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Effect of hyperoxia on the intestinal IgA secretory component in neonatal rats and on intestinal epithelial cells *in vitro*

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Abstract

Oxygen therapy is essential for the treatment of some neonatal critical care conditions but its extrapulmonary effects have not been adequately investigated. We therefore studied the effects of various oxygen concentrations on intestinal epithelial cell function. In order to assess the effects of hyperoxia on the intestinal immunological barrier, we studied two physiological changes in neonatal rats exposed to hyperoxia: the change in intestinal IgA secretory component (SC, an important component of SIgA) and changes in intestinal epithelial cells. Immunohistochemistry and Western blot were used to detect changes in the intestinal tissue SC of neonatal rats. To detect intestinal epithelial cell growth, cells were counted, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Giemsa staining were used to assess cell survival. Immunohistochemistry was used to determine SC expression. The expression of intestinal SC in neonatal rats under hyperoxic conditions was notably increased compared with rats inhaling room air ($P < 0.01$). *In vitro*, 40% O₂ was beneficial for cell growth. However, 60% O₂ and 90% O₂ induced rapid cell death. Also, 40% O₂ induced expression of SC by intestinal epithelial cells, whereas 60% O₂ did not; however, 90% O₂ limited the ability of intestinal epithelial cells to express SC. *In vivo* and *in vitro*, moderate hyperoxia brought about increases in intestinal SC. This would be expected to bring about an increase in intestinal SIgA. High levels of SC and SIgA would serve to benefit hyperoxia-exposed individuals by helping to maintain optimal conditions in the intestinal tract.

Key words: Intestinal epithelial cells; Cell growth; Secretory component; Hyperoxia; Secretory IgA

Introduction

Oxygen therapy (continuously inhaling a high concentration of oxygen, resulting in hyperoxia) is essential for the treatment of some neonatal critical care conditions, but its extrapulmonary effects have not been adequately investigated. We, therefore, studied the effects of various oxygen concentrations on intestinal epithelial cell functions. The lungs are the primary targets of hyperoxia. Pathophysiological manifestations of pulmonary oxygen toxicity have been extensively studied in neonatal rats (1), such as hyperoxia-induced pulmonary injury, retinopathy (2,3) and neurological disorders (4). There have been some reports about the effect of hyperoxia on intestinal epithelial cells *in vitro* (5-7). *In vivo*, hyperoxia creates intestinal serosal and submucosal vasodilation, vascularization, and growth retardation in neonatal rats (8). Because the intestinal villi and mucosa continue to grow and differentiate after birth, the long-term effects of postnatal hyperoxia exposure may

be most relevant to the human infant (9).

Secretory IgA (SIgA) plays a critical role as an immunological barrier in the intestine. It is the predominant intestinal immunoglobulin, and acts as the first line of defense for the intestinal mucosa. SIgA adheres mainly to bacteria or viruses on the intestinal epithelial surface. It guarantees both immune exclusion and neutralization of translocated bacteria, thus preserving the integrity of the intestinal barrier by preventing bacterial-induced inflammation. The anti-inflammatory properties of SIgA are well-known (10-12). SIgA secreted into the intestinal mucus is an important regulatory factor for maintaining the intestinal barrier (13). Also, SIgA may weaken bacterial translation (14-16). The secretory component (SC) in association with polymeric IgA (pIgA) forms SIgA. The SC is secreted by intestinal epithelial cells and is responsible for the transcytosis of newly synthesized pIgA and the formation of SIgA (17).

