The Impact of Oxygen and Pentoxifylline in Hypoxic Condition

Young-Duck Cho

Korea University Guro Hospital Department of emergency medicine

Sung-Hyuk Choi (✉ kuedchoi@korea.ac.kr)

Korea University Guro Hospital <https://orcid.org/0000-0003-3865-7318>

Sung-Jun Park

Korea university guro hospital

Woo-Sung Yu

Korea university

Jung-Youn Kim

korea university guro hospital

Han-Jin Cho

Korea University Ansan Hospital

Chae-Seung Lim

Department of laboratory medicine Korea university Guro hospital

Kyung-Hwan Kim

Department of emergency medicine Inje university Ilsan baik hospital

Tae-Gun Shin

Department of emergency medicine samsung medical center

Research

Keywords: Hypoxia, Trauma, Pentoxifylline, Hypertonic saline, coculture

Posted Date: December 28th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-131767/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at European Journal of Inflammation on January 1st, 2022. See the published version at <https://doi.org/10.1177/20587392211056508>.

Abstract

Background: Among major trauma patients in the emergency department (ED), the leading cause of morbidity and mortality is a hemorrhagic shock. The low oxygen flow with hypovolemia in trauma patients is believed to play a significant role. Hence, oxygen supply is essential in severe trauma patients with massive hemorrhage. This study aimed to investigate the effect of different oxygen environments (hypoxia 1%, normoxia 20%, hyperoxia 80% oxygen) and variable treatments such as pentoxifylline (PTX), glycerol, hypertonic saline (HTS), protease inhibitor and dexamethasone (DEXA) in macrophage and T cells.

Methods: Nitric oxide synthase (iNOS) and macrophage migration inhibitory factor (MIF) were measured for macrophage. MIF, interleukin (IL)-2 and 8 were measured for T cells. MTT assay was done for measuring T cell viability. Coculture was done on T cells on top of macrophages and the same protocols were carried out.

Results: PTX decreased iNOS expression mostly followed by Glycerol under hypoxia. Under the hyperoxia, PTX and other treatments decreased iNOS expressions. MIF expression was lowered with PTX under hypoxia. PTX, glycerol, HTS, and protease inhibitor were effective under hyperoxia. PTX increased T cell survival under hypoxia. Under the hyperoxia, IL-2 expressions were upregulated with PTX, Glycerol, and HTS. PTX and other treatments were effective for IL-8, too. Our results indicate that PTX, HTS, and other medications with oxygen showed an effect on macrophage and T cells under various insults and stimulations.

Conclusion: Our study demonstrated potential usefulness in improving immune systems during severe inflammatory conditions similar to septic shock possibly caused by massive hemorrhage.

Background

Among major trauma patients admitted to the emergency department, hemorrhage is the leading cause of morbidity and mortality. Hemorrhagic shock is one of the most common types of shock [1]. Shock is a disequilibrium of the circulatory system. During a shock, a significant difference between vascular loading capacity and intravascular volume distribution leads to decreased tissue perfusion, cellular hypoxia and microvascular aberrations causing metabolic damage, such as anaerobic glycolysis and lactic acidemia [2]. Production of reactive oxygen species (ROS) by neutrophils and tissue hypoxia is a significant phenomenon in hemorrhage shock-induced inflammatory response [3]. As hypovolemia progresses, cardiac insufficiency leads to diminished blood supply to organs and tissues. Compensatory mechanisms, including catecholamine, antidiuretic hormone, and atrial natriuretic peptide release compensate for the insufficiency and redistribute blood flow to vital organs. Nevertheless, cellular hypoxia worsens without imminent efforts, and result in an irreversible state [4]. Despite successful resuscitation after hemorrhage, shock results in diffuse reperfusion injury, and is associated with endogenous oxidant production causing direct cytotoxicity and activation of potent inflammatory cells

such as neutrophils and macrophages [5]. The diminished flow of oxygen associated with hypovolemia in trauma patients is believed to play a significant role in shock and cell damage, however, the specific mechanism has yet to be identified. Hence, oxygen supply is an essential step in the management of severe trauma patients with massive hemorrhage.

This study aimed to investigate the effect of different oxygen environments and variable treatments such as pentoxifylline (PTX), glycerol, hypertonic saline (HTS, 7.5% NaCl), protease inhibitor, and dexamethasone (DEXA) in THP-1 derived macrophage and T cells.

Methods

Cell culture, cell stimulation and co-culture

Human acute monocytic leukemic cell line (THP-1) cells (ATCC, Manassas, VA, USA) and lymphocytic leukemic cell line (Jurkat) cells (ATCC, Manassas, VA, USA) each were maintained in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 mM HEPES, 100 U/mL penicillin/streptomycin at 37°C in 5% carbon dioxide incubator. Cells were cultured to a density of 5×10^5 cells/mL. Cell viability, as determined by trypan blue dye exclusion, was > 99%. For macrophage differentiation, the THP-1 cells (5×10^5 cells/mL) were prepared in a 75T-flask (Corning Co, USA) and 1 µL/mL of PMA (Sigma-Aldrich Co., St. Louis, MO, USA) was added for 3 days. The supernatant was discarded and washed with 10 mL of phosphate buffer saline (PBS), followed by the addition of 5 mL of tryple™ express (Gibco Co, Denmark) to take the attached cells off the floor. After the addition of 10 mL of fresh medium and the reaction mixture was centrifuged for 5 min at $500 \times g$ get the differentiated THP-1 cells. Therefore, THP-1-derived macrophages and Jurkat cells were used for the experiments. Prostaglandin E₂ (PGE₂) (1 µg/mL) (Sigma-Aldrich Co., St. Louis, MO, USA) stimulation was used to demonstrate the effect of immunosuppression. Lipopolysaccharide (LPS) (1 µg/mL) (Sigma-Aldrich Co., St. Louis, MO, USA) induction was used to simulate the effect of endotoxin..

