

The oxidative effect of Hyperbaric Oxygen Therapy on Human retinal pigment epithelium cells

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Abstract

Background: Hyperbaric oxygen (HBO) therapy has been widely used in various diseases, which is considered safe and effective. Whereas recent studies discovered that HBO therapy could result in oxidative damage to tissues. The goal of our study was to investigate the oxidative effect of hyperbaric oxygen therapy on human retinal pigment epithelium (RPE) cells. **Method:** Human RPE cells (ARPE-19) were cultured in vitro, and divided into normoxic group (incubated with DMEM/F12 broth) and hypoxic group (incubated with DMEM/F12 broth containing 200 μ mol/L CoCl₂) randomly. The experimental groups were exposed to 100% pure oxygen under different pressures (0.15MPa, 0.2MPa, and 0.25MPa) for 60 and 90 minutes thrice, with 24 hours interval. Then the cell viability, 8-OHdG expression and hOGG1 expression of RPE cells were detected by MTT assay, immunocytochemistry (ICC) and western blot separately. **Result:** After HBO exposure, cell proliferation decreased, 8-OHdG and hOGG1 expression increased in normoxic RPE cells compared with control group, whereas in hypoxic RPE cells, cell proliferation increased, 8-OHdG and hOGG1 expression decreased compared with hypoxic control group. **Conclusion:** HBO therapy could suppress the cell proliferation and cause oxidative DNA damage of RPE cells in normoxic status. Conversely, in hypoxic status, HBO therapy could promote the proliferation and ameliorate oxidative DNA damage of human retinal pigment epithelium cells. Meanwhile, HBO therapy could trigger the oxidative DNA damage repair of RPE cells in both normoxic and hypoxic statuses.

Background

Hyperbaric oxygen (HBO) therapy is used as a medical treatment, where it delivers 100% pure oxygen at a level that is higher than the atmospheric pressure. HBO induces a state of hyperoxia, which is beneficial at a molecular, cellular and biochemical level, and leads to an increase of dissolved oxygen in the blood, which allowed oxygen to distribute throughout the whole body and maintained the tissues in a hyperoxygenated state.⁽¹⁾ This technology has been widely used in treatment of various diseases, especially for the management of conditions related to hypoxia, such as, decompression sickness, air embolism, carbon monoxide poisoning, enhance in healing, delay radiation injury. In addition, HBO has been considered to be effective for many eye diseases, including central/branch retinal artery occlusion, cystoid macular edema of vascular origin, orbital infections of mycotic and anaerobic origin, scleral necrosis, anterior segment ischemia and non-healing corneal edema.⁽²⁾ However, while increasing the dissolved oxygen in the blood, exposure to HBO results in an increase of reactive oxygen species (ROS) in tissues, which related to oxidative stress. The higher pressure, higher concentration and longer exposure time of HBO can cause the increasing production of ROS.⁽³⁾

ROS is produced in organisms as byproducts of aerobic metabolism, including superoxide ($\cdot\text{O}_2^-$), hypochlorous acid (HClO), hydroxyl ($\cdot\text{OH}$), and hydrogen peroxide (H_2O_2).⁽⁴⁾ Low concentration of ROS can defend against infectious organisms, induct mitogenic response and serve as signaling molecules in transduction cascades.⁽⁵⁾ In contrast, the imbalance between production and elimination of ROS, which caused by an excess of ROS, can lead to oxidative stress.⁽³⁾ Retina is susceptible to oxidative damage

due to its high metabolic activity under high level of oxygen consumption and polyunsaturated fatty acids.^(6, 7) Therefore, to many eye diseases including age-related macular degeneration, retinitis pigmentosa and diabetic retinopathy, oxidative stress is a contributing factor.⁽⁸⁾