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SC is the extracellular component of the poly Ig receptor (pIgR), which is expressed on the intestinal epithelial surface. SC prevents proteolytic degradation of SIgA, and also increases the viscosity of the mucus enough to allow mucosal adhesion and mucosal immunological defense. SC is a nonspecific scavenger of microorganisms and plays a key role in the protection of the intestinal mucous membrane, and also in limiting the inflammation process there (18). SC is important for defense against bacteria (19-22) and parasites (23). Moreover, SC can play a critical role in the immunologically mediated neutralization of cholera toxin (24). We have found that ileal SIgA was remarkably increased in neonatal rats under conditions of hyperoxia (25). In the present study, neonatal rats inhaled continuously high concentrations of oxygen (hyperoxia) for 14 and 21 days. We then assayed for changes in the expression of intestinal SC induced by the hyperoxia. We used cell counting, along with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Giemsa staining, to detect the growth, cell division, and survival of intestinal epithelial cells treated with hyperoxia, and immunohistochemistry to detect SC expression in intestinal epithelial cells treated with hyperoxia. Our aim was to gain a better understanding of the effects of hyperoxia on the immunological barrier of the gut.

Material and Methods

Animal model

Wistar rats were obtained from the China Medical University (Shenyang, China). The study was approved by the animal Ethics Committee of China Medical University. Timed pregnant Wistar rats were transported to our laboratory one week before delivery and were individually housed in transparent cages. Room temperature, humidity, and daily light-dark cycles were automatically controlled. All pregnant rats delivered within a 12-h period and their pups were randomly divided into an air-inhaling group (exposed to room air, N = 20) and hyperoxia group (90-95% O₂, N = 40). All pups were individually weighed and numbered on the back with a permanent ink marker and then placed in environmentally controlled chambers. In the hyperoxia group, oxygen was continuously delivered into sealed environmental chambers to achieve a constant concentration of 90-95% oxygen, as confirmed by an oxygen monitor (OM-25ME, MAXTEC, USA) daily. The air-inhaling group was exposed to similar environmental conditions, except for inhaling room air. Oxygen and room air were filtered through natrolite to keep the CO₂ concentration below 0.5% (as confirmed using a DapexGas Monitor, USA). To equalize the effect of nursing on the pups' development, at 24 h the mothers were cross-fostered to the opposite litter (the air-inhaling group to the hyperoxia group and vice versa). The dams were switched every 24 h throughout the protocol. Each pup was weighed daily. Pups were sacrificed on the 14th and 21st day and their intestinal tissues were dissected.

Immunohistochemistry for the detection of intestinal tissue SC

Paraffin-embedded sections of the intestinal tissues of rats were deparaffinized, rehydrated, and incubated with rabbit anti-rat SC (Bethyl, USA). The slides were rinsed three times with PBS between incubations, and sections were counterstained with hematoxylin. The primary antibody was replaced with PBS as a negative control. The median absorbance value of SC was determined with the Image analysis software (Shanghai, China) after scanning.

Western blot analysis of SC protein of intestinal tissues

Proteins extracted from intestinal tissues were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked with Tris-buffer containing 50 ng/L skim milk and probed with rabbit anti-rat SC antibody (Bethyl) or β -actin (Sigma, USA) followed by a peroxidase-conjugated secondary antibody. They were then incubated with an enhanced chemiluminescent substrate and exposed to X-OMAT film (Perkin Elmer, USA). Images were scanned and analyzed with the Tanon GIS-2020 software (Tanon, China).

Cell culture

The Caco-2 cell line was obtained from the Cell Biology Institute of Shanghai, China. Caco-2 cells were cultured on two 5-mm plastic Petri dishes at 37°C in air and 5% CO₂ in 1640 medium (Gibco, Invitrogen Co., USA), supplemented with 20% fetal calf serum (FCS), 100 μ g/mL streptomycin and 100 IU/mL penicillin, pH 7.4 (regulated with 3.7 g/L NaHCO₃). We selected the Caco-2 cell line because the Caco-2 cell monolayer is a model of intestinal epithelial cells, with the same enzymes, transporters, and morphology as intact human intestinal epithelial cells, with remarkable morphological and biochemical similarity to the columnar epithelium of the small intestine (26).