First, the differentiated THP-1 cells were plated at a density of 1×10^4 cells/mL in 24 well plates and were placed under normoxia (20% O₂) followed by hypoxia (1% O₂) for 30 min. After that, macrophages were stimulated with LPS (1 µg/mL) followed by variable treatments such as PTX, glycerol, HTS, protease inhibitor, and DEXA, followed by normoxia (20% O₂) or hyperoxia (80% O₂) for 2 hrs. After 20 hrs or overnight under normoxic conditions, the levels of macrophage migration inhibiting factor (MIF) and inducible nitric oxide synthase (iNOS) expression were measured by western blots and concentration of MIF in supernatant was measured by ELISA. Second, Jurkat cells (2×10^6 cells/mL) were subjected to normoxia followed by hypoxia for 30 min. After that, Jurkat cells induced with PGE₂ (1 µg/mL) with various treatments such as PTX, glycerol, HTS, protease inhibitor, and DEXA, followed by either normoxia or hyperoxia for 2 hrs. After 20 hrs or overnight, their metabolic state, as indicated by cell survival, was measured using an MTT assay (with dimethyl thiazolyl diphenyl tetrazolium salt). The levels of MIF, interleukin 2 (IL-2), and interleukin 8 (IL-8) expression were measured by western blots. Third, in the event

of hypoxia and trauma or infection, macrophages that are important to the initial responses were cocultured with T cells to understand the effects of T cells that are important to the immunity in the hypoxic condition. In addition, we want to find out the changes in oxygen supply and injection of variable treatments such as PTX, glycerol, HTS, protease inhibitor, and DEXA in normoxia or hyperoxia. For coculture, differentiated THP-1 cells (1×10^4 cells/mL $\times$ 0.5ml) / well were prepared on a bottom floor of 24-well plate and Jurkat cells (2×10^6 cells/mL $\times$ 0.5mL) / well were prepared in a top floor of transwell plate. In order to determine the proper incubation time for the coculture of macrophages and T cells in hypoxia, it was cocultured for 1 minute, 5 minutes, 30 minutes, 1 hour, and 2 hours based on the values of the appropriate MTT value and IL-2 of the T cells that are important for immunity. And cells were incubated under hypoxic conditions for 30 min. After that, cells in transwell plate were stimulated with LPS (1 μ g/mL) followed by variable treatments such as PTX, glycerol, HTS, protease inhibitor, and DEXA, followed by normoxia or hyperoxia for 2 hrs and cells were incubated overnight under normoxic conditions. The concentrations of MIF in the supernatant was measured, and MTT, IL-2, IL-8 were measured using the western blots method using the Jurkat cells at the top of the transwell plate (Fig. 1). Hypoxic insult is referred to as cells cultured under hypoxic conditions. Hyperoxic treatment is administered to cells with hypoxic insult followed by hyperoxia. This study was carried out with the approval of the Korea University Guro Hospital Institutional Review Board (approval number: 2019GR0188).

Enzyme-linked immunosorbent assay (ELISA) for MIF.

The MIF concentration in the culture supernatants was measured by sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, 2 μ g/ml of monoclonal capture antibody (R&D Systems, Minneapolis, MN, USA) was added to a 96-well plate and incubated for one day at room temperature. After incubation, the plates were incubated in a blocking solution of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.05% Tween 20 for 2hrs at room temperature. Test samples and standard recombinant MIF (R&D Systems) were added to the plates and incubated for 2hrs at 4°C. Plates were washed three times with PBS containing Tween 20, and after the addition of 200ng/ml of biotinylated detection monoclonal goat-antihuman antibodies (R&D Systems), the plates were incubated for 2hrs at room temperature. The plate were washed, streptavidin-alkaline-phosphatase (1:2000; Sigma-Aldrich Co.) was added and the reaction was allowed to proceed for 20min at room temperature. The plates were washed three times and 1mg/ml of p-nitrophenylphosphate dissolved in diethanolamine (Sigma-Aldrich Co.) was added to induce a color reaction, which was stopped with 50 μ l of 1M NaOH. The optical density at 450nm was measured on an automated microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). A standard curve was generated by plotting the optical density vs. the log of the MIF concentration.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) viability assay

The tetrazolium dye, MTT, is widely used to assess the viability or the metabolic state of the cells. The MTT-colorimetric monocyte mediated cytotoxicity assay, is based on the ability of living cells to reduce

MTT into formazan by mitochondrial succinate dehydrogenase in viable cells. After treatment at the different culture conditions, Jurkat cells were plated in 96-well flat-bottom tissue culture plates to attain a final concentration of 2×10^6 cells/mL. After incubation for 12 hours at 37°C, the resultant Jurkat cell viability was determined by the MTT viability assay (ATCC, Manassas, VA, USA)

