

# Reactive oxygen species induce injury of the intestinal epithelium during hyperoxia

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**Abstract.** Long-term therapeutic hyperoxia may exert serious toxic effects on intestinal epithelial cells *in vitro* and *in vivo*. The aim of the present study was to investigate the cause of this intestinal injury under conditions of hyperoxia. Caco-2 cells were treated with different concentrations of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and 85% hyperoxia for 24 h. Higher rates of injury of Caco-2 cells were observed in the hyperoxia and H<sub>2</sub>O<sub>2</sub> groups compared with the control group. The reactive oxygen species (ROS) level of the hyperoxia group was significantly higher compared with that of the 400  $\mu$ M H<sub>2</sub>O<sub>2</sub> group. The protein and gene levels of RelA, RelB, hypoxia-inducible factor-1 $\alpha$ , tumor necrosis factor- $\alpha$  and apoptosis signal-regulating kinase 1 were significantly higher in the hyperoxia and H<sub>2</sub>O<sub>2</sub> groups compared with those in the control group. In conclusion, during hyperoxia, intestinal epithelial cells were destroyed and the levels of ROS were increased. Therefore, ROS may play an important role in intestinal injury in a hyperoxic environment.

## Introduction

Hyperoxia is an indispensable therapeutic measure in the clinical intensive care of certain neonatal conditions; however, long-term therapeutic hyperoxia may exert serious toxic effects on several organs. Early exposure to hyperoxia results in progressive lung disease in premature infants (1), and hyperoxia may induce bronchopulmonary dysplasia,

inhibit cell proliferation and decrease cell viability (2). Hyperoxia-induced epithelial disruption, such as weakening of tight junctions and induction of a pro-inflammatory environment, is the main cause of hyperoxia-related organ injury (3). Recently, the clinical use of hyperoxia was reported to exacerbate organ injury by increasing the production of reactive oxygen species (ROS) (4). ROS include superoxide radicals and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), as well as its downstream products, such as peroxide and hydroxyl compounds. ROS affect cell viability, proliferation, differentiation, aging, apoptosis, and a number of physiological and pathological processes. ROS, primarily oxygen ions and H<sub>2</sub>O<sub>2</sub>, are produced in mitochondria (5). Under normal conditions *in vivo*, the generation and removal of ROS are in dynamic balance, and ROS are beneficial to the organism without causing harm. However, excess generation of ROS in epithelial cells is harmful. It has been reported that ROS coordinate the inflammatory response of tissues (6), and that H<sub>2</sub>O<sub>2</sub> significantly reduces the activities of superoxide dismutase, glutathione peroxidase, catalase and lipase (7). When the intestinal mucosal barrier is damaged, intestinal mucosal Th1 cytokines, such as tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1, stimulate epithelial cells to produce ROS. TNF- $\alpha$  is a central mediator of the inflammatory response (8). ROS are cytotoxic, but they may also act as second messengers in intracellular signal transduction and control the action of several signaling pathways, including mitogen-activated protein kinases (MAPKs) (9). Apoptosis signal-regulating kinase 1 (ASK1) is a MAPKKK of the c-Jun N-terminal kinase (JNK) and p38 MAPK pathways (9). ASK1 is a member of the MAPK family, and it plays an important role in the regulation of cellular apoptotic processes.

Additionally, MAPKs are known to be important for the transcriptional activation of nuclear factor (NF)- $\kappa$ B (10), which is a transcription factor that has been shown to be a central regulator of inflammatory response (11). The NF- $\kappa$ B family of transcription factors comprises 5 subunits, designated RelA (p65), RelB, c-Rel, p50 (NF- $\kappa$ B1) and p52 (NF- $\kappa$ B2). Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is a key regulatory factor in the induction of the hypoxia gene and the repair of the cellular oxygen environment. HIF-1 $\alpha$  may also provide information on hypoxia induction and oxidative stress (12). Long-term treatment with hyperoxia may exert serious toxic effects on intestinal epithelial cells *in vitro* and *in vivo* (2,13-16). Therefore, the aim of the present study was

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**Abbreviations:** ROS, reactive oxygen species; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; ASK1, apoptosis signal-regulating kinase 1; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; HIF-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$

**Key words:** hyperoxia, reactive oxygen species, hydrogen peroxide, nuclear factor- $\kappa$ B, apoptosis signal-regulating kinase 1, tumor necrosis factor- $\alpha$ , hypoxia-inducible factor-1 $\alpha$

to investigate the cause of intestinal injury during hyperoxia, and determine whether ROS generation is a main factor in intestinal injury under hyperoxic conditions.

## Materials and methods

**Cell culture.** The human colon adenocarcinoma cell line Caco-2 was obtained from the Cell Biological Institute of Shanghai, Chinese Academy of Sciences (Shanghai, China). The cells were grown at 37°C in air and 5% CO<sub>2</sub> in sterile basal medium/DMEM-H (Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Genview, Beijing, China), 1% L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B. The culture medium was changed every 2-3 days. Prior to treatment, the cells were plated with fresh medium (1×10<sup>6</sup> cells/ml) and cultured. On the second day after plating, Caco-2 cells were incubated with different concentrations of H<sub>2</sub>O<sub>2</sub> (100, 200 and 400 μM) and 85% oxygen [cells were cultured in three gas incubators (CBI60; Binder GmbH, Tuttlingen, Germany), with 85% oxygen and 5% CO<sub>2</sub>] for 24 h. A group of control cells received no treatment. Subsequently, the cultured cells were harvested, and the RNA and protein were extracted. All experiments were repeated 6-8 times.