Growth curve constructed by counting cells

Prior to counting, cells were plated with fresh medium and cultured at 37°C in air and 5% CO₂ for 24 h. On the 2nd day after plating, all nonadherent cells were discarded and the remaining cells were equally divided into groups and gassed with 5% CO₂:40% O₂, 5% CO₂:60% O₂, or 5% CO₂:90% O₂. Then, cells were counted on the 1st, 2nd, 3rd, 4th, 5th, and 6th days. Each sample was tested six times.

MTT

Prior to treatment, cells were plated onto 96-well microtiter plates with fresh medium and cultured at 37°C in air and 5% CO₂ for 24 h. On the 2nd day after plating, all nonadherent cells were discarded and the remaining cells were equally divided into groups gassed with 5% CO₂:40% O₂, 5% CO₂:60% O₂, or 5% CO₂:90% O₂. Then, on the 3rd and 6th days, cells were

treated with 20 μL MTT for 4 h at 37°C. The reactions were stopped by adding DMSO. The absorbance of each well was determined at 450 nm. Each sample was tested six times.

Cell division experiment

Caco-2 cells were cultured on coverslips at 37°C in air and 5% CO_2 for 24 h. On the 2nd day, cells on coverslips were divided equally into groups gassed with 5% CO_2 :40% O_2 , 5% CO_2 :60% O_2 , or 5% CO_2 :90% O_2 . Then, on the 3rd and 6th days, cells were fixed on coverslips with methanol/glacial acetic acid (3:1) for 30 min and counterstained with Giemsa for 10 min. Mitotic cells were counted. The mitotic index was calculated as mitotic cells/total cell number. Each sample was tested six times.

Immunohistochemistry for the detection of SC expression by Caco-2 cells

Cells on coverslips were fixed with 4% paraformaldehyde and incubated with 3% H_2O_2 . Then, sections were treated with 10% goat serum for 30 min and incubated with goat anti-rat SC (Bethyl) overnight at 4°C. The sections were counterstained with hematoxylin. The primary antibody was replaced with PBS as a negative control.

Statistical analysis

Data are reported as means \pm SD. Statistical differences between treatment groups were determined by the *t*-test. Growth curves were calculated as the best fit for exponential curves, and ANOVA with Bonferroni's correction was used for coupled categories.

Results

Mortality rate and growth rate of neonatal rats exposed to hyperoxia

Weight loss was observed in neonatal rats after 7-10 days of hyperoxia exposure. The body weight of neonatal rats in the

hyperoxia group was lower than that of the air-inhaling group (Figure 1). The mortality of the neonatal rats in the hyperoxia group was $45.9 \pm 4.2\%$ at day 14 and $62.5 \pm 4.2\%$ at day 21. Weight gain and mortality of the air-inhaling group of neonatal rats were normal.

SC was enhanced in ileal tissue of neonatal rats exposed to hyperoxia

The expression of SC in the cytoplasm and on the membranes of intestinal epithelial cells was remarkably enhanced in the hyperoxia group compared to the air-inhaling group (Figure 2). In the hyperoxia group, the median absorbance values for SC were 6.5 ± 1.2 at day 14 and 7.0 ± 1.5 at day 21. In the air-inhaling group, the median absorbance

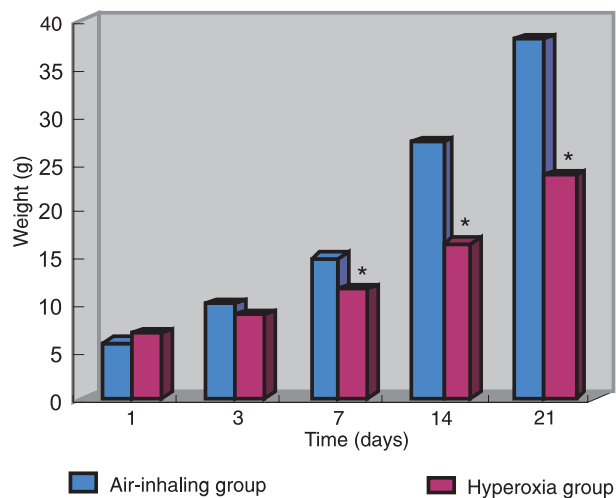


Figure 1. Weight of rats in the air-inhaling and hyperoxia groups. The body weight of the hyperoxia rats started to be lower than that of the air-inhaling rats on the 3rd day. From the 7th to the 21st day, the body weight of the hyperoxia rats was significantly lower compared to that of the air-inhaling group (* $P < 0.01$, *t*-test).