Western blot analysis for iNOs, MIF, IL-2 and IL-8 expression

The cells were washed 2 times in cold PBS and then centrifuged for 10 minutes. Cells pellet were suspended in 10 µL per 2×10^6 cell/mL pro prep protein extraction buffer. Incubated on ice for 10 minutes, and then centrifuged at $3,000 \times g$ for 15 minutes at 4°C. The supernatant was then transferred to a new tube and used for assay. The total protein concentration was determined by the Bradford method using a Bradford solution (Sigma Co.). The prepared protein were used for western blot analysis. Expression of iNOs, MIF, IL-2 and IL-8 protein was quantified by western blot analysis. Proteins (20µg/sample) were fractionated on a 15% sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad Laboratories Inc.) and transferred onto a nitrocellulose membrane. Membranes were blocked for 1 hour in 5% skim milk (Bio-rad Co.), and then incubated with a primary antibody, anti-human IL-8, iNOS, MIF, IL-2 (1:500; R&D systems). After washing, membranes were incubated with 1:1000 horseradish peroxidase-labeled anti-rabbit antibody (R&D systems) as the secondary antibody. The proteins were detected using ECL (Cyanagen) chemiluminescence kit.

Data and Statistical analysis

All results were expressed as the mean $\pm$ SD. Statistical significance was performed using a t-test, one-way ANOVA, and the Mann-Whitney U test using SPSS 18.0 (SPSS Inc, Chicago, IL). Each experiment was repeated twelve times at least. $p < 0.05$ was considered statistically significant.

Results

1. The effect of oxygen and medicines in the iNOs expression in macrophages in hypoxic condition

The mean iNOs expression ratio (iNOs/Actin ratio) was increased significantly when cells were exposed to hypoxia, compared with normoxic conditions ($p < 0.05$). After hypoxic insult and LPS stimulation, PTX and glycerol treatment significantly restored iNOS expression, compared to hypoxia and LPS injection ($p < 0.05$). The oxygen supply under LPS and hypoxic condition, the expression of iNOS was decreased significantly in the presence of PTX, glycerol, HTS, or protease inhibitor, compared to oxygen and LPS injection (Fig. 2). Therefore, oxygen supply reduced the iNOs expression compared to hypoxia, and most medicines restored iNOs expression which were increased by LPS injection when oxygen was supplied in the hypoxic condition, however, PTX or glycerol restored iNOs expression even in hypoxia.

2. The effect of oxygen and medicines in the MIF expression in macrophage in hypoxic condition.

The mean MIF expression ration (MIF/Actin ratio) was significantly elevated after the hypoxic insult compared to normoxic conditions ($p < 0.05$), and was significantly lowered with PTX compared to hypoxic conditions ($p < 0.05$). The MIF expression was significantly lowered with PTX, glycerol, HTS, and protease inhibitor when oxygen was administered to LPS-stimulated macrophages under hypoxic insult, compared to oxygen and LPS injection ($p < 0.05$) (Fig. 3). Therefore, Similar to iNOS expression, hypoxia increased MIF expressions, and LPS injections in hypoxia did not affect. Unlike other drugs, however, PTX restored the increased MIF expression in the hypoxic condition. In addition, oxygen supply restored the increased MIF expression in hypoxic condition, and most drugs reduced MIF expression in oxygen supply state.

3. The effect of oxygen and medicines in the cell viability of Jurkat cells (MTT)

Jurkat cells survival was decreased significantly by hypoxic condition ($p < 0.05$), and was further decreased under stimulation with PGE₂ without statistical significance. PTX and glycerol significantly increased survival in PGE₂-stimulated Jurkat cells under hypoxia ($p < 0.05$). Also, oxygen supply in hypoxic condition restored Jurkat cells survival, and a significant increase ($p < 0.05$) was observed in Jurkat cells treated with PTX and oxygen supply in cells exposed to hypoxia and PGE₂ stimulation ($p < 0.05$) (Fig. 4). Therefore, oxygen supply restored cell viability in hypoxic condition and PTX restored cell viability regardless of oxygen state.

4. The effect of oxygen and medicines in the IL-2 expression in Jurkat cells

After hypoxic insult and PGE₂ stimulation, the IL-2 expression was a little decreased without statistical significance. However, various medicines did not restored IL-2 expression in hypoxic condition. In the oxygen supply under hypoxic conditions, the IL-2 expression was restored significantly by the addition of PTX, glycerol, and HTS in cells subjected to hypoxia and PGE₂ stimulation ($p < 0.05$) (Fig. 5). Therefore, hypoxia a little decreased IL-2 expression and various medicines have restored IL-2 expression reduced by PGE₂ in state of oxygen supply.

5. The effect of oxygen and medicines in the IL-8 expression in Jurkat cells

The hypoxia increased a little the IL-8 expression without statistical significance. PTX, glycerol, HTS and Dexa significantly downregulated IL-8 expression in PGE-stimulated Jurkat cells under hypoxia ($p < 0.05$). PTX and Dexa downregulated IL-8 in Jurkat cells under oxygen supply following hypoxic insult and PGE₂ stimulation ($p < 0.05$) (Fig. 6). Therefore, hypoxia a little increased IL-8 expression and various medicines have restored IL-8 increased by PGE₂ in hypoxic condition. PTX or Dexa restored IL-8 expression significantly increased by PGE₂ in state of oxygen supply.

6. The effect of oxygen and medicines in the MIF expression in Jurkat cells

The hypoxia increased a little the MIF expression without statistical significance. PTX, protease inhibitor and Dexa significantly downregulated MIF expression in PGE-stimulated Jurkat cells under hypoxia ($p <$

0.05). Dexamethasone downregulated MIF in Jurkat cells under oxygen supply following hypoxic insult and PGE₂ stimulation ($p < 0.05$) (Fig. 7).