**Cell survival detected by MTT assay.** Prior to treatment, cells were plated onto 96-well microtiter plates with fresh medium and cultured at 37°C in air and 5% CO<sub>2</sub> for 24 h. On the 2nd day after plating, the cells were treated with either 100, 200 or 400 μM H<sub>2</sub>O<sub>2</sub>, and 85% hyperoxia for 24 h. Cells without any treatment were used as the control group. Subsequently, the cells were treated with 20 μl MTT for 4 h at 37°C. The reactions were stopped by adding DMSO and the absorbance of each well at 450 nm was determined. Each sample was tested 6-8 times.

**Determination of intracellular ROS level.** Cells were plated at the same cell density in a culture flask. Cells without any treatment were used as the control group. Cells were treated with H<sub>2</sub>O<sub>2</sub> (100, 200 and 400 μM) or 85% hyperoxia exposure for 24 h. ROS kits (E004; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used to measure ROS levels (including O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>), according to the manufacturer's instructions. Dichloro-dihydro-fluorescein diacetate (DCFH-DA; 10 μM) was added to the cells and incubated for 30 min at 37°C. The cells were then digested and suspended. The cell suspensions were centrifuged at 1,000 × g for 10 min and washed twice with phosphate-buffered saline (PBS). The cells were collected after centrifugation for fluorescence detection. Flow cytometry (FACSCalibur; Becton-Dickinson, San Jose, CA, USA) was used to measure fluorescence intensity. The positive area of DCFH-DA was ROS fluorescence intensity.

**Immunohistochemistry analysis.** The cells on coverslips that were treated with 100, 200 and 400 μM H<sub>2</sub>O<sub>2</sub>, or 85% hyperoxia for 24 h were fixed with 4% paraformaldehyde. The cells were then treated with 10% goat serum for 30 min and incubated with mouse anti-human RelA (1:2,000; cat. no. SAB1404309) and rabbit anti-human RelB (1:2,000;

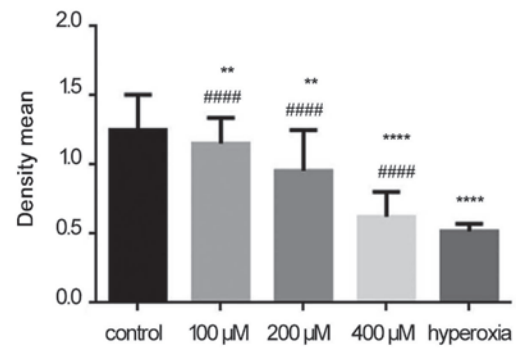


Figure 1. Survival of Caco-2 cells exposed to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hyperoxia. Compared with the control group, the cell survival rates in the hyperoxia and H<sub>2</sub>O<sub>2</sub> groups were significantly lower. The survival rates of the H<sub>2</sub>O<sub>2</sub> groups were higher compared with that of the hyperoxia group. The results are presented as mean ± standard deviation. \*\*P<0.01 and \*\*\*\*P<0.0001 compared with the control group; ####P<0.0001 compared with the 85% hyperoxia group, unpaired Student's t-test, n≥6.

cat. no. SAB4300501) (both from Sigma-Aldrich, Merck KGaA) (the antibody was diluted with PBS and 5% bovine serum albumin) overnight at 4°C, then incubated with a secondary antibody [biotin-labeled goat anti-mouse IgG (cat. no. SP-9002); biotin-labeled goat anti-rabbit IgG, (cat. no. SP-9001); ZSGB-BIO, Beijing, China] for 40 min at 37°C. Finally, the cells were stained by diaminobenzidine counterstained with hematoxylin. The primary antibody was replaced with PBS as a negative control. The median absorbance values of RelA and RelB were determined using image analysis software (Prism; Shanghai, China) after scanning.

**Protein determination.** The BCA Protein Assay kit (cat. no. P0013C; Beyotime Institute of Biotechnology, Shanghai, China) was used to determine protein concentrations in Caco-2 cells, according to the manufacturer's instructions.

**Western blot analysis.** Proteins (40 ng) extracted from Caco-2 cells were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were then incubated with Tris-buffered saline-Tween-20 containing 5% skimmed milk for 2 h at room temperature. The membranes were incubated overnight at 4°C with mouse anti-human RelA (1:1,000; cat. no. SAB1404309) and rabbit anti-human RelB (1:1,000; cat. no. SAB4300501) (both from Sigma-Aldrich, Merck KGaA), rabbit anti-human HIF-1α (1:1,000; cat. no. ab51608) and rabbit anti-human TNF-α (1:1,000; cat. no. ab6671) (both from Abcam, Cambridge, UK), rabbit anti-human ASK1 (1:1,000; cat. no. SAB1306399; Sigma-Aldrich, Merck KGaA), or GAPDH (1:10,000; cat. no. KC-5G5; Kangcheng Bioengineering Co., Shanghai, China), respectively. The membranes were then incubated with the appropriate secondary antibodies (1:5,000; peroxidase-conjugated goat anti-mouse IgG (cat. no. ZB-5305) and peroxidase-conjugated goat anti-rabbit IgG (cat. no. ZB-5301); ZSGB-BIO). Finally, the membranes were incubated with the SuperEnhanced chemiluminescence (Applygen Technologies Inc., Beijing, China) and the images were scanned by C300