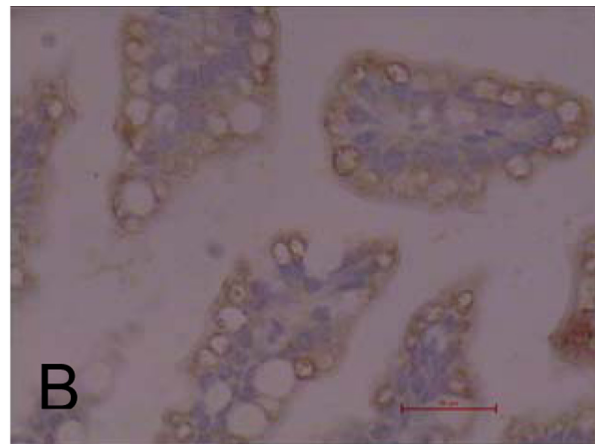
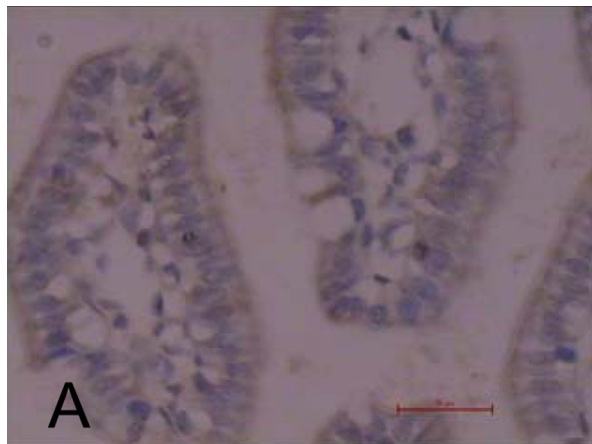


Figure 2. Staining of the secretory component (SC) in intestinal epithelial cells (40X). A, The SC of intestinal epithelial cells stained pale brown in the air-inhaling group and deep brown in the hyperoxia group (B).

values for SC were 3.1 ± 1.1 at day 14 and 3.2 ± 1.3 at day 21. Also, the median absorbance value for SC in the hyperoxia group was notably increased compared with the air-inhaling group ($P < 0.01$; Figure 3).

SC protein was increased in ileal tissue of neonatal rats exposed to hyperoxia

SC protein expression was increased in the hyperoxia group relative to the air-inhaling group. In the hyperoxia group, the relative expression rate of SC (absorbance value of SC/absorbance value of β -actin) was 0.919 ± 0.060 at day 14 and 0.934 ± 0.044 at day 21. In the air-inhaling group, the relative expression rate of SC (absorbance value of SC/absorbance value of β -actin) was 0.702 ± 0.032 at day 14 and 0.704 ± 0.022 at day 21. The relative expression rate of SC was notably increased in the hyperoxia group compared to the air-inhaling group at days 14 and 21 ($P < 0.01$; Figure 4).

Effect of hyperoxia on Caco-2 cell growth

The growth of Caco-2 cells was significantly affected by 60 and 90% O_2 , as shown in Figure 5. Growth in 40% O_2 was increased at day 2 and was exponentially increased at days 4 and 5. When the gas was changed to 60% O_2 , the growth rate decreased. Cells in 90% O_2 had arrested growth followed by rapid death. Significant differences in cell numbers were found between 21% O_2 and both 60 and 90% O_2 ($P < 0.001$). Significant differences in cell numbers were also found between 40% O_2 and both 60 and 90% O_2 ($P < 0.001$). There was also a significant difference in cell numbers for 60 and 90% O_2 ($P < 0.001$). These results suggest that cells can adapt to 40% O_2 , but 60 and 90% O_2 cause severe cell damage and eventually cell death.

