The effects of oxygen and medicines on T cells in hypoxic co-culture

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Abstract

Objectives: Many patients with massive hemorrhage, respiratory failure due to trauma admit the emergency department, and further that the experience can fall into shock, inducing to sepsis, multiple organ failure due to hyperinflammation or immunosuppression. In the these patients, the low oxygen flow with immunosuppression is believed to play a significant role. Hence, oxygen supply and medicines is essential in severe trauma patients. Therefore, this study aims to investigate the effects of oxygen and variable medicines in hypoxic condition.

Methods: T cells and macrophages were plated into trans-well plate for co-culture for 30 minutes in hypoxia. After that, the cells were stimulated with lipopolysaccharide (LPS) followed by variable medicines by normoxia or oxygen supply for 2 hrs and cells were incubated overnight under normoxic conditions. The T cell viability was measured by MTT, and the expression of interleukin-2 (IL-2), interleukin-8 (IL-8) and macrophage migration inhibitory factor (MIF) were measured by western blots using the T cells with co-culture with inflammatory macrophages. Also, the concentration of MIF was analyzed by ELISA.

Results: The T cell viability was decreased in hypoxia with LPS stimulation, however, pentoxifylline (PTX) effectively restored cell viability regardless of oxygen state (p < 0.05). Besides, PTX in oxygen supply status restored the decreases in IL-2 expression of T cells and the increases MIF in the LPS stimulation with hypoxia (p < 0.05).

Conclusions: PTX has more effectively restored the T cells immunosuppression in hypoxia during oxygen supply, and has an immunomodulation effect by controlling hyperinflammation.

Keywords

Hypoxia; Trauma; Pentoxifylline; Co-culture; Immunosuppression

1. Introduction

Most major trauma patients visiting trauma centers are accompanied by hemorrhagic shock, and despite proper treatment, complications such as sepsis and multiple organ failure often occur in the later stages [1]. In other words, hemorrhage is caused by excessive hyper-inflammatory conditions and the occurrence of immunosuppression, i.e., the destruction of homeostasis, resulting in multiple organ failure, sepsis, etc. [2, 3]. In addition, a lack of oxygen accompanied by hemorrhage leads to cell damage, resulting in various cell changes [4]. Cell immunologically, the role of T lymphocytes, which play an important role in immune system and mononuclear cells that cause hyperinflammation in the event of damage, is important [5]. However, there have been no reported effects of hyperinflammatory monocytes initially responding to T lymphocytes that are critical to the development of sepsis later in hypoxia accompanying damage. Pentoxifylline (PTX), hypertonic saline (HTS), and dexamethasone (DEXA), which have recently been known to alleviate hyperinflammation, have also been reported to affect T lymphocytes, which play important roles in immunity [6, 7]. However, there have been no study of intercellular effects.

Therefore, this study was to investigated to the effects of oxygen supply and variable treatments such as PTX, HTS, and DEXA in the T cell viability (MTT), interleukin-2 (IL-2), interleukin-8 (IL-8), macrophage migration inhibitory factor (MIF) on the T cells in hyper-inflammatory condition by using co-culture whether oxygen supply or not. In other words, we would like to check the intercellular mechanism of macrophages and T-cells and find out the usefulness of the oxygen and variable treatments.
2. Material-method

2.1 Cell culture, cell stimulation and co-culture

Human acute monocytic leukemia cell line (THP-1) cells (ATCC, Manassas, VA, USA) and lymphocytic leukemia 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 $5 \times 10^5$ cells/mL. Cell viability, as determined by trypan blue dye exclusion, was >99%. For macrophage differentiation, the THP-1 cells ($5 \times 10^5$ 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™ expression (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 × g to get the differentiated THP-1 cells. Therefore, THP-1-derived macrophages and Jurkat cells were used for the experiments. Lipopolysaccharide (LPS) (1 μg/mL) (Sigma-Aldrich Co., St. Louis, MO, USA) induction was used to simulate the effect of endotoxin.