HBO has been confirmed safe and efficient for many diseases. However, due to the toxicity of ROS, this therapy may damage the eyes, especially some unreasonable use of oxygen partial pressure, oxygen concentration and exposure time. Retinal toxicity from HBO has been investigated in many studies. Nachman-Cewner et al discovered a major loss of photoreceptor cells in the central retinas of mice exposure to HBO, but no effect on the peripheral retina. The degree of photoreceptor cell damage and cell death increased with duration of HBO exposure.⁽⁹⁾ Another study detected constriction of retinal arterioles and venules during HBO treatment, and the constriction was constant through the series of treatments.⁽¹⁰⁾ In newborn rats, a sustained HBO induced retinal vasoconstriction, which followed by a hypoxic-ischemic injury, might result in ROP development on return to air.⁽¹¹⁾ Whereas, in another study HBO treatment didn't cause ROP.⁽¹²⁾ The most common complication of HBO is nuclear cataract, which is proved to be associated with oxidative stress. Seven of 15 patients with clear lens before treatment developed nuclear cataract during HBO therapy.⁽¹³⁾ Schaal et al studied the effects of different combinations of oxygen concentration and pressure in an organ culture for 7 days on bovine lenses. They inferred that the longer the time of exposure and the higher the oxygen partial pressure, the more severe the changes observed in the lenses.⁽¹⁴⁾ Nevertheless, there are few studies about whether the HBO therapy would lead to oxidative damage to retina.

RPE cells locate between the photoreceptor layer and choroid, where is characterized by hyperoxia and high polyunsaturated fatty acid concentrations. Moreover, RPE cells play critical roles in phagocytosis of photoreceptor outer segments, formation of the blood-retinal barrier, transporting of nutrients and waste products, and protection against light and free radicals.⁽¹⁵⁾ Therefore, RPE is vulnerable to oxidative stress and plays a critical role in pathogenesis of many eye diseases. The goal of this study was to investigate the oxidative effect of HBO therapy on human RPE cells in both normoxic and hypoxic conditions.

Methods

1. Cell culture and treatment

The ARPE-19 cells were purchased from American Type Culture Collection. Cells were cultured in DMEM-F12 (Dulbecco's MEM: Ham's Nutrient Mixture F12,1:1 Mix) containing 100U/mL penicillin-streptomycin (1:1 Mix) and 10% fetal bovine serum, grown at 37°C in an atmosphere of 5% CO₂. The cells were divided into two experimental groups: normoxic group and hypoxic group which was mimicked by CoCl₂. For experiments, the cells, when they were in the exponential growth phase, were harvested and transferred to wells of 96-well plates, coverslips which had been put into 24-well plates and petri dishes. When the cells had cover about 80% of the well, petri dish and the coverslip, changed the broth into bovine serum free

DMEM-F12 for normoxic group and bovine serum free DMEM-F12 containing 200 μ mol/L CoCl₂ for hypoxic group, and treated for 12 hours. Then, the cells were exposed to HBO intervention of different pressure (0.15MPa, 0.2MPa, 0.25MPa) for different time (60min,90min).

2. HBO exposure method

The chamber was disinfected with ultraviolet light for 20 minutes, the surface was wiped down with 75% alcohol. The petri dishes were placed inside the chamber, which filled with 100% pure oxygen. Slowly increased the pressure to desired amount (0.15MPa, 0.2MPa, 0.25MPa), maintained for 60 minutes and 90 minutes separately, and then slowly decompressed. The cells were exposed thrice with a 24 hours interval.

3.MTT assay for cell viability

4. Immunocytochemistry assay for 8-OHdG

Exponentially growing APRE-19 cells in a coverslip, were washed with PBS twice and fixed with cold paraformaldehyde for 10 min. Then, the coverslips were treated with 3%H₂O₂ at 37 °C for 10 min, goat serum at room temperature for 15 min. After that, the cells were incubated with primary antibody: monoclonal mouse anti-human 8-OHdG antibody at 4 °C overnight, washed with PBS three times, and then incubated with secondary antibody for 15 min at room temperature and horseradish peroxidase for 15 min at room temperature. Finally, the coverslips were stained with diaminobenzidine (DAB) for 3-10 min, counterstained with hematoxylin, dehydrated and mounted on a glass slide. The images were taken by microscope. The stained samples were quantified by using Image-Pro Plus 4.5.1.