Effect of hyperoxia on Caco-2 cell survival

MTT was used to detect cell survival. At day 3, cells that were cultured at 40 and 60% O_2 were unchanged in population, but at 90% O_2 , cells underwent rapid death ($P < 0.01$). At day 6, cells that were cultured at 40% O_2 increased in number ($P > 0.01$), but at 60 and 90% O_2 their numbers were rapidly decreased compared to cells cultured at 21% O_2 ($P < 0.01$; as shown in Figure 6). This showed that moderate oxygen induced intestinal epithelial cell growth.

Effect of hyperoxia on Caco-2 cell division

At day 3, the mitotic indices of cells grown in 40, 60 and 90% O_2 were significantly decreased compared to those grown in 21% O_2 . However, at day 6, the mitotic index of cells grown in 40% O_2 was increased compared with those grown in 21% O_2 . At 60% O_2 , the mitotic index was decreased, and some cell nuclei were larger than those seen under the 21% O_2 conditions. At day 6, cell splitting was not observed at 90% O_2 and the nuclei of living cells

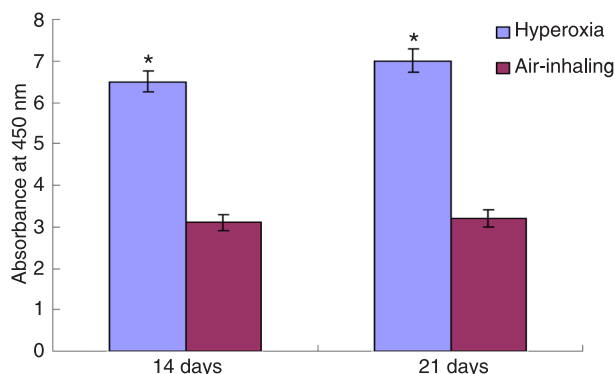


Figure 3. Absorbance of the intestinal secretory component (SC) in the hyperoxia and air-inhaling groups. The mean absorbance value of SC was notably increased in the hyperoxia group compared to the air-inhaling group (* $P < 0.01$, *t*-test).

