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The impact of hypoxic conditions on apoptosis and Toll-like receptor 4 expression in polymorphonuclear neutrophils

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Abstract

Many patients are admitted to the emergency department due to trauma. Trauma patients suffer from hypoxia due to massive hemorrhage, respiratory failure, and hypovolemic shock. Further damage is caused by reduced immune function and over-expression of inflammatory response. We conducted an experiment to determine the effects of hyperoxia and hypoxia on apoptosis and expression of Toll-like receptor 4 (TLR_4) in polymorphonuclear neutrophils (PMNs). Initially, the PMNs were placed in normoxic and hypoxic conditions, and these PMNs were divided into two groups as stimulated or not stimulated with lipopolysaccharide (LPS). Levels of apoptosis and TLR_4 expression were measured under normoxic, hypoxic, and hyperoxic conditions. Apoptosis decreased in the hypoxic group than in the normoxic group. With LPS stimulation, apoptosis was decreased in all three treatment groups and even more reduced in the hypoxic group. TLR_4 expression increased in all three treatment groups with LPS stimulation, increased further in the hypoxic group, and to a lesser degree in the hyperoxic group. Unlike the cells exposed to hypoxic conditions, the cells exposed to the hyperoxic condition reacted similarly to the cells in the control (normoxic) group. Therefore, the inflammatory reactions can be stronger in the hypoxic group than in the other two groups.

Keywords

apoptosis, hyperoxia, hypoxia, inflammation, neutrophils

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Introduction

Many patients are admitted to the emergency department (ED) because of severe external injuries. Trauma patients account for approximately 25%–35% of ED patients, and trauma is the leading cause of acquired disability. Trauma can cause significant damage to personal lives, as it has strong socioeconomic impacts, since it accounts for a considerable proportion of mortality and disability in people under the age of 44.[1] Injured patients may experience massive hemorrhage,

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respiratory failure, and go into hypovolemic shock. Secondarily, hypoperfusion due to external injuries and hemorrhagic shock causes hypoxia and systemic inflammatory response syndrome (SIRS). It also induces compensatory anti-inflammatory response syndrome (CARS) because of the immunocompromised state of the patient. Patients can sustain further injury caused by acute respiratory distress syndrome (ARDS) due to lack of maintenance of homeostasis, multiple organ failure (MOF), decreased immune function, over-expression of inflammation, and so on and may die even after surviving the initial trauma because recovery becomes difficult. Trauma patients may be exposed to hypoxia and hyperoxia during various stages of complications and treatment of their injuries.[2,3]

In the treatment of trauma patients, airway maintenance and oxygen supply are known to be of paramount importance. These are also emphasized in pre-hospital treatment. It is important to prevent the occurrence of secondary complications in trauma patients through maintenance of homeostasis, and, to this end, inflammation must be controlled. SIRS, the main mechanism of early MOF, is caused by monocytes, multinucleated polymorphonuclear neutrophils (PMNs), vascular endothelial cells, and several resulting inflammatory mediators. Several studies on the maintenance of the appropriate inflammatory response have been conducted in recent years. PMNs are known to be important in causing organ damage after shock resuscitation. Hypoxia and hyperoxia are considered to affect inflammation after injury via PMNs, and therefore determining the effect of oxygen supply to injured patients is thought to be essential for effective treatment.[4,5] However, few studies have demonstrated the effects of oxygen supply on PMNs, an inflammatory cell that plays an important role in immune function after trauma. Thus, to determine the damage in the cell unit by hypovolemic shock and respiratory failure during the early stages of trauma, we conducted experiments to determine the effect of hypoxia and hyperoxia on apoptosis and expression of Toll-like receptor 4 (TLR_4) of PMNs.

Materials and methods

Whole blood samples from healthy adult volunteers were collected in tubes containing EDTA and PMNs were isolated using a modified Böyum method.[6,7] Separated PMNs were divided into normoxic (control) and hypoxic groups, and further separated into groups treated for 5 min, 30 min, 1 h, and 2 h. Cells were exposed to hypoxia (1% O_2) and then cells from each group were collected to measure the levels of apoptosis. Next, the separated PMNs were divided into a group not stimulated with lipopolysaccharide (LPS) and a group stimulated with LPS, and each was exposed to normoxic, hypoxic, or hyperoxic conditions; the levels of apoptosis and TLR_4 were determined. The study protocol and the written informed consent form were reviewed and approved by Korean University Guro Hospital (institutional review board No. KUGH 13243).