5. Western blot assay for hOGG1

Cells were harvested in PBS and lysed with cold lysis buffer. The total concentration of protein was detected by bicinchoninic acid assay. 20 μ g protein extracts were electrophoresed on a 10% polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) transfer membranes, which were initially blocked in 3% BSA for 1h. Then the blots were incubated overnight with the primary antibody: monoclonal mouse anti-hOGG1 (1:2000), mouse monoclonal anti- β -actin (1:5000). After that, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10000). The signals were detected by enhanced chemiluminescence. The signals were visualized by exposure to light sensitive films. Semiquantitation was determined by analyzing band intensity using the software of BandScan 5.0, and hOGG1 relative levels were normalized to β -actin values.

6. Statistical Analysis

All experiments were repeated three times. Statistical analyses were performed using the SPSS 19.0 software program. The results are expressed as mean \pm standard deviation (SD). The statistical significance of differences among groups was performed with one-way analysis of variance (ANOVA). The statistically significant difference was determined when $p < 0.05$.

Results

1. Cell viability/proliferation tested by MTT assay

For both 60 and 90 minutes with HBO intervention, the OD570 value decreased with all three different pressure respectively (0.15 MPa, 0.20 MPa, 0.25 MPa group vs. control, $P < 0.01$, in both 60min and 90min) in normoxic group (Fig1.a), and it decreased as the pressure increased with statistical significance different ($P < 0.01$). In hypoxic group (Fig1.b), the OD570 value increased with all three different pressure respectively (0.15 MPa, 0.20 MPa, 0.25 MPa group vs. CoCl_2 group, $P < 0.01$, in both 60min and 90min) and increased as the pressure increased with statistical significance different ($P < 0.01$).

2. The changes of 8-OHdG expression before and after ARPE-19 cells undergone HBO intervention

Before HBO intervention

8-OHdG mainly locates in cytoplasm, a small amount of localization in the nucleus, as shown in Fig2. Compared with the normoxic group, there was a significant increase expression of 8-OHdG in hypoxic group hypoxic group vs. normoxic group, $P < 0.01$, in both 60min and 90min (Fig3.a).

After HBO intervention (0.15MPa, 0.20MPa, 0.25MPa)

In normoxic group, for both 60 and 90 minutes with HBO intervention (Fig3.b), the expression of 8-OHdG all increased with all three different pressure (0.15 MPa, 0.20 MPa, 0.25 MPa group vs. control, $P < 0.01$, in both 60min and 90min), and it increased as the pressure increased ($P < 0.01$). In addition, there was an increase trend of 8-OHdG expression with an increase of duration. In hypoxic group (Fig3.c), a decrease of the 8-OHdG expression was detected with all three different HBO pressure intervention for both 60 and 90 minutes (0.15 MPa, 0.20 MPa, 0.25 MPa group vs. CoCl_2 group, $P < 0.01$, in both 60min and 90min). When comparing the 0.2MPa with 0.25MPa groups for 60min exposure, there was no significant difference between the two groups ($P > 0.05$). While the 8-OHdG expression decreased as the pressure increased ($P < 0.01$) for 90min exposure.

3. The changes of hOGG1 expression after ARPE-19 cells undergone HBO intervention

For both 60 and 90 minutes with HBO intervention, the expression of hOGG1 protein increased with all three different pressure respectively (0.15 MPa, 0.20 MPa, 0.25 MPa group vs. control, $P < 0.01$, in both 60min and 90min) in normoxic group Fig4.b, and it decreased as the pressure increased with statistical significance ($P < 0.01$). In hypoxic group, the hOGG1 protein expression increased with all three different pressure respectively (0.15 MPa, 0.20 MPa, 0.25 MPa group vs. CoCl_2 group, $P < 0.01$, in both 60min and 90min) for both 60 and 90 minutes with HBO intervention Fig4.c, and the hOGG1 protein expression increased as the pressure increased with statistical significance ($P < 0.01$).