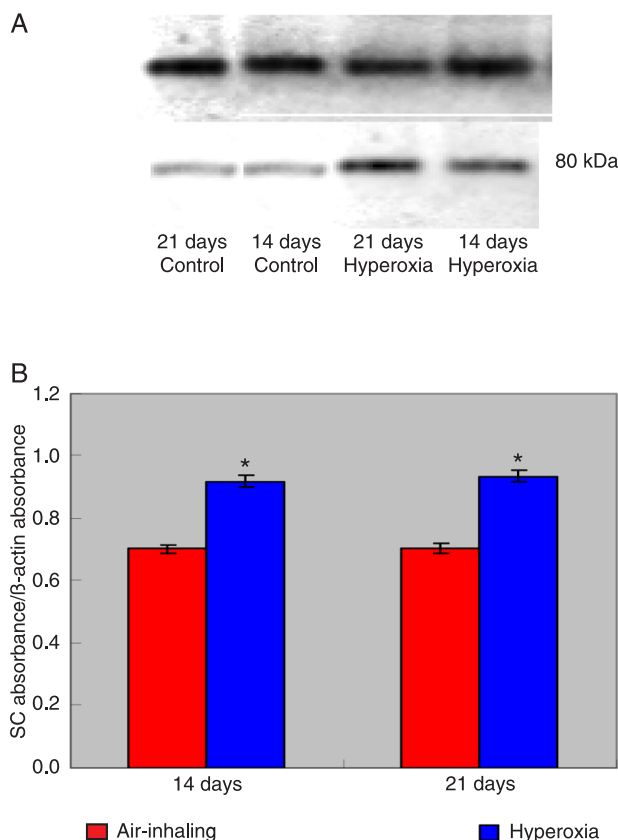


Figure 4. The intestinal secretory component (SC) protein of rats was notably increased in the hyperoxia group. A, SC protein was detected by Western blot. B, Densitometric analysis using image software. Absorbance value of SC/absorbance value of β -actin was notably increased in the hyperoxia group compared to the air-inhaling group (* $P < 0.01$, *t*-test).

were larger than those seen under 21% O₂ conditions (as shown in Figure 7).

Immunochemical analysis of the effects of hyperoxia on SC-positive cells

SC is mainly localized in the cytoplasm and cell mem-

branes of Caco-2 cells, as shown in Figure 8. SC-positive cells constituted about 0.1-0.5% of the population of cells grown in 21% O₂ and about 0.4-0.8% of the population of cells grown in 40% O₂. SC-positive cells constituted about 0.2-0.7% of the population of cells grown in 60% O₂. The expression of SC in Caco-2 cells was remarkably

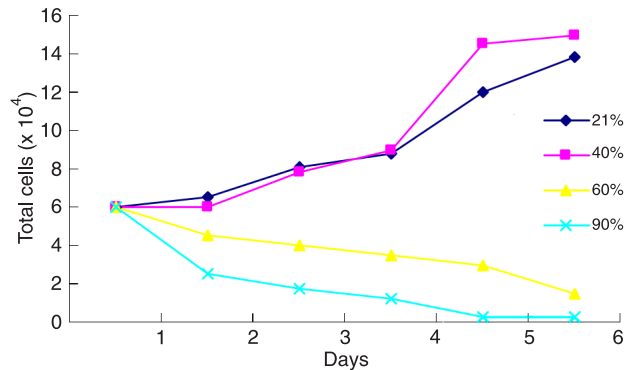


Figure 5. Influence of O₂ concentration on Caco-2 cell growth. Each data point represents the mean cell number of 5-6 plastic Petri dishes from each of six preparations of mixed Caco-2 cells. Cells were maintained in 21, 40, 60, and 90% O₂.

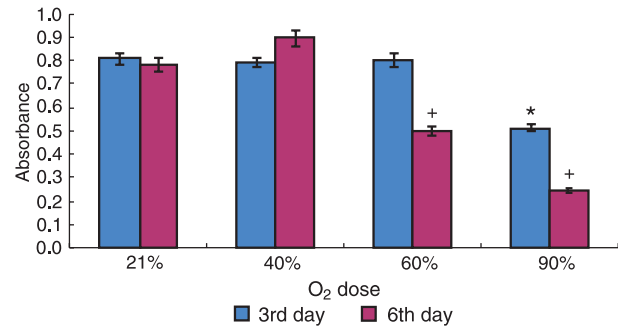


Figure 6. Effect of hyperoxia on Caco-2 cell survival. On the 3rd day, cells rapidly died in the presence of 90% O₂ (*P < 0.01, t-test) and on the 6th day, at 60 and 90% O₂ cell numbers were rapidly decreased compared to 21% O₂ (*P < 0.01, t-test). At 40% O₂, cell growth was good.

