In vitro study on the inhibitory effect of ketamine to LPS-induced apoptosis in HUVEC culture

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ABSTRACT

Objective: To determine the effect of lipopolysaccharide (LPS) exposure and treatment with ketamine to the levels of nitric oxide, intracellular calcium and caspase-3 of human umbilical vein endothelial cell culture.
Methods: This research used the human umbilical vein endothelial cell culture with LPS induction. ELISA was conducted to measure the levels of apoptosis markers such as nitric oxide, intracellular calcium and caspase-3.
Results: The results showed significant elevation of all parameters after LPS induction. Ketamine addition resulted in various effects. Measurement of intracellular calcium and caspase-3 showed similar result, in which the inhibitory effect of ketamine was more effective when added at the same time as LPS. *Vice versa*, in nitric oxide measurement, the highest inhibitory effect of ketamine was obtained when added 6 h after LPS induction.
Conclusions: At least in part, ketamine was suggested to have inhibitory effect in apoptosis mechanism.

1. Introduction

Sepsis is the host response to infection of microbiological products[1]. Sepsis is one of the most significant problems in the medical field. Worldwide, approximately 13 million people suffered and 4 million people had died from sepsis. The incidence of severe sepsis and septic shock reached 46%. The number of patients admitted to the intensive care unit from sepsis continues to increase[2].

In sepsis and multiorgan dysfunction syndrome, the cytokine production from innate and adaptive immune system such as tumor necrosis factor-α (TNF-α) and interleukin-1β are induced by lipopolysaccharide (LPS). TNF-α and interleukin-1β are released 30–90 min after exposing to LPS[3,4], then it would induce the activation of inflammation cascade related to the release of cytokine, lipid mediator, and reactive oxygen species[3,5]. This condition could elevate intracellular calcium and free radicals that lead to apoptosis[6]. Elevation of intracellular calcium has been described in later stages of apoptosis. Glucocorticoids mobilized Ca^{2+} stores and triggered apoptosis by early transient elevation of Ca^{2+}[7]. Sepsis is also characterized by up-regulation of inducible nitric oxide (NO) synthase that could increase the NO production. A study of serum from septic patients showed significantly higher levels of NO as compared to normal patients[8].

Proinflammatory cytokines and endotoxin could initiate the activation of a cascade leading to apoptosis[9]. Apoptosis activation through intrinsic and extrinsic pathway is pointed to caspase-3 activation and caspase-3 act as the executor of cascade[10]. Those apoptosis processes also occur in endothelial cells[11]. A study to septic human umbilical vein endothelial cell (HUVEC) medium showed up-regulation of caspase-3 activity from 3 to 12 h after LPS exposure. The highest concentration of caspase-3 was obtained at 12 h after exposing to LPS[12]. Endothelial dysfunction caused by apoptotic cells could increase the permeability of blood vessel that promotes hypovolemic, hypotension, and perfusion disorder[9,11].

Surviving sepsis campaign in 2012 was recommended to provide treatment as early as possible in 6 h after diagnosis of severe sepsis[13]. Administration of ketamine HCl has been reported to increase the survival rate of septic mice. This effect is suggested due to the inhibition of proinflammatory cytokines interleukin-6[14]. In septic rats, ketamine HCl has proven to suppress activation of nuclear factor-κB and TNF-α in the evaluation of 1, 4, and 6 h after exposing to LPS[15], as well as to suppress the increase of toll-like receptor 4 and the activity of nuclear factor-κB in the evaluation of 1, 3 and 5 h after LPS exposure[16]. This study aimed to determine the effect of LPS exposure and treatment with ketamine to the levels of NO, intracellular calcium and caspase-3 of HUVEC culture.

2. Materials and methods

2.1. Sample preparation

Umbilical sample was taken immediately after birth. About 20 cm

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umbilical cords of healthy patients who already signed the informed consent forms were cut then put into bottle that contained cord solution with the temperature of 4 °C. Endothelial cells from HUVEC were isolated and cultured before 4 h after birth.