For co-culture, differentiated THP-1 cells ($1 \times 10^4$ cells/mL × 0.5 mL) well were prepared on a bottom floor of 24-well plate and Jurkat cells ($2 \times 10^6$ cells/mL × 0.5 mL) well were prepared in a top floor of transwell plate. In order to determine the proper incubation time for the co-culture of macrophages and T cells in hypoxia, it was co-cultured 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 μg/mL) followed by variable treatments such as PTX, HTS, and DEXA, followed by normoxia or hypoxia 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 and MIF expression 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 cultivated under hypoxic conditions. By using modular incubator chamber (Billups-Rothenburg Inc., Del Mar. CA, USA). The connector tube from a gas tank containing 1% oxygen was connected to a flow meter then connected to an inlet port with outlet port left open. The desired mixture of gas was flushed through the chamber for 40 minutes, then both inlets were closed and the outlet port was tightened using the attached plastic clamp. Hyperoxic treatment is administered to cells with hypoxic insult followed by hyperoxia. It was performed by injecting 80% oxygen, the same as oxygen injection for hypoxic conditions.

This study was carried out with the approval of the Korea University Guro Hospital Institutional Review Board (approval number: 2019GR0188).

2.2 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 $2 \times 10^6$ 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).

2.3 Western blot analysis for 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 $\times 10^6$ cell/mL pre prep protein extraction buffer. Incubated on ice for 10 minutes, and then centrifuged at 3000 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 MIF, IL-2 and IL-8 protein was quantified by western blot analysis. Proteins (20 μg/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, 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.

2.4 Enzyme-linked immunosorbent assay (ELISA) for MIF

The MIF concentration in the culture supernatants was measured by sandwich enzyme-linked immunosorbent assay (ELISA). The optical density at 450 nm 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.

2.5 Data and statistical analysis

All results were expressed as the mean ± 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.
FIGURE 1. Co-culture 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.

3. Results

3.1 Cell viability of Jurkat cells in co-culture with macrophages under various hypoxic times

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 co-cultured with macrophages; the values decreased gradually (p = 0.045) (Fig. 2). And IL-2 expression of Jurkat cells co-cultured with macrophage also gradually decreased as exposure time increased (p = 0.042) (Fig. 3). Considering the usefulness of the experiment, the most appropriate time was set at 30 minutes (Fig. 4).

3.2 The effects of oxygen and medicines in the cell viability of Jurkat cells in co-culture with macrophages (MTT)

PH and partial pressure of carbon dioxide (pCO₂) of each culture medium were similar in all of the groups. The partial pressure of oxygen (pO₂) was lowest in hypoxia group (72.3 ± 2.4 mmHg), and highest in the oxygen supply after hypoxia group (261.3 ± 70.7 mmHg). The hypoxia decreased a little the MTT value without statistical significance. However, PTX treatment restored the decreases the MTT levels in Jurkat cells co-cultured with macrophages after hypoxic injury with LPS stimulation regardless of oxygen state (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 = 0.035) (Fig. 8). Measurement of MIF concentration using ELISA method showed similar results to MIF expression by western blots, however, PTX restored significantly the concentration of MIF regardless of hypoxia (p = 0.005) and oxygen supply state (p = 0.03) (Fig. 9).

3.3 The effects of oxygen and medicines in the IL-2 expression of Jurkat cells co-cultured with macrophages

After hypoxic insult, the IL-2 expression was a little decreased without statistical significance and 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 in cells subjected to hypoxia and LPS stimulation (p = 0.043) (Fig. 6).

3.4 The effects of oxygen and medicines in the IL-8 expression of Jurkat cells co-cultured 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 co-cultured with macrophages. However, PTX downregulated IL-8 expression in Jurkat cells under oxygen supply after hypoxic injury with LPS stimulation (p = 0.02) (Fig. 7).

3.5 The effects of oxygen and medicines in the MIF expression and concentration of MIF in Jurkat cells co-cultured with macrophages

The hypoxia a little increased the MIF expression without statistical significance. PTX (p = 0.008), HTS (p = 0.042), and DEXA (p = 0.018) significantly restored MIF expression which were increased in Jurkat cells co-cultured 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 = 0.035) (Fig. 8). Measurement of MIF concentration using ELISA method showed similar results to MIF expression by western blots, however, PTX restored significantly the concentration of MIF regardless of hypoxia (p = 0.005) and oxygen supply state (p = 0.03) (Fig. 9).