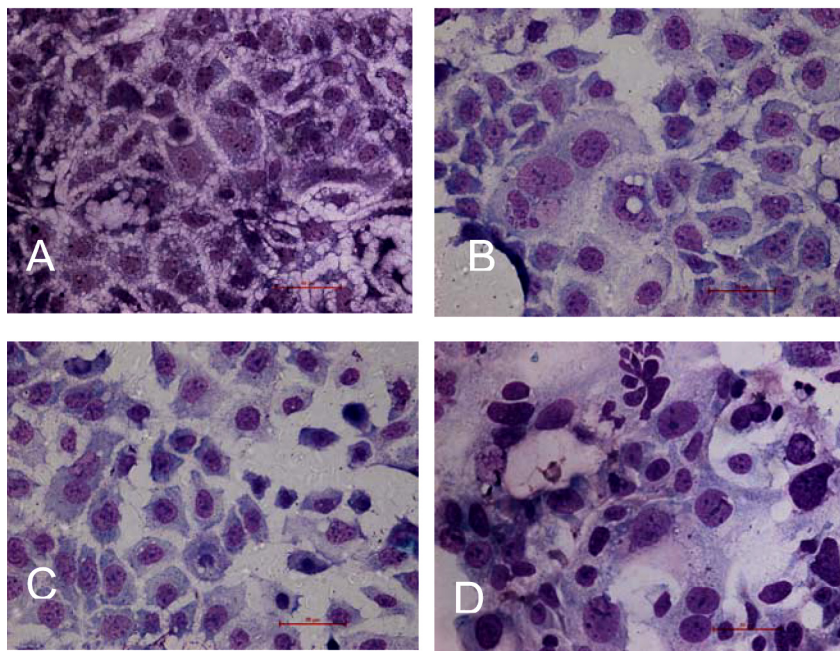


Figure 7. Effect of hyperoxia on Caco-2 cell division (40X). A, 21% O₂: cells distributed densely, some cells were splitting, and the mitotic index was 2.5%; B, 40% O₂: compared to 21% O₂, cell splitting was increased and the mitotic index was 3.3%; C, 60% O₂: compared to 21% O₂, cell splitting was decreased, the mitotic index was 1.3% and many larger cell nuclei were observed; D, 90% O₂: cell splitting was not observed and only larger nuclei were seen.

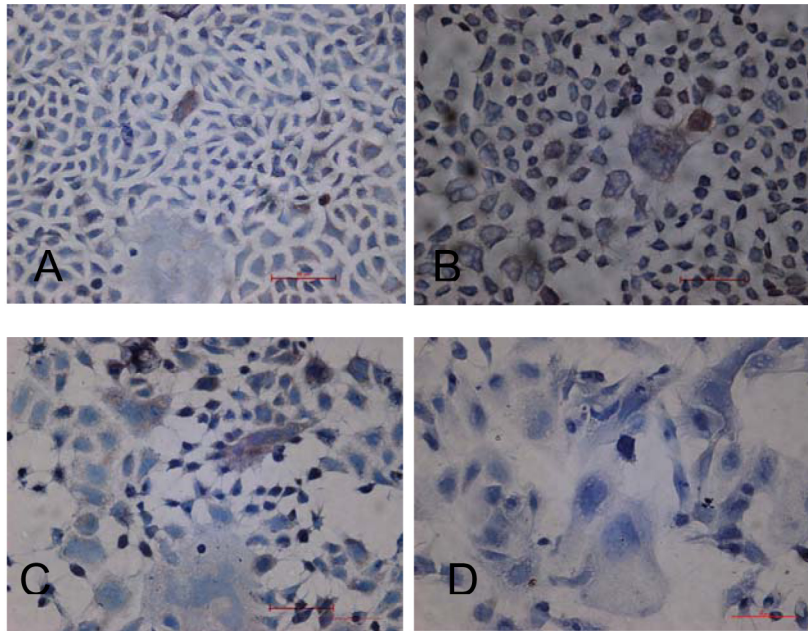


Figure 8. Effect of hyperoxia on the intestinal secretory component (SC) expression of Caco-2 cell (40X). *A*, The expression of SC in the cytoplasm and on the membranes of Caco-2 cell was pale brown. *B*, The expression of SC in the cytoplasm and on the membranes of Caco-2 cell was deep brown. *C*, The expression of SC in the cytoplasm and on the membranes of Caco-2 cell was brown. *D*, The expression of SC of Caco-2 cell was remarkably decreased. SC-positive cell was not seen in 90% O₂.

decreased as the percentage of oxygen was increased above 60%, and SC-positive cells were not demonstrable in cells grown in 90% O₂.