Separation of PMNs

PMNs were separated using the abovementioned modified Böyum method.[6,7] First, whole blood samples of healthy adult volunteers were collected in tubes containing EDTA. In a sterile hood, 5 mL of the collected whole blood sample was added to 5 mL of Polymorphprep™ (Nycomed Pharma AS, Oslo, Norway) in 15-mL test tubes without mixing and then centrifuged for approximately 35 min at 500*g*. Of the layers produced by centrifugation, the PMN layer, located between the monocyte and erythrocyte layers, was collected using a pipette. To lyse any red blood cells remaining among the collected PMNs, 0.2% saline solution was administered for 30 s and then the cells were centrifuged at 450*g* for 10 min. An equivalent volume of 1.8% saline solution was added to normalize the osmotic pressure and the cells were washed twice with phosphate-buffered solution (PBS). Isolated PMNs were cultured in RPMI 1640 (Roswell Park Memorial Institute 1640; Sigma, St. Louis, MO, USA) containing penicillin, 10% fetal bovine serum (FBS), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The cultured cells were maintained at a density of more than 2×10^6 cells/mL and a survival rate of >95% was confirmed using trypan blue staining.

Different oxygen environment settings

1. *Normoxic environment.* The neutrophil dish was placed at 37°C in room oxygen tension.

2. *Hypoxic environment.* The neutrophil dish was placed in a modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA, USA). The connector tube from a gas tank containing 1% oxygen was first connected to a flow meter, then to an inlet port, and the outlet port was left open. The desired mixture of gas was flushed through the chamber for 20 min, both inlets were closed, and the outlet port was tightened using the attached plastic clamp.
3. *Hyperoxic environment.* A neutrophil dish was placed in the modular incubator chamber (Billups-Rothenberg). The connector tube from the gas tank containing 80% oxygen was connected to the flow meter, then to the inlet port, and the outlet port was left open. The desired gas mixture was flushed through the chamber for 20 min and both the inlet and outlet ports were closed tightly using the attached plastic clamp. The injection of LPS was carried out simultaneously with the onset of various concentrations of oxygen (1% O_2, 80% O_2) to prevent changes in the assessment of hypoxia and hyperoxia.

Apoptosis of PMNs under hypoxic and normoxic conditions

The separately obtained PMNs were divided into two groups: a normal oxygen group (20% O_2) and a hypoxic group (1% O_2); the cells were incubated in their respective conditions for 5 min, 30 min, 1 h, and 2 h and stained with Annexin V-fluorescein isothiocyanate (AnV-FITC) and propidium iodide (PI) (ApoScan™ Annexin V-FITC Apoptosis Detection Kit; BioBud, Gyeonggi-Do, Korea). Flow cytometric analysis was conducted to determine the levels of apoptosis. In normal cells, phosphatidylserine (PS) was located on the cytoplasmic surface of the cell membrane; when apoptosis occurs, the phospholipid layer of cells suddenly changed and PS was exposed on the cell surface. Macrophages recognize PS and remove these cells. Annexin V (AnV) binds to PS on the surface of apoptotic cells with high affinity. When using PI (fluorescent DNA chromosome), cells with viable cell membranes and apoptotic cells are not stained; only necrotic cells are stained. High uptake of PI was indicative of dead cells; those that did not uptake AnV were regarded as necrotic cells; cells that did not uptake either PI or AnV were regarded as viable cells.

Apoptosis of PMNs under normoxic, hypoxic, and hyperoxic conditions, with or without LPS stimulation

The separately obtained PMNs were divided into two groups: a group stimulated with 1 μg/mL LPS and a group not stimulated with LPS (Sigma-Aldrich, St. Louis, MO, USA);[8] each group was exposed to normoxic (20% O_2), hypoxic (1% O_2), or hyperoxic (80% O_2) conditions for 2 h. The time of exposure to hypoxia was determined to be the same as the time of exposure to hyperoxia used clinically. Each group was incubated and stained with AnV-FITC and PI (ApoScan Annexin V-FITC Apoptosis Detection Kit), then flow cytometric analysis was carried out, and the results were determined. The injection of LPS was carried out simultaneously to prevent changes in the assessment of hypoxia and hyperoxia.