7. Cell viability of Jurkat cells in coculture with macrophage varying duration of hypoxia

The MTT values were measured at five different hypoxic exposure times (1 min, 5 min, 30 min, 1 h, and 2 h) in Jurkat cells cocultured with macrophages; the values decreased gradually (Fig. 8). And IL-2 expression of Jurkat cells cocultured with hypoxia under different exposure times. IL-2 expression was measured at five different times of hypoxic exposure (1 min, 5 min, 30 min, 1 h, and 2 h) in Jurkat cells cocultured with macrophages. A gradual downregulation of IL-2 expression was observed (Fig. 9). Considering the usefulness of the experiment, the most appropriate time was set at 30 minutes and also the appropriate number of cells was determined by our previous study (Fig. 10)

8. The effect of oxygen and medicines in the cell viability of Jurkat cells cocultured with macrophages (MTT)

The hypoxia decreased a little the MTT value without statistical significance. However, PTX treatment increased the MTT levels in Jurkat cells cocultured with macrophages after hypoxic injury with LPS stimulation ($p < 0.05$). The MTT value was increased with PTX treatment in Jurkat cells under oxygen supply after hypoxic injury with LPS stimulation ($P < 0.05$) (Fig. 11). Therefore, oxygen supply restored cell viability in hypoxic condition without statistical significance. Of the three medicines that worked in the previous study, PTX most effectively restored cell viability regardless of oxygen state.

9. The effect of oxygen and medicines in the IL-2 expression of Jurkat cells cocultured with macrophages

After hypoxic insult, the IL-2 expression was a little decreased without statistical significance. However, various medicines did not restore IL-2 expression in hypoxic condition. In the oxygen supply under hypoxic conditions, the IL-2 expression was restored significantly by the addition of PTX in cells subjected to hypoxia and LPS stimulation ($p < 0.05$) (Fig. 12).

10. The effect of oxygen and medicines in the IL-8 expression of Jurkat cells cocultured with macrophages

The hypoxia did not affect the IL-8 expression. And also none of the treatments showed a significant change in IL-8 expression under hypoxic injury in LPS-stimulated Jurkat cells cocultured with macrophages. However, PTX downregulated IL-8 expression ($p < 0.05$) in Jurkat cells under oxygen supply after hypoxic injury with LPS stimulation (Fig. 13).

11. The effect of oxygen and medicines in the MIF expression and concentration of MIF in Jurkat cells cocultured with macrophages

The hypoxia a little increased the MIF expression without statistical significance. PTX, HTS and Dexamethasone significantly restored MIF expression which were increased in Jurkat cells cocultured with macrophages after hypoxic injury with LPS stimulation ($p < 0.05$). Although most medicines have restored MIF expression, HTS in particular has decreased statistically MIF expression in Jurkat cells under oxygen supply after hypoxic injury with LPS stimulation ($p < 0.05$) (Fig. 14). Measurement of MIF concentration using ELISA method showed similar results to MIF expression by western blots, however, PTX only restored the concentration of MIF regardless of hypoxia and oxygen supply state (Fig.15). Therefore, hypoxia a little increased MIF expression and various medicines have restored MIF increased by LPS regardless of hypoxic condition and oxygen supply state. However, PTX was more useful in restoring MIF regardless of oxygen presence.

Discussion

Massive hemorrhage due to trauma is a major cause of septic shock in the later, which can trigger life-threatening conditions. It is very closely related to multi organ failure due to defective immune response against pathogen infections [6]. Data derived from recent studies showed that sepsis was associated with a higher risk of long-term mortality [7]. In the past, inflammation was believed to cause early mortality and the anti-inflammatory compensatory response caused organ failure, immune suppression, and mortality subsequently [8]. Nevertheless, analysis of septic tissue samples and severe trauma revealed sustained and concurrent inflammatory and anti-inflammatory state caused by dysfunctional innate and suppressed adaptive immunity, which together resulted in prolonged organ injury and mortality [9]. Our previous study showed the ameliorating effect of PTX in neuronal cells subjected to hypoxia. Further, the combined exposure to HTS and PTX resulted in a possible neuroprotective effect in neuronal cells after hypoxic injury [10]. Regulation and balancing have been known to play an important role in sepsis control. Massive blood loss triggers hemorrhagic and septic shock and eventually leads to death in the absence of appropriate intervention in severe trauma patients.

Macrophages, which are derived from bone marrow monocytes, play a vital role in the inflammatory response following hypoxia or infection. Macrophage induce the production of high levels of pro-inflammatory cytokines. Macrophages synthesize high levels of reactive nitrogen and oxygen intermediates. They are microbicidal and enhance the ability to resist pathogens. In vitro, macrophages are classically activated by the bacterial cell wall component LPS. Recognition of pathogen-related molecular patterns such as endotoxin (LPS) ignites the activation [11]. Also, the reduced capacity of blood monocytes after endotoxin stimulation in sepsis is associated with poor short- and long-term outcomes [12]. T cells play a major role in immunity, which has been reduced in sepsis and multiple organ failure [13,14]. For the experiment, PGE₂ was used as a representative of immunosuppressive substances. PGE₂ is an inflammatory mediator derived from arachidonic acid following the catalytic action of PGE synthases and cyclooxygenase isoenzymes (COX-1 and -2) [15]. The PGE₂ suppression on T cells, natural killer cells and macrophages is mediated via specific receptors (EP2 and EP4). The

interaction between PGE₂ and specific receptors activates multiple pathways underlying the induction of anti-inflammatory and immunosuppressive genes [16].

