 μmol/L CoCl₂ study groups was significantly higher than in the normoxia group (p ≤ 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 ≤ 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. 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 µmol/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 cleaved PARP-1 significantly increased in 1,000 µmol/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 casapse-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 ± 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 ± 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. 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 ± SD, n = 5, ** p < 0.05, *** p < 0.001.
Several CoCl₂ 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 CoCl₂ 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 CoCl₂ 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 CoCl₂ or to 1 % oxygen causes decreased PDX phosphorylation in HepG2 cells (Borcar et al., 2013). CoCl₂ treatment has been reported to up-regulate HIF-1 transcription factor and HO-1 in many cell types (Ameresi 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 CoCl₂ increases expressions of the hypoxic markers HIF-1α and HO-1 (Ameresi 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 CoCl₂, and particularly by 500 µmol/L and 1,000 µmol/L CoCl₂, ROS production, rates of apoptosis as well as expressions of caspase-3, PARP-1, VEGF and MMP-2 were also significantly increased by 1,000 µmol/L CoCl₂. Several studies have reported increases in HIF-1α expression induced by different concentrations of CoCl₂, exposure times and cell types, respectively: 500 µmol/L, 4 h, human endothelial cells (Kim et al., 2006); 100-1,000 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 CoCl₂ (relatively high concentrations) to induce hypoxia. Additionally, because CoCl₂ 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 CoCl₂ 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). CoCl₂-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 CoCl₂-induced hypoxia increased ROS production. A previous study reported that CoCl₂-induced hypoxia promoted apoptosis in PC12 cells, through ROS production accompanied by AP-1 activation (Zou et al., 2001). CoCl₂-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, CoCl₂-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 CoCl₂-induced hypoxia promotes apoptosis.

In the present study, CoCl₂-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; Sadaghiannoo et al., 2017). MMP’s 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 collagen 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 CoCl₂-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). CoCl₂-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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**References**


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.


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.