4. Discussion

Many patients admit the emergency department due to trauma. These patients with massive hemorrhage, respiratory failure, and further that the experience can fall into hemorrhagic shock [1]. And massive hemorrhage due to trauma is a major cause of septic shock in the later, which can trigger life-threatening conditions [2]. Therefore, it is important to maintain the homeostasis of trauma patients until surgical treatment in order to alleviate hyper-inflammatory and immune-paralysis conditions to prevent the occurrence of posttraumatic secondary complications, such as sepsis, multiple organ failure [3]. The diminished flow of oxygen associated with hypovolemia in trauma patients is believed to play in shock and cell damage, hence, oxygen supply is an essential step in management of severe trauma patients with massive hemorrhage [4]. PTX
FIGURE 2. The cell viability of Jurkat cells in co-culture 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 ± SD.*p = 0.045.

FIGURE 3. The IL-2 expression of Jurkat cells in co-culture 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 ± SD. *p = 0.042. IL-2; Interleukin 2.

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 [8]. HTS improved microcirculatory blood flow, decreased neutrophil adhesiveness and attenuated inflammatory responses. And several studies have been shown that HTS has a immunomodulatory effects [9]. Corticosteroids are commonly used drugs for a wide range of inflammatory conditions and have been shown neuroprotective and anti-inflammatory effects [10]. Therefore, this study investigated the effects of oxygen supply and variable medicines on T cells in co-culture with macrophages.
In order to determine the concentration of two cells for co-culture, the concentration of THP-1 cells was determined to be $1 \times 10^4$ cells/mL, and Jurkat cells to be $2 \times 10^6$ cells/mL, based on the MTT value. Data are presented as mean ± SD. *$p = 0.04$.

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 co-cultured and LPS stimulated under hypoxia. The MTT value was increased significantly with PTX when oxygen was administered to Jurkat cells in co-culture with LPS-stimulated macrophage under hypoxia, compared to co-cultured Jurkat cell under hypoxia (from 9.03 to 28.71). Data are presented as mean ± SD. *$p < 0.05$. LPS, lipopolysaccharide; PTX, pentoxifylline; HTS, Hypertonic saline; Dexa, Dexamethasone.

Macrophages, which are derived from bone marrow monocytes, play a vital role in the inflammatory response following hypoxia or infection. In vitro, macrophages are classically activated by the bacterial cell wall component LPS. Activated macrophages promote the secretion of proinflammatory cytokines and activate T cells, which play a major in immunity and have been reduced in sepsis and multiple organ failure [11, 12]. We used the two cells were co-cultured to find out the effects of macrophages on T cells under hypoxia, which play an important role in the immune system. Generally, the number of lymphocytes in normal healthy people was about...
FIGURE 6. The IL-2 expression of Jurkat cells in co-culture with macrophage under hypoxia and oxygen supply. The IL-2 expression was little decreased without statistical significance after hypoxic insult (1% O\textsubscript{2}) (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 = 0.032, †p = 0.043. IL-2, Interleukin 2; LPS, lipopolysaccharide; PTX, pentoxifylline; HTS, Hypertonic saline; Dexa, Dexamethasone.

FIGURE 7. IL-8 expression of Jurkat cell in co-culture 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 co-culture with LPS-stimulated macrophage under hypoxia, compared to co-cultured Jukat cell under hypoxia (from 0.89 to 0.76). Data are presented as mean ± SD. *p = 0.02. IL-8, Interleukin 8; LPS, lipopolysaccharide; PTX, pentoxifylline; HTS, Hypertonic saline; Dexa, Dexamethasone.

7 to 20 times that of monocyte, but in order to determine the appropriate number of cells for co-culture between two
**Figure 8.** The MIF expression of Jurkat cell in co-culture with macrophage under hypoxia and oxygen supply. The MIF expression slightly increased after co-cultured with macrophage under hypoxia (1% O$_2$) (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 co-cultured 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 ± SD. *p < 0.05, †p = 0.035.

MIF, macrophage migration inhibiting factor; LPS, lipopolysaccharide; PTX, pentoxifylline; HTS, Hypertonic saline; Dexa, Dexamethasone.