TLR_4 expression under normoxic, hypoxic, and hyperoxic conditions (flow cytometry analysis)

The separately obtained PMNs were divided into two groups: a group stimulated with LPS and a group not stimulated with LPS; each was exposed to normoxic, hypoxic, or hyperoxic conditions for 2 h. Expression of cell surface TLR_4 receptor was determined by flow cytometry analysis. The cells were stained with 5 μL (2 μg) of PE-conjugated anti-TLR_4 monoclonal antibody (Clone: HTA125, eBioscience, Inc., USA) in the dark for 1 h on ice. After staining, the cells were analyzed by flow cytometry using Cytomics FC 500 and CXP software (Beckman Coulter, USA).

Data analysis and statistics

Each measured value is expressed as mean ± standard deviation (SD). Statistical significance was performed using *t* test, two-way analysis of variance (ANOVA), and Mann–Whitney *U* test using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Each experiment was repeated 12 times. $P < 0.05$ was considered statistically significant.

Results

Apoptosis depending on the time of exposure to hypoxia

When PMNs were exposed to hypoxia for 5 min, 30 min, 1 h, or 2 h, the apoptosis levels were measured. In each graph, the left lower quadrant shows a viable cell, the right lower quadrant shows an apoptotic cell, and the right upper quadrant shows a necrotic cell (Figure 1(a)). The number of apoptotic cells observed is expressed as a percentage. In all hypoxic groups, apoptosis decreased when the cells were exposed to hypoxia (normoxic: 58.3%±3.2% vs 5 min: 48.0%±2.3%, 30 min: 49.1%±4.5%, 1 h: 48.5%±3.4%, 2 h: 52.1%±3.2%); the groups did not differ significantly with respect to the exposure time until 2 h ($P < 0.05$; Figure 1(b)).

Apoptosis of PMNs under normoxic, hypoxic, and hyperoxic conditions with or without LPS stimulation

In the group stimulated with LPS, apoptosis decreased significantly in all groups. Compared to the normoxic group, apoptosis decreased in the hypo- and hyperoxic groups, regardless of stimulation with LPS (normoxic without LPS: 59.7± 4.8, hypoxic without LPS: 48.4± 2.5, hyperoxic without LPS: 52.0± 0.3, normoxic with LPS: 37.4± 1.9, hypoxic with LPS: 27.8± 1.4, hyperoxic with LPS: 31.6± 0.4). Apoptosis decreased further in the hypoxic group than in the hyperoxic group ($P < 0.05$; Figure 2).

TLR_4 expression under normoxic, hypoxic, and hyperoxic conditions (flow cytometry analysis)

In the group stimulated with LPS, TLR_4 expression increased significantly in all groups. TLR_4 expression was higher in the hypoxic group than in the normoxic group, regardless of LPS stimulation (normoxic without LPS: 8.7± 2.8, hypoxic without LPS: 20.3 ± 17.4, hyperoxic without LPS: 8.3± 4.7, normoxic with LPS: 28.4± 12.4, hypoxic with LPS: 43.6± 26.4, hyperoxic with LPS: 24.4± 0.91; Figure 3(a)). TLR4 expression increased the most in the hypoxic group among all the groups under different conditions ($P < 0.05$) (Figure 3(b)).