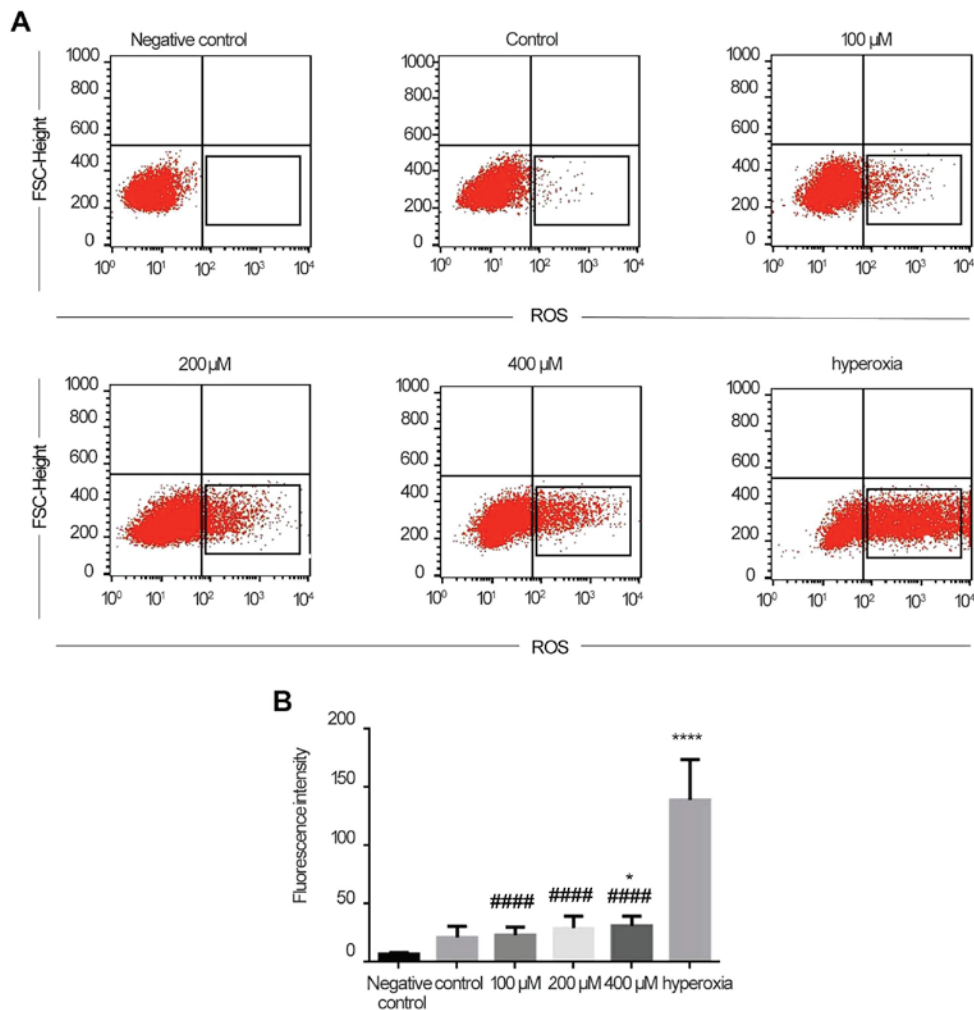


Figure 2. Levels of reactive oxygen species (ROS) in Caco-2 cells in a hyperoxic environment and different concentrations of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). (A) Flow cytometry scatter diagrams. (B) Levels of ROS in Caco-2 cells. The control group exhibited a small amount of fluorescence. The fluorescence intensity increased with increasing concentrations of H<sub>2</sub>O<sub>2</sub>. In the hyperoxia group, the fluorescence intensity was stronger. The results are presented as mean  $\pm$  standard deviation. \*P<0.05 compared with the control group; \*\*\*\*P<0.0001 compared with the 85% hyperoxia group, unpaired Student's t-test, n $\geq$ 6.

(Azure Biosystems, Inc., Dublin, CA, USA). Image-Pro Plus 6.0 software was used to analyze densitometry for RelA, RelB, HIF-1 $\alpha$ , TNF- $\alpha$  and ASK1 protein levels normalized to GAPDH.

**Quantitative polymerase chain reaction (qPCR) analysis.** Total RNA was extracted from Caco-2 cells using an RNA Mini kit (RR047A; Takara Biotechnology Co., Ltd., Dalian, China). cDNA was synthesized using 100 ng RNA (RR420A; Takara Biotechnology Co., Ltd.). qPCR was performed using the LightCycler<sup>®</sup> 480 Real-Time PCR system (Roche Applied Science, Mannheim, Germany). The primers for RelA, RelB, HIF-1 $\alpha$ , TNF- $\alpha$  and ASK1 were as follows: RelA forward, 5'-GGAGCACAGATACCACCAAGA-3' and reverse, 5'-CGGAGTCCTTCTCTACAAG-3'; RelB forward, 5'-TGTGGTGAGGATCTGCTTCCAG-3' and reverse, 5'-GGCCGCTTTCCTTGTTAATTC-3'; HIF-1 $\alpha$  forward, 5'-GCAGCAACGACACAGAACT-3' and reverse, 5'-AGCGGTGGGTAATGGAGAC-3'; TNF- $\alpha$  forward, 5'-GGCGTGAGCTGAGAGATAA-3' and reverse, 5'-GTGTGGGTGAGGAGCACAT-3'; ASK1 forward, 5'-TTCACACAAAACGGATGTAACATT-3' and reverse,

5'-CCTAAACAGTTATGGTCACATTTTGG-3'; and GAPDH forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-TGGTGAAGACGCCAGTGA-3'.

The primers and fluorescent probes for RelA, RelB, HIF-1 $\alpha$ , TNF- $\alpha$ , ASK1 and the internal reference (GAPDH) were purchased from Takara Biotechnology Co., Ltd. The PCR conditions were as follows: A preliminary cycle at 95°C for 10 sec, followed by 45 cycles at 95°C for 5 sec and 60°C for 20 sec, followed by 1 min at 60°C and 5 sec at 95°C. The efficiency of amplification for each target gene (GAPDH) was confirmed to be 100% in the exponential phase of PCR. The mRNA levels were normalized to GAPDH mRNA according to the following formula: Active levels of pIgR mRNA =  $2^{-(C_{pIgR} - C_{GAPDH})} \times 100\%$ . The levels of mRNA in the Caco-2 cells exposed to hyperoxia were compared with those of the control group.