Discussion

Oxidative stress caused by high concentration ROS can lead to damage to cell structures, including membrane lipids, proteins and DNA, particularly mitochondrial DNA. 8-OHdG, the DNA guanine base oxidation product, is considered to be one of the most abundant DNA lesions resulting from oxidative stress and is a biomarker of the oxidative DNA damage. It is highly mutagenic causing GC to TA transversions.^(16, 17) The DNA repair include base excision repair (BER), double strand break repair (DSBR), mismatch repair (MR) and nucleotide excision repair (NER). BER is the main pathway for repair of oxidative DNA damage. Human 8-OHdGuanine DNA glycosylase (hOGG1), an enzyme involving in BER, can specifically recognize and excise 8-OHdG.⁽¹⁸⁾

Mitochondria is a major producer of ROS, thus it is susceptible to direct injury of ROS.⁽¹⁹⁾ In this study, we mainly observed the oxidative DNA damage of RPE cells after HBO exposure. Our data showed that in the normal RPE cells, the level of 8-OHdG increased and the proliferation was suppressed after exposure to HBO, which are likely associated with oxidative damage. In addition, the damage was aggravated with increased duration and pressure of HBO. Conversely, HBO therapy could ameliorate oxidative DNA damage of RPE cells in hypoxic status. Meanwhile, the increased expression of DNA repair enzymes hOGG1 in the normoxic and hypoxic RPE cells demonstrated that HBO could induce the oxidative DNA damage repair.

Previous experiment demonstrated that in rats after inspired hyperoxia, the expression of DNA repair enzymes and the level of 8-OHdG in the rat lens increased.⁽²⁰⁾ In our study, in normoxic group, HBO could cause oxidative DNA damage to RPE cells, which may related to the generation of ROS. Meanwhile, the increased expression of hOGG1 in RPE cells after HBO therapy reflected the stimulation of oxidative DNA damage. Nevertheless, increased duration and pressure of HBO caused decreased expression of hOGG1, which may in connection with accumulation of ROS and damage in DNA leading to the decline in capability of repair of DNA. There is evidence to support that. Santos JH et al established a feed-forward cascade of ROS production and mtDNA damage, which demonstrated persistence of lesions in the mtDNA involving a drop in mitochondrial membrane potential, secondary ROS generation, and loss of repair capacity. In addition, the feed-forward cascade at last result in the cell apoptosis and death.⁽²¹⁾ As in our study, HBO suppressed the proliferation of RPE cells in normoxic status.

In our study, we used 200 μ mol/L CoCl₂ to mimic hypoxic status of RPE cells. CoCl₂, a chemical substance, is usually used to mimic hypoxia. On one hand, CoCl₂ can inhibit heme oxygenation by replacing Fe²⁺ in heme with Co²⁺ thus result in the hypoxic status of cells.⁽²²⁾ On the other hand, CoCl₂ can cause the stabilization of hypoxia-inducible factor- α (HIF- α) in cells, which can activate the expression of genes that contain a hypoxia response element.⁽²³⁾ Moreover, previous study detected that, after cells incubated with CoCl₂, the production of ROS increased.⁽²⁴⁾ The increasing expression of 8-OHdG in RPE cells treated with CoCl₂ in our study may involve in the generation of ROS.