Discussion

The mortality rate among patients with severe trauma requiring emergency surgery is estimated to be particularly high; thus, the maintenance of homeostasis and prevention of secondary complications, such as sepsis and MOF, are important in trauma patients until surgical treatment can be performed and patients recover.[9] In addition, immunocompromised state and hemorrhagic shock are well-known trauma manifestations, resulting in complications such as infection, septicemia, and, in severe cases, even MOF.[10] Trauma patients experience hypoxia at all stages of hemorrhage, shock, and secondary complications, which can adversely affect recovery. Supply of oxygen is essential for trauma patients and continues to be emphasized in treatment. By delaying apoptosis of cells, hypoxia is thought to cause SIRS due to an increase in reactive oxygen species (ROS) via oxidative stress and to increase chemotaxis, resulting in MOF in severe cases. In addition, hypoxic damage causes subsequent damage via ischemia, as well as subsequent ischemic–reperfusion injury.[11,12]

SIRS, the main mechanism of early MOF, is caused by monocytes, PMNs, vascular endothelial cells, and so on and increased expression of several inflammatory mediators. By immune signals for basic inflammation, PMNs play an important role in defense mechanisms and not only control the cell cycle through apoptosis but also cause cytotoxicity through ischemia–reperfusion.[13] Thus, PMNs play an important role in immunity and also participates in the inflammatory response. In other words, PMNs are important in defense mechanisms, but their uncontrolled long-term survival may lead to inflammatory responses overly. Therefore, it may be important to regulate apoptosis in an appropriate and balanced manner. PMNs, at the end of their normal functional life, undergo apoptosis and are terminated by macrophages. If the cells become necrotic, dying cells may cause damage by leaking out precursors of inflammation because of the loss of cell membrane. And the inflammatory response can persist and develop into systemic inflammation syndrome as a result of the prolongation of the neutrophils' life span. When PMNs are stimulated with LPS, apoptosis is delayed, and this may cause over-expression of the inflammatory response.[14,15] The results of this study