**Statistical analysis.** For each experiment, at least 6 generations of each group were tested. The data from all groups were reported as the means  $\pm$  standard deviations. The t-test was used to determine significant differences between treatment groups. P<0.05 was considered to indicate a statistically significant difference.

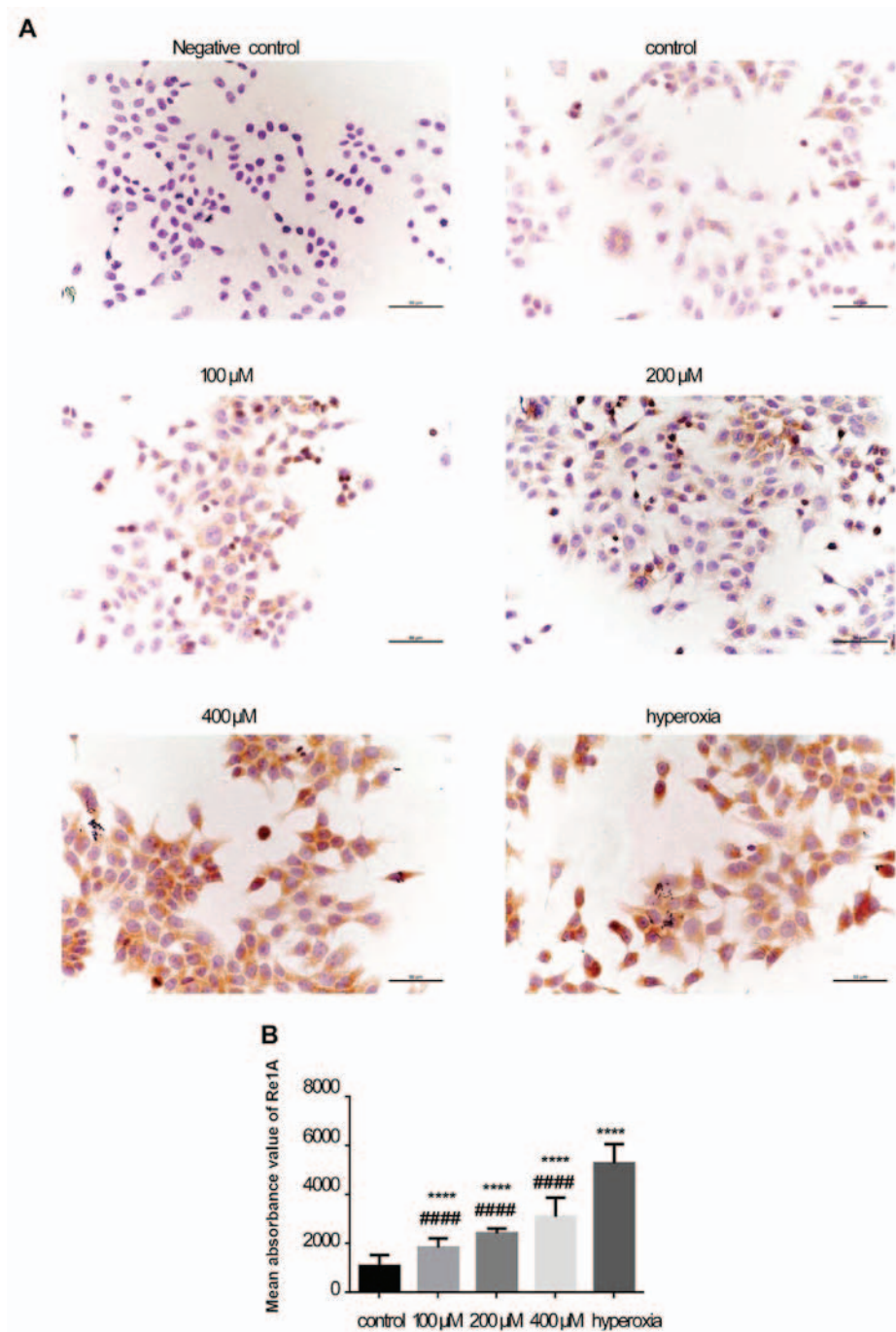


Figure 3. ReIA immunochemistry staining of Caco-2 cells treated by hyperoxia and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). (A) ReIA staining. Magnification, x400. (B) Integrated optical density of ReIA. In the control group, ReIA staining was located in the cytoplasm. In the  $\text{H}_2\text{O}_2$  and hyperoxia groups, ReIA staining was observed in the cytoplasm and nucleus. Compared with the control group, the integrated optical density of ReIA was increased in the  $\text{H}_2\text{O}_2$  group and notably increased in the hyperoxia group. The results are presented as mean  $\pm$  standard deviation. \*\*\*\* $P$ <0.0001 compared with the control group; ### $P$ <0.0001 compared with the hyperoxia group, unpaired Student's *t*-test,  $n \geq 6$ .

## Results

**Cell survival.** The cytotoxic effects of  $\text{H}_2\text{O}_2$  are often attributed to ROS release. We investigated the cytotoxicity of  $\text{H}_2\text{O}_2$  in Caco-2 cells. As shown in Fig. 1, the survival rates of cells exposed to 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  ( $P$ <0.01), 200  $\mu\text{M}$   $\text{H}_2\text{O}_2$  ( $P$ <0.0001), 400  $\mu\text{M}$   $\text{H}_2\text{O}_2$  ( $P$ <0.0001), and hyperoxia ( $P$ <0.0001), were significantly lower compared with the control group. However, the survival rates of the 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  ( $P$ <0.0001), 200  $\mu\text{M}$   $\text{H}_2\text{O}_2$  ( $P$ <0.0001), and 400  $\mu\text{M}$   $\text{H}_2\text{O}_2$  ( $P$ <0.0001) groups were

higher compared with the hyperoxia group. These findings indicate that ROS generation may be responsible for cell injury during hyperoxia.