It is well known that tissue injury and multiple organ dysfunction occur in sepsis when excessive iNOs production induces nitric oxide (NO) synthesis [17-19]. In our research, the increased iNOS after hypoxia and LPS stimulation was restored to PTX and glycerol injection, and the production of iNO was restored to PTX, HTS, protease inhibitors, glycerol when oxygen was supplied. Therefore, hypoxia increased the iNOs that were important to the occurrence of shock, was able to see the efficacy of oxygen injection and PTX, and had similar effects on MIF, indicating that they had an effect on inflammatory reactions.

As in other studies, cell proliferation in hypoxia has decreased and further decreased by PGE₂ [20]. However, PTX and glycerol treatments restored Jurkat cell viability under PGE₂ stimulation, which simulates immunosuppression. Interestingly, the results of IL-2 expression differed under hypoxia and hyperoxia. In hypoxia, the PGE₂ stimulation decreased the expression of IL-2, and the IL-2 expression was not restored by variable medicines, whereas in the hypoxia-oxygen supply group, the IL-2 expression was restored by PTX, glycerol and HTS. IL-2 plays an important role in immune homeostasis, especially in determining the magnitude and duration of primary and memory immune responses and plays an essential role in downregulating immune responses. The loss of IL-2 leads to severe autoimmunity due to abnormal activation of T cell elimination [21,22]. IL-2 is known for anti-apoptotic signaling, and effects on glycolysis and cellular metabolism, which are essential for the long-term survival of T cells [23,24]. The contrasting results under hypoxia and hypoxia-oxygen supply are attributed to the effect of oxygen. Therefore, it is estimated that there are various multiple factors other than IL-2 in hypoxia when it comes to the proliferation of T cells. In hypoxia-insulted Jurkat cells, stimulation with PGE₂ increased the IL-8 expression, whereas treatment with various medications decreased the IL-8 expression. Following hypoxia-oxygen supply, exposure to PTX and Dexa significantly decreased IL-8 expression. IL-8 is used as an evaluation factor of immunity that results in sepsis and multiple organ failure in traumatic patients and is a chemokine and angiogenic factor produced by alveolar macrophages, T cells, and epithelial cells in response to a variety of stimuli, including LPS, IL-1, and hypoxia. IL-8 increased in hypoxia, as in other studies, to show that it was related to the suppression of cell proliferation [25,26]. PTX in our study shows meaningful results by restoring IL-8 during hypoxia and hypoxia-oxygen supply. MIF antagonizes glucocorticoid inhibition of T cells proliferation in vitro by restoring IL-2 and IFN- γ production, as other studies, and plays a critical role in the host control of inflammation and immunity [27]. Although there was no change in hypoxia in our study, we could see that there was also a change in hypoxia condition as it was increased in injection of PGE₂ and restored by PTX, protease inhibitor and Dexa. In hypoxia-oxygen injection conditions, the results are similar to hypoxia, which is different from IL-2, so research on hypoxia will be needed in the results are different from normal oxygen groups.

Coculture of macrophages and Jurkat cells induced simultaneous inflammatory and anti-inflammatory states. The concentration of cells for coculture has been set by our previous study as Jurkat cells 2X10⁶ cells/mL, and differentiated THP-1 cells 1X10⁴ cells/mL, which resulted in the same decrease in T cells in

coculture in other studies [28]. In order to determine the time of hypoxia in coculture, cell viability (MTT), IL-2 and MIF at various times were determined to be 30 minutes of statistical significance. In coculture, decreased Jurkat cell viability and diminished IL-2 levels were observed under prolonged hypoxia. Regardless of statistical significance, the results demonstrate cellular injury caused by hypoxic insult, and the improved cell viability with PTX in both hypoxia and oxygen supply groups. Also, the results of IL-8, MIF expression and concentration of MIF under coculture were similar to their individual cultures. PTX is effective in enhancing cell viability, iNOs production, and the expression of IL-2, 8, and MIF. In addition to altered red cell deformity and improved microcirculatory blood flow, the attenuation of inflammatory response after the hemorrhagic shock has been established by the activity of PTX as an adjuvant to conventional fluid resuscitation in hemorrhagic shock [29]. HTS demonstrated equivalent effectiveness in attenuating inflammatory conditions in this study. HTS has been used widely as a volume expander, and the number of studies showed enhanced microcirculatory blood flow and neutrophil adhesiveness after HTS resuscitation [30]

Conclusions

Our results indicate the effect of PTX, HTS, and other medications administered with oxygen therapy on macrophage and Jurkat cells under various insults and stimulations. Also, our study demonstrated the potential usefulness in improving both innate and adaptive immune systems in severe inflammatory conditions similar to septic shock possibly caused by massive hemorrhage. Clinical studies in the future are required to overcome the limitations associated with this in vitro study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data and materials used in the current study are all available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (2018R1A2B6004283) and partially supported by a Korea University Grant.

Authors' contributions

YDC, SJP, and SHC conceived the idea. JYK, SHC, CSL, TGS designed the experiments and interpreted the data. YDC, WSY, HJC, KHK, and SHC performed the experiments. YDC and SHC wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We are grateful to our colleague Qiuyu C, Bang IY, and Korean shock society member for participation in the study and helpful discussion and we appreciated Song DJ who conducted a statistical review.