Discussion

The intestinal tract faces physiological challenges in the human body. It must provide ready access to ingested nutrients and also protect against food-borne microbes. This challenge is met by multiple specific and nonspecific host defenses that establish and maintain a selective intestinal barrier. This immunological intestinal barrier is critical for defense against intestinal microbes and their toxins.

Oxygen therapy, leading to hyperoxia, is a necessary method for the treatment of some critical care conditions. The high concentration of oxygen inhaled, however, may be both a friend and a foe of the body. An adequate oxygen supply serves to maintain the physical integrity of the mucosa, thus decreasing invasion of the lumen by microbes. An adequate supply of oxygen is also important for effective bactericidal function of resident neutrophils and other phagocytic cells in the gut (7). However, hyperoxia also enhances the inflammatory response in adult mice infected with influenza A virus (27). It has been proposed that normobaric hyperoxia might affect certain peripheral organs (kidney, ileum) (8). The thickness of the ileal mucosa of

neonatal rats under conditions leading to hyperoxia was significantly greater (9). Hyperoxia may affect the barrier function of the newborn rat's intestine, rendering it susceptible to bacterial insult (9). In this study, the expression of SC protein in neonatal rats under conditions leading to hyperoxia was increased compared to that of air-inhaling neonatal rats. The rise of SC may be due to the following causes: first, hyperoxia renders the intestinal mucosa of neonatal rats susceptible to bacterial insult. Bacterial invasion of the intestine stimulates intestinal epithelial cells to secrete more SC. Second, a direct effect of oxidative stress may induce changes in intestinal immunity cells, such as greater secretion of cytokines. Some cytokines regulate the expression of SC (28-30). Third, secondary systemic inflammation may be another cause of the rise of SC. These possibilities require further investigation. Increased SC expression is beneficial to the transcytosis of pIgA and the formation of SIgA. This is favorable to the defense against oxygen toxicity from hyperoxia and bacterial insults. Then, SC and SIgA both exert immunological functions in the exclusion and neutralization of bacteria.

In order to better understand the influence of hyperoxia on intestinal epithelial cells, Caco-2 cells were treated *in vitro* with 40, 60 and 90% O₂. Our results showed that Caco-2 cells rapidly grow at 40% O₂, but cell growth slows at 60% O₂ and rapid death occurs at 90% O₂. At 40% O₂, the cell

division rate reached 3.3%. At 60% O₂, the cells continued to divide, although at a reduced rate. However, at 90% O₂, there was both growth arrest and cell death. This demonstrates that severe hyperoxia may check cell growth and kill intestinal epithelial cells, which in turn would facilitate the invasion of the gut by bacteria. *In vitro*, the optimal concentration of inhaled oxygen (about 0.4-0.8% for SC-positive cells at 40% O₂ and about 0.2-0.7% for SC-positive cells at 60% O₂) induced intestinal mucus to secrete SC, but severe hyperoxia (90% O₂) limited the secretion of SC. However, *in vivo*, the expression of intestinal SC protein in neonatal rats induced by hyperoxia was increased. The reason for this may be that, *in vitro*, 90% O₂ acts directly on intestinal epithelial cells, but *in vivo*, 95% O₂ acts indirectly on the intestine. We speculated that sublethal O₂ exposure was associated with induction of antioxidant defense systems

and the change in the intestinal environment. The change in intestinal environment may lead to intestinal bacterial disorders (9), which in turn may induce the intestinal epithelial cells to secrete SC.

In vivo and *in vitro* increases in intestinal SC in response to exposure to moderate hyperoxia would be expected to cause an increase in intestinal SIgA. High levels of SC and SIgA are beneficial to maintaining the optimal state of the intestinal tract.

Acknowledgments

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