**ROS detection in Caco-2 cells.** As shown in Fig. 2A, there was no fluorescence in the negative control, whereas the control group exhibited a small amount of fluorescence. The fluorescence intensity increased with increasing concentrations of  $\text{H}_2\text{O}_2$ . In the hyperoxia group, the fluorescence intensity was stronger, and significantly higher compared with that in the



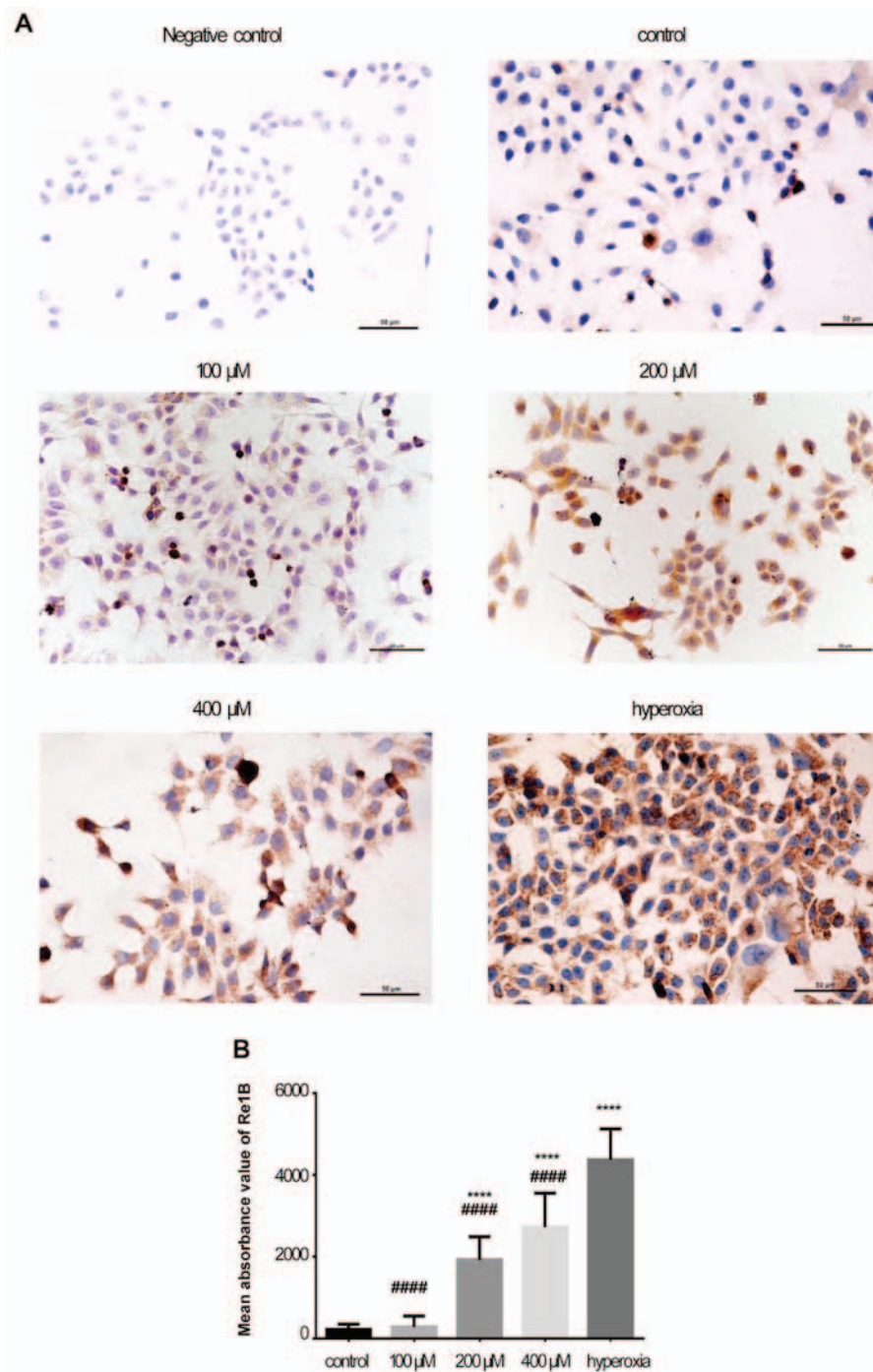


Figure 4. RelB immunocytochemistry staining of Caco-2 cells treated by hyperoxia and hydrogen peroxide ( $H_2O_2$ ). (A) RelB staining. Magnification, x400. (B) Integrated optical density of RelB. In the control group, RelB staining was observed in the cytoplasm. In the  $H_2O_2$  and hyperoxia groups, RelB staining was observed in the cytoplasm and nucleus. Compared with the control group, the integrated optical density of RelB was increased in the  $H_2O_2$  group and notably increased in the hyperoxia group. The results are presented as mean  $\pm$  standard deviation. \*\*\*\* $P$ <0.0001 compared with the control group; #### $P$ <0.0001 compared with the hyperoxia group, unpaired Student's t-test,  $n \geq 6$ .

$H_2O_2$  ( $P$ <0.0001) and control ( $P$ <0.0001) groups (Fig. 2B). Therefore, it was initially concluded that Caco-2 cells produced more ROS under hyperoxic conditions rather than in a normal environment.