In hypoxic group, HBO treatment ameliorated oxidative DNA damage of RPE cells. That may be concerned with the increased expression of hOGG1 in RPE cells. Furthermore, a number of experiments have proved that, HBO can upregulate antioxidant gene expression and enhance the activity of antioxidant enzymes in cells.⁽²⁵⁻²⁷⁾ There are some studies about preconditioning of HBO on mouse with focal cerebral ischemia, and they discovered upregulated HIF-1 α and erythropoietin in rats after HBO \square which induced ischemic tolerance and reduced infarct volume.^(28, 29)

In conclusion \square we have demonstrated that HBO treatment may lead to oxidative DNA damage in normal RPE cells, and there is an increase trend of the damage with the increase of duration and pressure. Meanwhile, HBO can protect from the oxidative damage in hypoxic PRE cells. These findings invite further exploration into the oxidative effect of HBO therapy on retina in vivo and suggest the reasonable and individual treatment applying in clinical work. Furthermore, antioxidant can be used during the HBO therapy to prevent from the oxidative damage.

Abbreviations

8-OHdG: 8-Hydroxy-2'-deoxyguanosine

CoCl₂: cobaltous chloride

HBO: hyperbaric oxygen

hOGG1: human 8-oxoguanine-DNA glycosylase

RPE: retinal pigment epithelium

ROS: reactive oxygen species

HIF- α : hypoxia-inducible factor- α

Declarations

[Ethics approval and consent to participate]

Not applicable

[Consent for publication]

Not Applicable

[Availability of data and materials]

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

[Competing interests]

The authors declare that they have no competing interests

[Funding]

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[Authors' contributions]

JLL designed the study protocol, conducted the study, analyzed the data and drafted the manuscript. ICC designed the study protocol and conducted the study. JL designed the study protocol and reviewed the manuscript. All authors read and approved the final manuscript.