**Figure 9.** The concentration of MIF of Jurkat cell in co-culture with macrophage under hypoxia and oxygen supply. The MIF expression slightly increased after co-cultured with macrophage under hypoxia (1% O$_2$) 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 co-cultured Jurkat cell under hypoxia. In the oxygen supply in hypoxic conditions, PTX have restored concentration of MIF increased by LPS. Data are presented as mean ± SD. *p < 0.05, †p = 0.005, ‡p = 0.034, †p = 0.03. MIF, macrophage migration inhibiting factor; LPS, lipopolysaccharide; PTX, pentoxifylline; HTS, Hypertonic saline; Dexa, Dexamethasone.
cells in the experiment, the Jurkat cells for MTT measurement evaluation were set at $2 \times 10^6$/mL. The differentiated THP-1 cells were co-cultured in various numbers and differentiated THP-1 cells were selected to be $1 \times 10^5$/mL by showing similar MTT as shown. Munn DH, et al. [13] also reported that macrophage is known to suppress T cell proliferation due to the influence of macrophage colony-stimulating factor (MCSF), and MCSF-derived macrophages were capable of depletion of the essential amino acid tryptophan from co-culture. Our study showed similar conclusions. In order to determine the time of hypoxia in co-culture, cell viability (MTT) and IL-2 at various times were determined to be 30 minutes of statistical significance. In co-culture, decreased Jurkat cell viability and diminished IL-2 levels were observed under prolonged hypoxia.

The MTT value of Jurkat cells viability were less affected by hypoxia, but further decreased when stimulated by LPS. However, PTX injection only were statistically significant, although there was a tendency to be restored on injections of PTX, HTS and DEXA. And the medicines in oxygen supply state made it easier to restore the MTT values. Therefore, PTX and oxygen supply more clearly restored Jurkat cell viability under LPS stimulation in co-culture state. Interestingly, the results of IL-2 expression differed under hypoxia and oxygen supply state. Only in the oxygen supply to hypoxia group, the IL-2 expression was restored by PTX. 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 immune responses. 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 [14, 15]. 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 viability of T cells. As in our previous study, which attenuated inflammation through appropriate apoptosis and attenuation of the expression of TLR_4 receptor when oxygen supply after hypoxia [16], hypoxia affects the T cells viability, but in the cases of IL-2, the medicines affected the case of oxygen supply after hypoxia.

The MIF has been shown to not only override the anti-inflammatory effects of glucocorticoid but also to induce TLR_4 expression on the surface of the cell, inhibit p53-mediated apoptosis and stimulate proliferation of cells [12]. Therefore, the MIF plays an important role in sepsis by controlling inflammatory reactions, including activations of various cytokines in macrophages, neutrophils, and T lymphocytes [17, 18]. In our study, 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, although HTS and DEXA were effective in western blot method. In our previous study, MIF was increased during the injection of LPS into macrophages, resulting in pro-inflammatory effects, and MIF was decreased in the injection of PGE_2 which indicates decreased immune function, along with the reduction of T cell proliferation, to indicate immune-paralysis [19]. It is estimated that more MIF in co-culture have a greater effect on inflammation response than immunosuppression and can be used clinically.

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. Other studies have shown that IL-8 was increased in hypoxia and reduced cell viability [20, 21], but in this study there has been no change in IL-8 in hypoxic co-culture, however PTX has somewhat reduced IL-8. This is estimated to be due to various effects in co-culture. And the medicines were presumed to affect the inflammation when oxygen supply after hypoxia. This study demonstrates T cells injury caused by hypoxic insult, and the improved cell viability with PTX. PTX is more effective in enhancing cell viability, and restored the expression of IL-2, 8, and MIF when oxygen supply after hypoxia. This research has some limitations. Instead of using the monocytes and T cells, we conducted an experiment using THP-1 cells and Jurkat cells. The THP-1 cell and Jurkat cell lines, a human acute monocytic leukemia cell and lymphocytic leukemic cells, is believed to be partially different from the true monocyte and lymphocyte cells, but is widely used in various studies such as cell proliferation, apoptosis, and cell therapy, which is thought to be suitable for use in various literature. Second, animal experiments or clinical trials have not been conducted, but it is expected if sufficient research is done; we will proceed later Third, clinically, HTS was difficult to use due to adverse effects due to elevated osmolality and severe immunosuppression of DEXA.

5. Conclusions
PTX has more effectively restored the T cells immunosuppression in hypoxia during oxygen supply, and has an immunomodulation effect by controlling hyperinflammation. Also, our study demonstrated the potential usefulness in improving immune systems in severe inflammatory conditions similar to septic shock possibly caused by massive hemorrhage. Clinical research will be conducted later as the potential for clinical usefulness of PTX, due to the difficulty of clinical use as significant adverse effects of HTS and DEXA.

AUTHOR CONTRIBUTIONS
YDC, SJP, and SHC conceived the idea. SHC, 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.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
This study was carried out with the approval of the Korea University Guro Hospital Institutional Review Board (approval number: 2019GR0188).

PARTICIPANTS

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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