Authors' information

¹ Institute for Trauma Research, Department of Emergency Medicine, Korea University Medical Center, Guro Hospital, 148 gurodong-ro, guro-gu, Seoul 08308, Korea. ² Department of Laboratory Medicine, Korea University Medical Center, Guro Hospital, 148 gurodong-ro, guro-gu, Seoul 08308, Korea. ³ Department of Emergency Medicine, Inje University Ilsanbaek Hospital, 170 Juwha-ro, Ilsanseo-gu, Goyang-city 10380, Kyunggi-do, Korea. ⁴ Department of Emergency Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 81 Irwon-ro, Gangnam-gu, Seoul 06351, Korea.

References

1. Nunez TC, Cotton BA. Transfusion therapy in hemorrhage shock. *Curr Opin Crit Care* 2009;15(6):536-41.
2. Fülöp A, Turóczi Z, Garbaisz D, Harsányi L, Szijártó A. Experimental models of hemorrhagic shock: a review. *Eur Surg Res* 2013;50(2):57-70.
3. Constantini TW, Deree J, Peterson CY, Putnam JG, Woon T, Loomis WH, et al. Pentoxifylline modulates p47 phox activation and downregulates neutrophil oxidative burst through PKA-dependent and -independent mechanisms. *Immunopharmacol Immunotoxicol* 2010;32(1):82-91.
4. Gutierrez G, Reines HD, Wulf-Gutierrez ME. Clinical review. Hemorrhagic shock. *Crit Care* 2004;8(5):373–81.
5. Vedder NB, Winn RK, Sharar SR, Harlan JM: Beta 2-integrins in neutrophil adhesion and their role in ischemia-reperfusion and trauma. IN Siess WLR, Lorenz R, Weber PC (eds). *Adhesion Molecules and Cell Signaling: Biology and Clinical Applications*. Vol 1. Frankfurt, Germany: Raven Press. 1995, pp 127-140.
6. Saito M, Inoue S, Yamashita K, Kakeji Y, Fukumoto T, Kotani J. IL-15 improves aging-induced persistent T cell exhaustion in mouse models of repeated sepsis. *Shock* 2020;53(2):228-35.

7. Prescott HC, Osterholzer JJ, Langa KM, Angus DC, Iwashyna TJ. Late mortality after sepsis: propensity matched cohort study. *BMJ* 2016;353:i2375.
8. Bone RC, Grodzin CJ, Balk RA. Sepsis: A new hypothesis for pathogenesis of the disease process. *Chest* 2009; 112(1):235-43.
9. Boomer JS, To K, Chang KC, Takasu O, Osborne DF, Walton AH, et al. Immunosppression in patients who die of sepsis and multiple organ failure. *JAMA* 2011;306(23):2594-605.
10. Cho YD, Choi SH, Yoon YH, Kim JY, Park SJ, Lim CS. The effect of oxygen and treatments in hypoxic conditions in SH-SY5Y cells. *Shock* 2018;50(4):449-54.
11. Mosser D, Edwards J. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 2008;8(12):958-69.
12. Munoz C, Carlet J, Fitting C, Misset B, Bleriot JP, Cavaillon JM. Dysregulation of in vitro cytokine production by monocytes during sepsis. *J Clin Invest* 1991;88(5):1747-54.
13. Yoon YH, Choi SH, Hong YS, Lee SW, Moon SW, Cho HJ, et al. Effect of hypertonic saline and macrophage migration inhibitory factor in restoration of T cell dysfunction. *J Korean Surg Soc.* 2011;81(4):229-34.
14. Junger WG, Hoyt DB, Hamreus M, Liu FC, Herdon-Remelius C, Junger W, et al. Hypertonic saline activates protein tyrosine kinases and mitogen-activated protein kinase p38 in T-cells. *J Trauma.* 1997;42(3):437-45.
15. Phipps RP, Stein SH, Roper RL. A new view of prostaglandin E regulation of the immune response. *Immunol Today* 1991;12(10):349-52.
16. Yao C, Hirata T, Soontrapa K, Ma X, Takemori H, Narumiya S. Prostaglandin E₂ promotes Th1 differentiation via synergistic amplification of IL-12 signaling by cAMP and PI3-kinase. *Nature Commun* 2013;4:1685.
17. Titheradge MA. Nitric oxide in septic shock. *Biochim Biophys Acta* 1999;1411(2-3):437-55.
18. Borutaite V, Moncada S, Brown GC. Nitric oxide from inducible nitric oxide synthase sensitizes the inflamed aorta to hypoxic damage via respiratory inhibition. *Shock.* 2005;23(4):319-23..
19. Chen IC, Lin YT, Huang JS, Wu BN, Hsu JH, Tan MS, et al. .Decreased Ambient Oxygen Tension Alters the Expression of Endothelin-1, iNOS and cGMP in Rat Alveolar Macrophages. *Int J Med Sci.* 2019;16(3):443-9.
20. Hubbi ME, Semenza GL. Regulation of cell proliferation by hypoxia-inducible factors. *Am J Physiol Cell Physical* 2015;309:C775-82.
21. Gaber T, Tran CL, Schellmann S, Hahne M, Strehl C, Hoff P, et al. Pathophysiological hypoxia affects the redox state and IL-2 signalling of human CD4⁺ T cells and concomitantly impairs survival and proliferation. *Eur J Immunol.* 2013;43(6):1588-97.
22. Kündig TM, Scorle H, Bachmann MF, Hengartner H, Zinkernagel RM, Horak I. Immune response in interleukin-2-deficient mice. *Science* 1993;262(5136):1059-61.