**Immunohistochemical staining of RelA and RelB.** As shown in Figs. 3A and 4A, RelA and RelB staining was mostly localized in the cytoplasm in the control group. In the  $H_2O_2$  and hyperoxia groups, RelA and RelB staining was observed in the

cytoplasm and nucleus. The expression of RelA was higher in the 100  $\mu$ M  $H_2O_2$  ( $P$ <0.0001), 200  $\mu$ M  $H_2O_2$  ( $P$ <0.0001), and 400  $\mu$ M  $H_2O_2$  ( $P$ <0.0001) groups compared with the control group (Fig. 3B). The expression of RelB was higher only in the 200  $\mu$ M  $H_2O_2$  ( $P$ <0.0001) and 400  $\mu$ M  $H_2O_2$  ( $P$ <0.0001) groups (Fig. 4B). In the hyperoxia group, the morphology of the Caco-2 cells changed and the expression of RelA and RelB was higher ( $P$ <0.0001) compared with that in the control and  $H_2O_2$  groups.

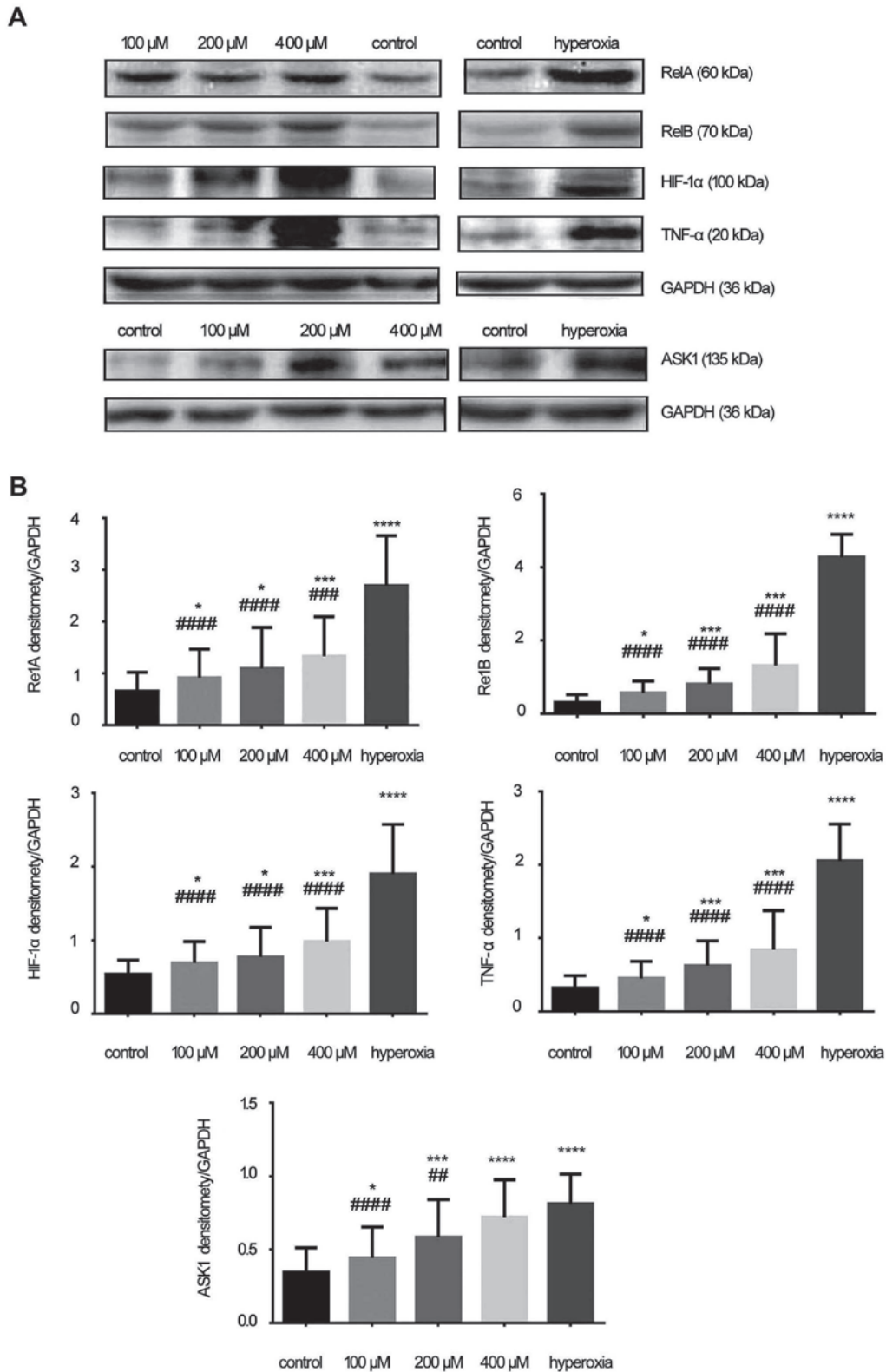


Figure 5. Expression of RelA, RelB, hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and apoptosis signal-regulating kinase 1 (ASK1) proteins. (A) The expression of RelA (60 kDa), RelB (70 kDa), HIF-1 $\alpha$  (100 kDa), TNF- $\alpha$  (20 kDa) and ASK1 (135 kDa). (B) Densitometric analysis of RelA, RelB, HIF-1 $\alpha$ , TNF- $\alpha$  and ASK1. In the hydrogen peroxide ( $H_2O_2$ ) group, expression of these proteins was higher compared with the control group, while in the hyperoxia group the proteins expression of RelA, RelB, HIF-1 $\alpha$ , TNF- $\alpha$  and ASK1 were significantly increased. The results were presented as mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$  compared with the control group; ### $P < 0.001$  and #### $P < 0.0001$  compared with the hyperoxia group, unpaired Student's  $t$ -test,  $n \geq 6$ .