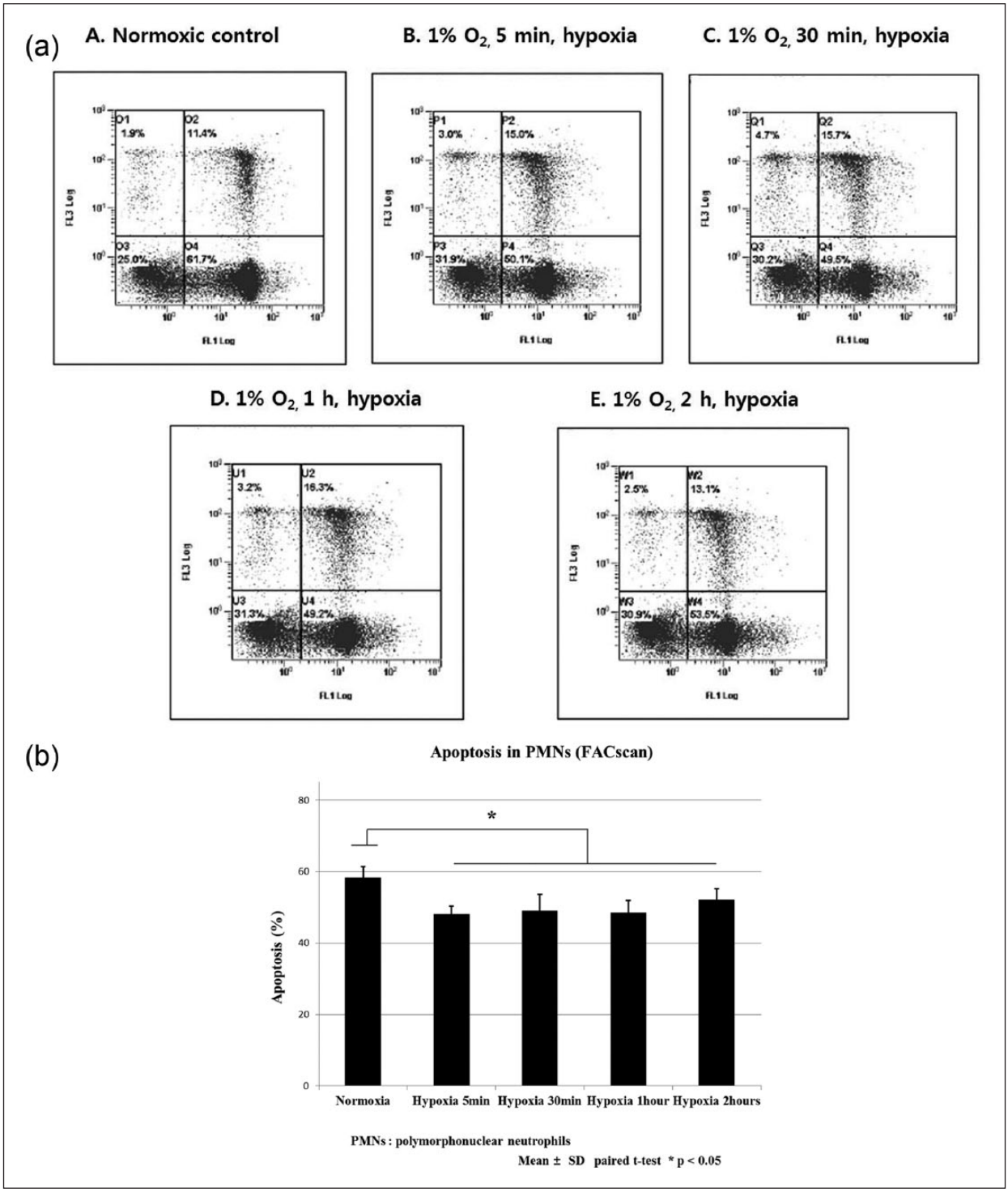

Figure 1. (a) Flow cytometric analysis of apoptosis in PMNs after incubation under hypoxic conditions. Apoptosis measured by flow cytometry using Annexin V and propidium iodide (PI) fluorochrome labels. Representative flow cytometry images from different experimental groups. Normoxic control (A), 5 min at hypoxic state (B), 30 min at hypoxic state (C), 1 h at hypoxic state (D), and 2 h at hypoxic state (E). Each panel shows cell distribution in different areas: right upper quadrant contains the nonviable, necrotic cells (AnV-positive, PI-positive), left lower quadrant shows viable cells (AnV-negative, PI-negative), and right lower quadrant shows apoptotic cells (AnV-positive, PI-negative). (b) Apoptosis in PMNs after incubation under hypoxic conditions. Graph shows the average percentage of apoptosis from Figure 1(a) (right lower quadrant). Apoptosis decreased when the cells were exposed to hypoxia (normoxic: 58.3% ± 3.2% vs 5 min: 48.0% ± 2.3%, 30 min: 49.1% ± 4.5%, 1 h: 48.5% ± 3.4%, 2 h: 52.1% ± 3.2%); groups did not differ significantly with respect to the exposure time until 2 h ($P < 0.05$).

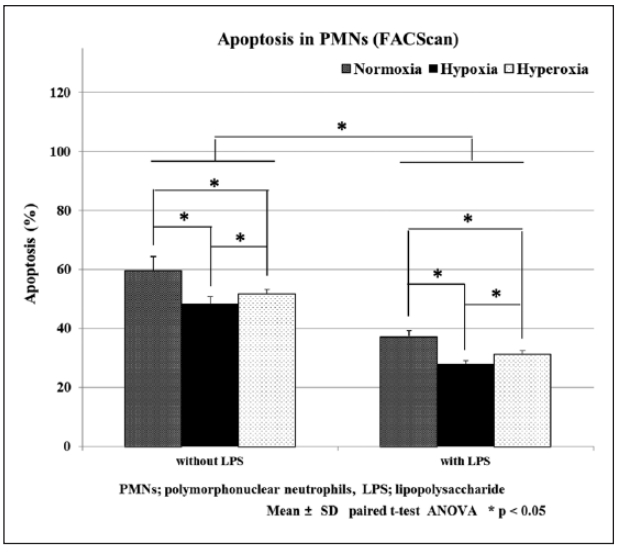

Figure 2. Comparison of apoptosis of PMNs after stimulation with or without LPS as measured by flow cytometry. In the group stimulated with LPS, apoptosis decreased significantly in all groups. Compared to the normoxic group, apoptosis decreased in the hypo- and hyperoxic groups, regardless of stimulation with LPS (normoxic without LPS: 59.7 ± 4.8, hypoxic without LPS: 48.4 ± 2.5, hyperoxic without LPS: 52.0 ± 0.3, normoxic with LPS: 37.4 ± 1.9, hypoxic with LPS: 27.8 ± 1.4, hyperoxic with LPS: 31.6 ± 0.4). Apoptosis decreased further in the hypoxic group than in the hyperoxic group ($P < 0.05$).