2.2. Isolation and culture of HUVEC

Endothelial cells were cultured and isolated using the method of collagenase as previously described with some modification in the process[17,18]. Supernatant contained endothelial cell from umbilical vein was cultured in medium tissue culture wells that contained 0.2% gelatin and cover glass. The culture was incubated for about 3–4 days in incubator (5% CO_2, 37 °C) to form monolayer cells (cobblestone).

2.3. LPS and ketamine HCl treatment

HUVEC was co-cultured with monocytes to trigger the LPS action. Endothelial cell culture was induced with 1 μg/mL of LPS. Ketamine was added in two different methods. First, ketamine was added directly with LPS (P1) and second, ketamine was added 6 h after LPS induction (P2). Ketamine dose used in this research was 50 μmol/L. The effect of treatment was observed 6 h after incubation.

2.4. Apoptosis analysis

ELISA was performed to measure several apoptosis parameters. Apoptosis parameters that measured in this research were NO, intracellular calcium, and caspase-3. ELISA method was conducted based on manufactured protocol.

2.4. Statistical analysis

Statistical analysis was performed using SPSS 19.0 software. One-way ANOVA test followed by Duncan's test with significances $P < 0.05$ was used in the analysis.
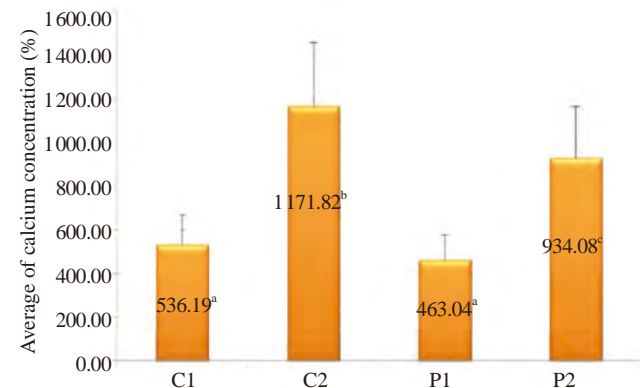
3. Results

Measurement of NO using ELISA method was shown in Figure 1. The level of NO in HUVEC after LPS induction was significantly higher than HUVEC without LPS induction (20.13 μmol/L). In comparison, P1 and P2 resulted in different effect. The level of NO was decreased significantly in ketamine treatment 6 h after LPS induction as compared to ketamine added immediately after LPS induction, with NO level 15.70 μmol/L.
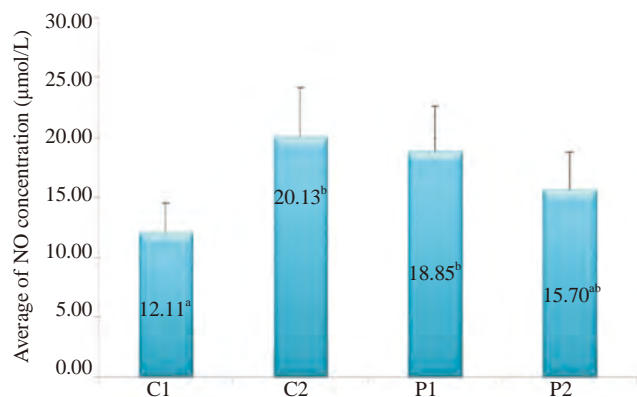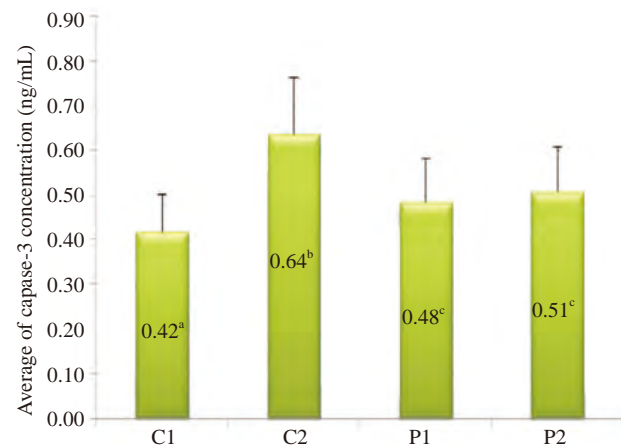

Figure 1. ELISA results of NO measurement.
C1: HUVEC; C2: HUVEC + LPS. Different superscript letters showed significant difference with $P < 0.05$.