23. Scorle H, Holtschke T, Hünig T, Scimpl A, Horak I. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature* 1991;352(6336):621-4.
24. Frauwirth KA, Thompson CB. Regulation of T lymphocyte metabolism. *J Immunol* 2004;172(8):4661-5.
25. Wang J, Huang M, Lee P, Komanduri K, Sharma S, Chen G, et al. Interleukin-8 inhibits non-small cell lung cancer proliferation: a possible role for regulation of tumor growth by autocrine and paracrine pathways. *J Interferon Cytokine Res.* 1996;16(1):53-60.
26. Rotondo R, Barisione G, Mastracci L, Grossi F, Orengo AM, Costa R, et al. IL-8 induces exocytosis of arginase 1 by neutrophil polymorphonuclears in nonsmall cell lung cancer. *Int J Cancer* 2009;125(4):887-93.
27. Calandra T, Bucala R. Macrophage Migration Inhibitory Factor (MIF): A Glucocorticoid Counter-Regulator within the Immune System. *Crit Rev Immunol* 2017;37:359-70.
28. Gornostaeva AN, Bobyleva PI, Andreeva ER, Yakubets DA, Buravkova LB. Adipose-derived Stromal Cell Immunosuppression of T Cells Is Enhanced Under "Physiological" Hypoxia. *Tissue Cell.* 2020;63:101320.
29. Coccia MT, Waxman K, Soliman MH, Tominaga G, Pinderski L. Pentoxifylline improves survival following hemorrhagic shock. *Crit Care Med* 1989;17(1):36–8.
30. Rizoli SB, Kapus A, Fan J, Li YH, Marshall JC, Rotstein OD. Rizoli SB, et al. Immunomodulatory effects of hypertonic resuscitation on the development of lung inflammation following hemorrhagic shock. *J Immunol.* 1998;161(11):6288-96.

Figures

Figure 1

Coculture method PMA; Phorbol 12-myristate 13-acetate, LPS; Lipopolysaccharide, PTX; Pentoxifylline, HTS; Hypertonic saline, MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, IL-2;

Interleukin 2, IL-8; Interleukin 8, MIF; macrophage migration inhibiting factor, Hypoxia: 1% O2 condition, Normoxia; 20% O2 condition, Hyperoxia; 80% O2 condition

Figure 2

The iNOs expression in macrophages under hypoxia and oxygen supply. After hypoxic insult (1% O2), macrophage was stimulated with LPS. The iNOS expression was significantly increased after hypoxic insult and LPS stimulation (from 1.00 to 3.79, 3.16) and the iNOS expression was most significantly restored with PTX and followed by glycerol (from 3.16 to 0.35, 0.98). The oxygen supply under LPS and hypoxic insult, the iNOS expression was restored with PTX and followed by HTS, protease and Glycerol, respectively, compared to oxygen and LPS stimulation (from 1.03 to 0.54, 0.68, 0.72 and 0.87). Data are presented as mean ± SD. *p < 0.05.

MIF expression in macrophage

Figure 3

The MIF expression in macrophage under hypoxia and oxygen supply. After hypoxic insult (1% O₂), macrophage was stimulated with LPS. The MIF expression was significantly increased after hypoxic insult (from 1.00 to 1.49) and the MIF expression was restored with PTX, compared to hypoxic injury (from 1.49 to 1.06). The MIF expression was lowered with PTX, glycerol, HTS, protease inhibitor when oxygen was administered to LPS-stimulated macrophage under hypoxic insult (from 0.90 to 0.65, 0.67, 0.75, 0.71). Data are presented as mean ± SD. *p < 0.05.

T cells viability (MTT) in Jurkat cells

Figure 4

The cell viability in Jurkat cell under hypoxia and oxygen supply. After hypoxic insult (1% O₂), Jurkat cell was stimulated with PGE₂. MTT value was significantly decreased after hypoxia and PGE₂ stimulation (from 1.00 to -13.99, -21.29) and MTT value was restored with PTX and followed by glycerol (from -21.29 to -1.69, -6.91). The MTT value was increased significantly with PTX when oxygen was administered to PGE₂-stimulated Jukat cell under hypoxic insult (from -1.86 to 10.63). Data are presented as mean ± SD. *p < 0.05.

IL-2 expression in Jurkat cells

Figure 5

The IL-2 expression in Jurkat cell under hypoxia and oxygen supply. The IL-2 expression was a little decreased without statistical significance after hypoxic insult (1% O₂) and PGE₂ stimulation (from 1.00 to 0.91, 0.82), however, various medicines did not restored the IL-2 expression. In the oxygen supply in hypoxic Jurkat cells, the IL-2 expression was restored by PTX, glycerol and HTS, compared to the oxygen supply in PGE₂-stimulated cells under hypoxia (from 0.79 to 0.93, 0.90 and 1.09). Data are presented as mean ± SD. *p < 0.05.

IL-8 expression in Jurkat cells

Figure 6

The IL-8 expression in Jurkat cell under hypoxia and oxygen supply. The IL-8 expression was increased after hypoxia and PGE2 stimulation (from 1.00 to 1.26) and the IL-8 expression was significantly decreased with Dexamethasone and followed by PTX, glycerol and HTS, respectively (from 1.26 to 0.93, 0.99, 1.00 and 1.05). PTX and Dexamethasone significantly further downregulated IL-8 expression when oxygen was administered to PGE2-stimulated Jurkat cell under hypoxic insult (from 1.08 to 0.73, 0.83). Data are presented as mean $\pm$ SD. *p < 0.05.