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Figures

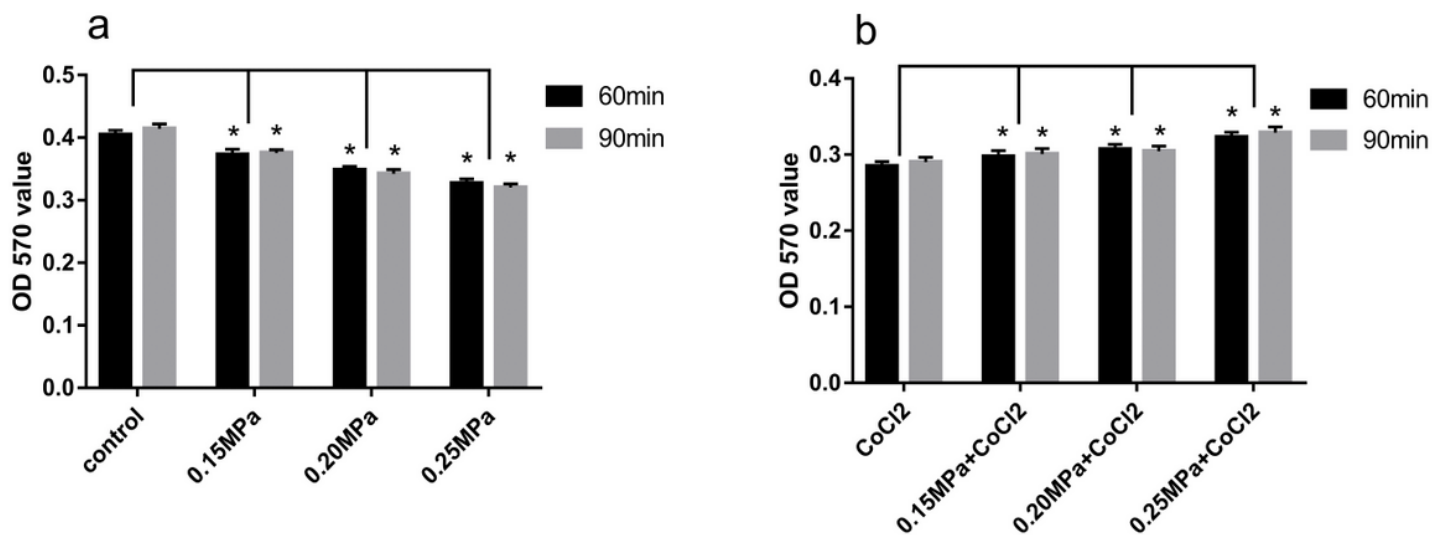