*Protein expression of RelA, RelB, HIF-1 $\alpha$ , TNF- $\alpha$  and ASK1.*  
The protein expressions of RelA (60 kDa), RelB (70 kDa), HIF-1 $\alpha$  (100 kDa), TNF- $\alpha$  (20 kDa) and ASK1 (135 kDa)

were measured (Fig. 5A). The protein expressions of RelA, RelB, HIF-1 $\alpha$ , TNF- $\alpha$  and ASK1 increased with increasing concentrations of  $H_2O_2$ ; their levels in the hyperoxia group

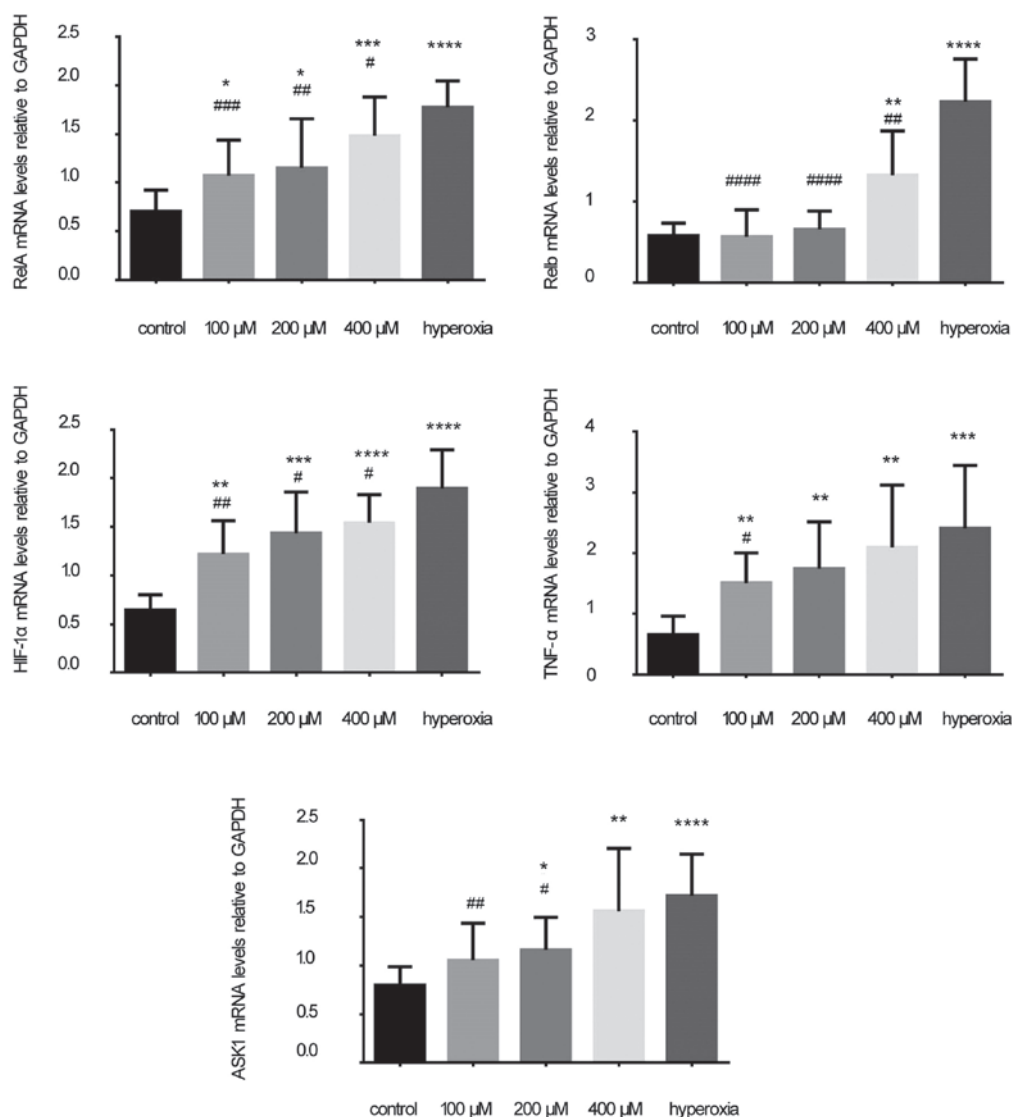


Figure 6. RelA, RelB, hypoxia-inducible factor-1α (HIF-1α), tumor necrosis factor-α (TNF-α) and apoptosis signal-regulating kinase 1 (ASK1) mRNA levels. The levels of RelA, RelB, HIF-1α, TNF-α and ASK1 mRNAs from the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) groups and hyperoxia group were significantly increased compared with the control group. The results are presented as mean ± standard deviation. \*P<0.05, \*\*\*P<0.001 and \*\*\*\*P<0.0001 compared with the control group; #P<0.05, ##P<0.01 and ####P<0.0001 compared with the hyperoxia group, unpaired Student's t-test, n≥6.

were higher compared with those in the H<sub>2</sub>O<sub>2</sub> and control groups. Densitometry analysis (RelA, RelB, HIF-1α, TNF-α and ASK1 densitometry/GAPDH densitometry) revealed that the intensity of all these proteins in the hyperoxia group was significantly higher compared with that in the control and H<sub>2</sub>O<sub>2</sub> groups (P<0.05; Fig. 5B).

**mRNA levels of RelA, RelB, HIF-1α, TNF-α and ASK1.** In the hyperoxia group, the mRNA levels of RelA, RelB, HIF-1α, TNF-α and ASK1 were significantly increased than in the control and H<sub>2</sub>O<sub>2</sub> groups (P<0.001; Fig. 6). The mRNA levels of RelA, RelB, HIF-1α, TNF-α and ASK1 also increased with increasing concentrations of H<sub>2</sub>O<sub>2</sub> (P<0.01; Fig. 6).