showed that stimulation with LPS or hypoxia decreased the levels of apoptosis. Furthermore, this shows that apoptosis decreases slightly under hyperoxic than hypoxic conditions, leading us to infer that the supply of oxygen will be important for preventing cell damage in trauma patients. Neither an increase nor a decrease in apoptosis can be said to be unconditionally good, but if apoptosis is reduced under the inflammatory condition, the damage to the surrounding tissue increases; an appropriate supply of oxygen may control tissue damage by increasing the levels of apoptosis and inducing anti-inflammatory functions. The reason may be that shortening the life span of the cells may cause less damage or activate anti-inflammatory action by promoting the cell cycle.[16] The results of this study showed that the survival of PMNs was affected by the concentration of oxygen. In PMNs stimulated with LPS, apoptosis decreased; however, apoptosis decreased more strongly under hypoxic conditions.

TLR_4, a member of the Toll-like receptor family, recognizes pathogens and plays a fundamental role in activating innate immunity. As a primary signaling receptor for bacterial endotoxins or LPS, TLR_4 is activated by various factors, such as autoimmune responses and allergic reactions, as well as microbial pathogenesis. The elevation of TLR_4 receptor was closely related to the inflammatory reaction and TLR_4–NADPH oxidase 4 (NOX4) signal-mediated ROS production might contribute to the damage.[17–20] In addition, the activity of TLR_4 can cause systemic damage via MOF.[17] LPS is known as the representative substance which serves to activate TLR_4. In the group stimulated with LPS, TLR_4 expression increased and under the hypoxic state it increased further regardless of LPS stimulation, compared to the normoxic state (control). However, the hyperoxic state restores the expression of TLR_4. It is thought that the hypoxic state often experienced by trauma patients may cause over-expression of inflammatory response. The results of this study show that when the inflammatory response of trauma patients increases, an appropriate supply of oxygen is expected to reduce the aggravating factors, such as MOF, by reducing the inflammatory response and future systemic inflammation.

The limitations of this study are as follows: this study was performed in vitro, so it may not accurately reflect the response of PMNs in vivo. Various other reactions that may occur in the plasma of patients could not be applied in this study totally because these results were obtained in vitro and stimulation via LPS may not be identical to the stimulation of PMNs in trauma patients. Future experiments in animal models are required to confirm our findings, and the findings of this research can serve as a baseline for such future studies. Additional research will be needed on the relationship between cell apoptosis and oxygen supply in ischemia–reperfusion model.

In conclusion, the apoptosis levels decreased and the TLR_4 expression increased in PMNs subjected to hypoxic conditions. In PMNs stimulated with LPS, apoptosis was observed to decrease more strongly under hypoxic conditions than under hyperoxic conditions. The expression of TLR_4, a mediator of the inflammatory response, increased when the cells were stimulated with LPS and was found to increase more strongly under hypoxic conditions than under hyperoxic conditions. It can be hypothesized that both hypoxia and hyperoxia may have an adverse impact on the neutrophil-mediated