Figure 1

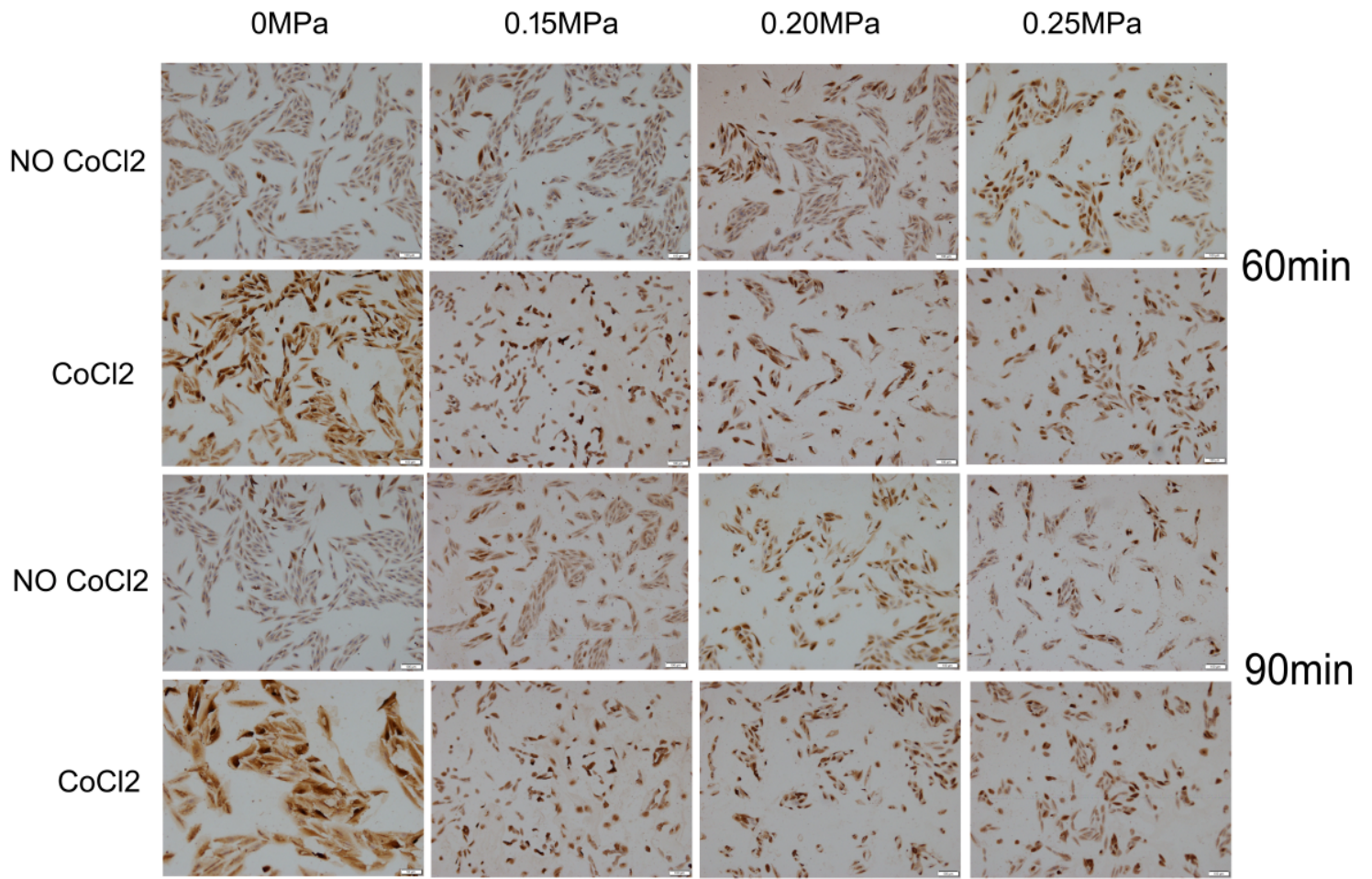


Figure 2