Intracellular calcium was measured as a parameter which also related to apoptosis mechanism (Figure 2). Similar results with NO measurement were obtained when we compared the level of intracellular calcium to culture cell with and without LPS induction. The level of intracellular calcium was elevated significantly to 1 171.82% in culture with LPS induction. In contrast, ketamine induction showed different results. The lowest decreasing level was obtained in the ketamine treatment coinciding with LPS induction, with intracellular calcium level 463.04%. Although the addition of ketamine 6 h after LPS induction resulted in the increase of intracellular calcium level as compared to the ketamine treatment that added at the same time with LPS induction, it was still significantly lower compared to the culture with LPS induction only.

Figure 2. ELISA results of intracellular calcium measurement.
ELISA analysis was performed to determine the level of intracellular calcium. C1: HUVEC; C2: HUVEC + LPS. Different superscript letters showed significant difference with $P < 0.05$.

The level of caspase-3 was measured as a downstream cascade of apoptosis mechanism (Figure 3). In general, similar result with the intracellular calcium level of all treatments was shown in caspase-3 measurements. The highest level of caspase-3, 0.64 ng/mL, was obtained in culture with LPS induction. It was significantly different as compared to other treatment. Treatment with ketamine could significantly decrease the level of caspase-3 in both treatment, coinciding with LPS (0.48 ng/mL) and 6 h after LPS induction (0.51 ng/mL). Although the caspase-3 level of ketamine addition 6 h after LPS induction was slightly higher than when ketamine added at the same time with LPS induction, there was no significant difference between those two treatments.

Figure 3. ELISA results of caspase-3 measurement.
ELISA analysis was performed to determine the level of caspase-3. C1: HUVEC; C2: HUVEC + LPS. Different superscript letters showed significant difference with $P < 0.05$.

4. Discussion

In all parameter measurements (NO, intracellular calcium, and caspase-3), the expression levels were increased in LPS induction compared to culture without LPS induction. In our research, monocytes were treated with LPS and the supernatant was added to HUVEC culture. Monocyte is an important regulator in inflammation and immune processes. Together with LPS, monocytes activate the inflammation mechanism and in turn also activate the apoptosis process. Apoptosis mechanism is closely related with the inflammation process. Activation of inflammatory process could stimulate the apoptosis mechanism[19,20].

Different levels of NO, intracellular calcium and caspase-3 measurements showed similar results after ketamine addition. In general, ketamine addition could decrease the level of NO, intracellular calcium and caspase-3. In relation to the inhibitory effect of ketamine on intracellular calcium, our results were almost similar to previous studies[21], they reported that ketamine concentration (100–1 000 µmol/L) could inhibit intracellular calcium signals and in turn suppress the proliferation of neural stem cell. Another study about the effect of ketamine on calcium homeostasis in cardiomyocytes with hypoxia also supported our results[22]. The slight elevation of intracellular calcium and caspase-3 levels were related to the mechanism of action of ketamine. The onset of ketamine was between 15 and 45 s with the duration of 10–15 min and elimination half-life was around 2–3 h. Besides that, the effectiveness of ketamine is dose dependent[23]. Based on previous study (Munshi), caspase-3 is activated between 6 and 12 h after LPS-induced endothelial injury[12]. Therefore, although in general caspase-3 level was decreased after ketamine addition, but it was slightly elevated in ketamine treatment 6 h after LPS induction.

The results of our study suggested the inhibitory effect of ketamine on LPS-induced apoptosis. We showed that ketamine inhibited the apoptosis process both in upstream (NO and intracellular calcium) and downstream (caspase-3) cascade mechanism. Further research related to the ketamine doses and the effects on the genetic level of apoptosis gene is still needed.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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