Figure 7

The MIF expression in Jurkat cell under hypoxia and oxygen supply. The MIF expression was increased after hypoxia and PGE₂ stimulation (from 1.00 to 1.25), however, DEXA, protease inhibitor and PTX restored the MIF expression in PGE₂-stimulated Jurkat cells under hypoxia (from 1.25 to 0.88, 0.88 and 0.93). DEXA downregulated MIF expression when oxygen was administered to PGE₂-stimulated Jurkat cell under hypoxic insult (from 1.24 to 0.93). Data are presented as mean $\pm$ SD. *p < 0.05.

Cells viability of Jurkat cells in coculture with macrophage

Figure 8

The cell viability of Jurkat cells in coculture with macrophage. The MTT value was decreased from 1.00 to 0.933, 0.950, 0.862, 0.757 and 0.715, respectively, under 1 minute, 5 minutes, 30 minutes, 1 hour and 6 hour hypoxia (1% O₂). Data are presented as mean $\pm$ SD. *p < 0.05.

IL-2 expression of Jurkat cells in coculture with macrophage

Figure 9

The IL-2 expression of Jurkat cells in coculture with macrophage. The IL-2 expression was decreased from 1.00 to 0.78, 0.80, 0.81, 0.67 and 0.53, respectively, under 1 minute, 5 minutes, 30 minutes, 1 hour and 6 hour hypoxia (1% O₂). Data are presented as mean $\pm$ SD. * $p < 0.05$.

Cell viability of Jurkat cells in coculture with macrophage

Figure 10

The determination of concentration of THP-1 cells and Jurkat cells for coculture In order to determine the concentration of two cells for coculture, the concentration of THP-1 cells was determined to be 1×10^4 cells/mL, and Jurkat cells to be 2×10^6 cells/ml, based on the MTT value. Data are presented as mean ± SD. *p < 0.05.

Cells viability of Jurkat cells in coculture with macrophage

Figure 11

Cell viability of Jurkat cells in coculture with macrophage under hypoxia and oxygen supply. After hypoxia and LPS stimulation, the MTT value was decreased (from 0.00 to -3.97, -12.95). The MTT value was significantly increased with PTX (from -12.95 to 8.93) compared to cocultured and LPS stimulated under hypoxia. The MTT value was increased significantly with PTX when oxygen was administered to Jurkat cells in coculture with LPS-stimulated macrophage under hypoxia, compared to cocultured Jurkat cell under hypoxia (from 9.03 to 28.71). Data are presented as mean $\pm$ SD. * $p < 0.05$.

IL-2 expression of Jurkat cells in coculture with macrophage

Figure 12

The IL-2 expression of Jurkat cells in coculture with macrophage under hypoxia and oxygen supply. The IL-2 expression was little decreased without statistical significance after hypoxic insult (1% O₂) (from 1.00 to 0.93) and various medicines did not restored the IL-2 expression in hypoxic condition. In the oxygen supply in hypoxic conditions, PTX have restored IL-2 expression reduced by LPS in state of oxygen supply (from 0.74 to 0.96). Data are presented as mean ± SD. *p < 0.05.

Figure 13

IL-8 expression of Jurkat cell in coculture with macrophage under hypoxia and oxygen supply. The IL-8 expression did not affected by hypoxia and none of the treatments showed a significant change in the IL-8 expression under hypoxia. PTX decreased IL-8 expression significantly when oxygen was administered to Jurkat cells in coculture with LPS-stimulated macrophage under hypoxia, compared to cocultured Jurkat cell under hypoxia (from 0.89 to 0.76). Data are presented as mean ± SD. *p < 0.05.

MIF expression of Jurkat cells in coculture with macrophage

MIF; Macrophage migration inhibitory factor, LPS; Lipopolysaccharide, PTX; Pentoxifylline, HTS; Hypertonic saline, Dexa; Dexamethasone Mean $\pm$ SD paired t-test * $p < 0.05$ $n=12$

Figure 14

The MIF expression of Jurkat cell in coculture with macrophage under hypoxia and oxygen supply. The MIF expression slightly increased after cocultured with macrophage under hypoxia (1% O₂) (from 1.00 to 1.36) and stimulation with LPS (1.00 to 1.40). MIF expression was restored most significantly with HTS and followed by Dexa and PTX (from 1.40 to 0.93, 0.94 and 1.07) compared to LPS stimulated cocultured Jurkat cell under hypoxia. In the oxygen supply in hypoxic conditions, most medicines have restored MIF expression increased by LPS. Data are presented as mean $\pm$ SD. * $p < 0.05$.

Concentration of MIF of Jurkat cells in coculture with macrophage

Figure 15

The concentration of MIF of Jurkat cell in coculture with macrophage under hypoxia and oxygen supply. The MIF expression slightly increased after cocultured with macrophage under hypoxia (1% O₂) with LPS stimulation (1.00 to 1.24). MIF expression was restored most significantly with PTX (from 1.24 to 0.79) compared to LPS stimulated cocultured Jurkat cell under hypoxia. In the oxygen supply in hypoxic conditions, PTX have restored concentration of MIF increased by LPS.