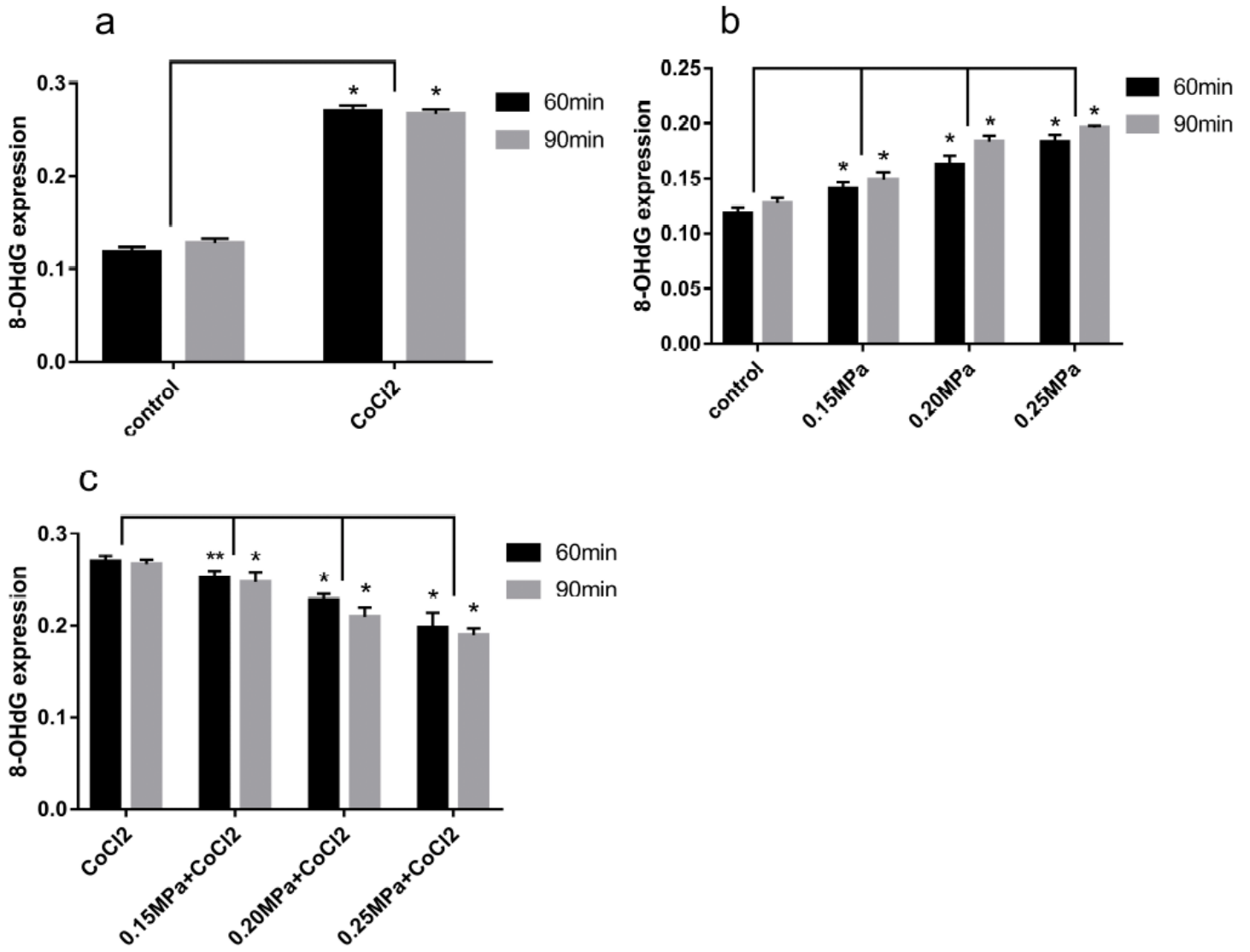


Figure 3

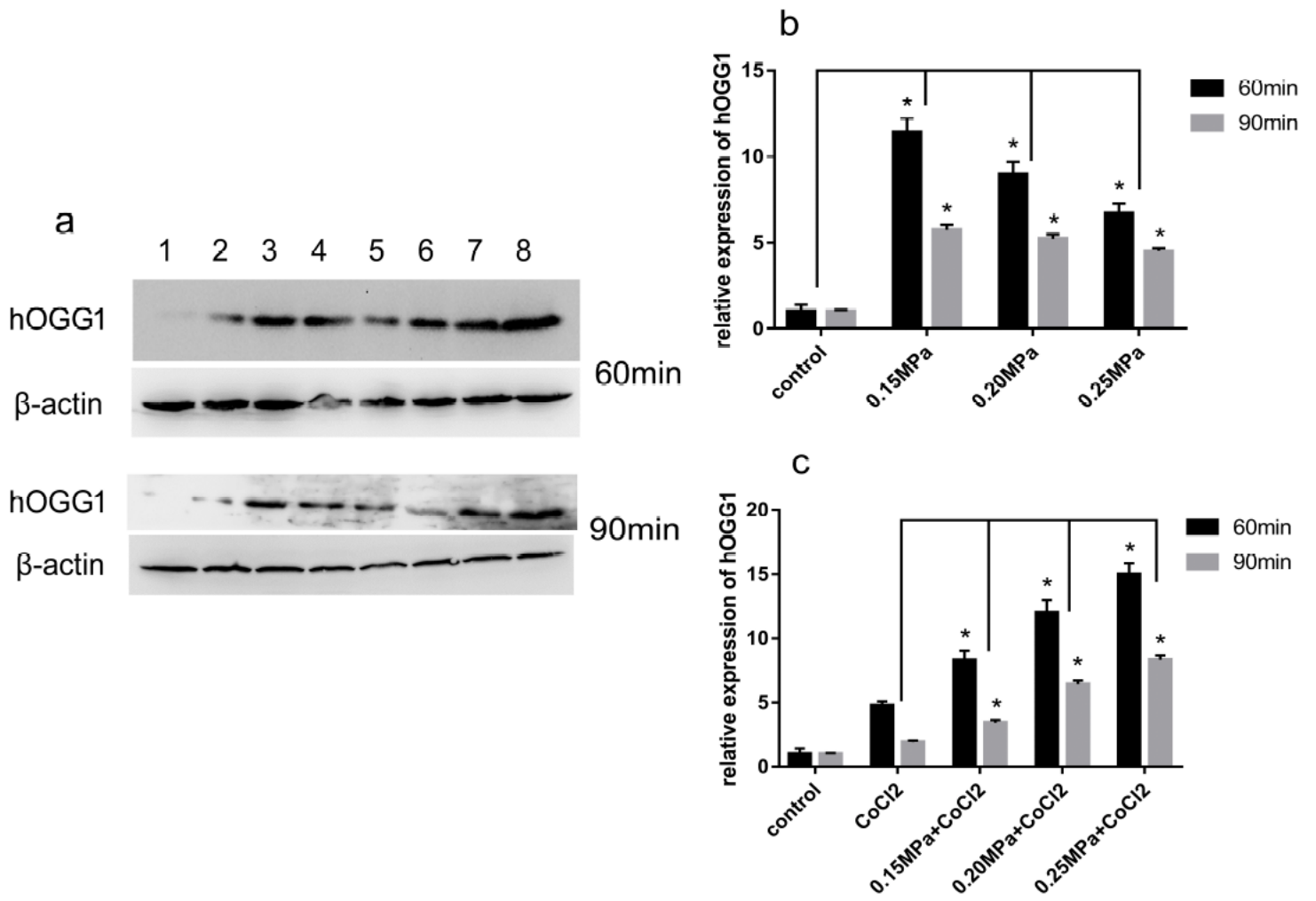


Figure 4