## Discussion

Cells are continuously exposed to ROS, which are generated by aerobic metabolism. Excessive generation of ROS causes severe damage to cells in the form of oxidative stress (17). ROS

are byproducts of oxygen metabolism, which plays a crucial role in cell signaling and in maintaining the balance of the organism (18). In complex biological systems, physiologically produced ROS act as second messenger signals that affect cell proliferation and differentiation (19). ROS play an important role as signaling intermediates that induce a variety of cellular responses, such as proliferation, differentiation, senescence and apoptosis (17). As the levels of ROS increase, the rate of apoptosis gradually increases (20). ROS include superoxide radicals, H<sub>2</sub>O<sub>2</sub>, hydroperoxyl radicals and hydroxyl radicals. H<sub>2</sub>O<sub>2</sub> significantly increases free radicals in the cells, resulting in critical DNA damage (7), and H<sub>2</sub>O<sub>2</sub>-induced oxidative stress increases cell apoptosis (21). During normal metabolism, ROS signal cells to stimulate proliferation or to induce cellular damage, depending on their concentration (22).

In the present study, the results of the MTT assay demonstrated that the amount of apoptotic cells significantly increased during hyperoxia and at higher doses of H<sub>2</sub>O<sub>2</sub>. The results of flow cytometry revealed that the levels of ROS under

conditions of hyperoxia were significantly higher compared with those under normal conditions. Initially, on the basis of these findings, it was concluded that hyperoxia may stimulate organs to produce more ROS (23). Hyperoxia may also cause oxidative damage and induce production of ROS in the mitochondria and expression of antioxidant proteins (24), and may also increase the antioxidant response to ROS (25). Therefore, increasing levels of ROS is the first step in the series of reactions that constitute intestinal inflammation (18). Hyperoxia-induced epithelial disruption is associated with tight junction weakening and the development of a pro-inflammatory environment (3).

ROS induce the production of cytokines during inflammation (26). Subsequently, TNF- $\alpha$  may lead to cell damage by inducing the production of intracellular mitochondrial ROS, thereby increasing intestinal inflammation and damage (27). High expression of TNF- $\alpha$  in intestinal epithelial cells suggests that TNF- $\alpha$  levels may be indicative of intestinal damage (27). Therefore, TNF- $\alpha$  expression was detected in the intestinal epithelium. The results revealed that the damage to the intestinal epithelial cells that was induced by H<sub>2</sub>O<sub>2</sub> or hyperoxia increased the expression of TNF- $\alpha$  at the protein and gene levels. TNF- $\alpha$  activates MLK3 and leads to JNK activation *in vivo* (28). As a member of the MAPK family, ASK1 is present in several physiological and pathological processes. ASK1 is required for apoptosis induced by oxidative stress and TNF (29). In normal cells, activation of ASK1 is strictly controlled by phosphorylation and dephosphorylation of serine/threonine. Since it may be activated by several stress and inflammatory factors, the overexpression of ASK1 may induce cell apoptosis through the MAPK signaling pathway, and ASK1 is activated in cells treated with TNF- $\alpha$  (30,31). ROS also directly or indirectly affect signaling molecules, such as protein kinases (MAPK), which cause oxidative damage to organs (1,32). During oxidative stress, H<sub>2</sub>O<sub>2</sub> catalyzes the phosphorylation of ASK1, and then activates the downstream JNK/p38 pathway, promoting cell apoptosis (33). Therefore, the role of ASK1 in the MAPK family was specifically investigated. Our results demonstrated that, with the increased oxidative stress induced by hyperoxia, the expression of ASK1 significantly increased at the protein and gene levels.

TNF- $\alpha$  mediates activation of the NF- $\kappa$ B survival pathway in the cytoplasm (34). The NF- $\kappa$ B pathway regulates genes encoding acute reactive proteins, cytokines, cell adhesion molecules, and immune regulatory molecules in inflammation. NF- $\kappa$ B is an important signaling molecule, and it is involved in physiological responses induced by hyperoxia in different cell types and tissues (35). NF- $\kappa$ B may also regulate inflammatory response and epithelial cell damage or death (36). It has also been reported that the activation of oxidative stress and NF- $\kappa$ B plays an important role in the pathogenesis of inflammatory bowel disease (IBD) (37). In the present study, RelA and RelB expression in the NF- $\kappa$ B signaling pathway were significantly increased at both the protein and gene levels during hyperoxia and at higher doses of H<sub>2</sub>O<sub>2</sub>. This proves that intestinal inflammation may occur in hyperoxia and that ROS play an important role in inflammation induced by hyperoxia.

NF- $\kappa$ B is involved in stimulating HIF-1 $\alpha$  mRNA expression. There is significant evidence on the need for an intact NF- $\kappa$ B pathway for proper oxygen- and ligand-induced HIF activity (38). HIF-1 $\alpha$  is key to the pathogenesis of IBD, and

the role of HIF-1 $\alpha$  in immune cells is becoming increasingly important (39). Therefore, the protein and mRNA levels of HIF-1 $\alpha$  were measured. The results demonstrated that 85% hyperoxia and high concentrations of H<sub>2</sub>O<sub>2</sub> exerted similar effects in terms of activating the HIF signaling pathway. This proves that ROS play an important role during hyperoxia.

In conclusion, intestinal epithelial cells were destroyed and the levels of ROS increased during hyperoxia. Thus, ROS may play an important role in intestinal injury in a hyperoxic environment. In the future, further research focusing on the intestinal mechanisms of hyperoxic injury may reveal an even more important role of ROS. Novel strategies for treating hyperoxia-induced intestinal injury must also be investigated.

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