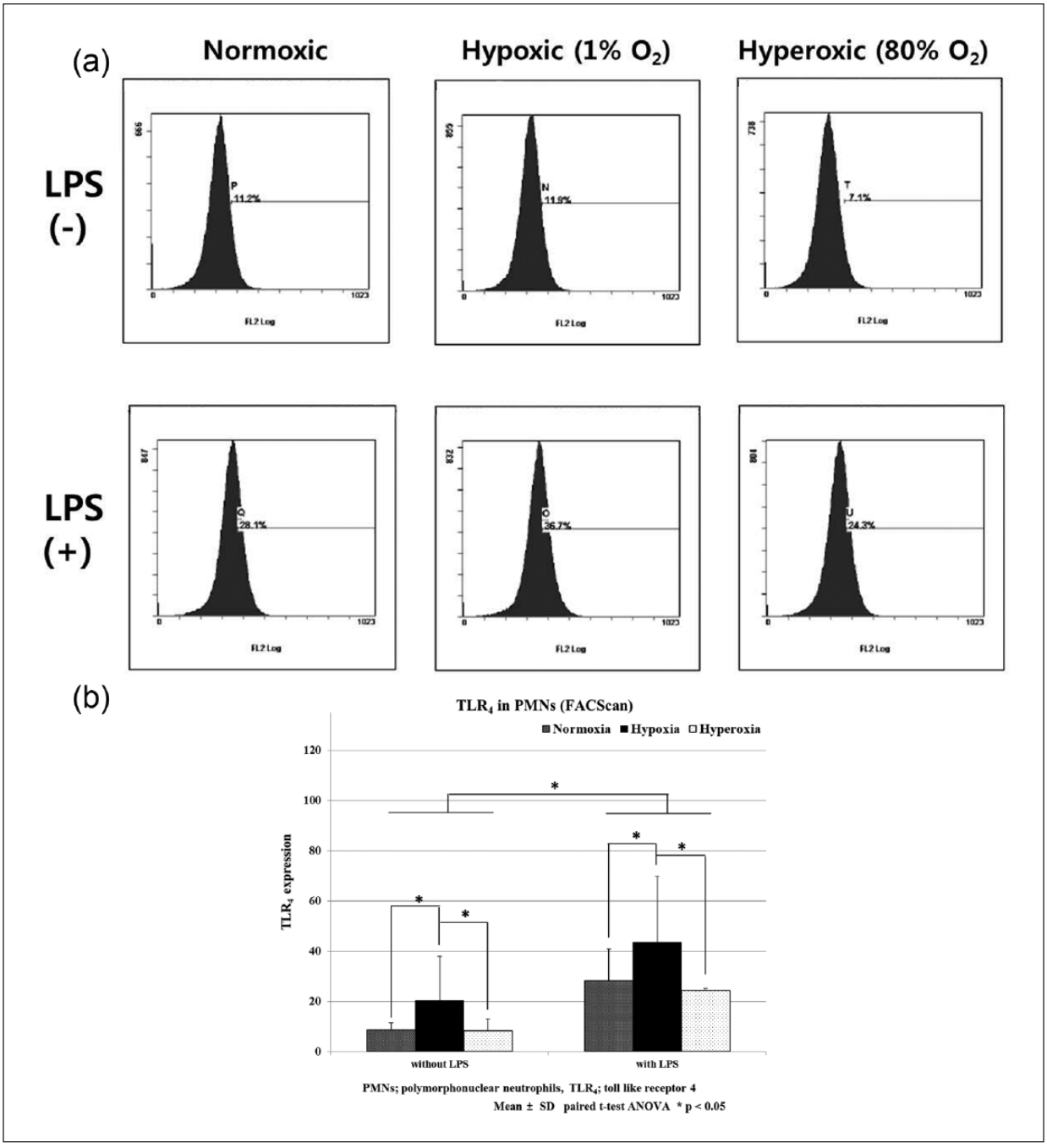

Figure 3. (a) TLR_4 expression by flow cytometry. TLR_4 expression in PMNs with or without LPS stimulation at normoxic, hypoxic, or hyperoxic state was measured by flow cytometry as described in section "Materials and methods." Numbers within histograms denote the percentage of TLR_4 expression. (b) TLR_4 expression after stimulation with or without LPS. In the group stimulated with LPS, TLR_4 expression increased significantly in all groups. TLR_4 expression was higher in the hypoxic group than in the normoxic group, regardless of LPS stimulation (normoxic without LPS: 8.7 ± 2.8, hypoxic without LPS: 20.3 ± 17.4, hyperoxic without LPS: 8.3 ± 4.7, normoxic with LPS: 28.4 ± 12.4, hypoxic with LPS: 43.6 ± 26.4, hyperoxic with LPS: 24.4 ± 0.91). TLR4 expression increased the most in the hypoxic group among all the groups under different conditions ($P < 0.05$).

inflammatory response via external factors, resulting in cell damage; this mechanism may have a stronger impact under hypoxic state than under hyperoxic state.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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