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## **Ketamine Improves Sepsis-induced Immunosuppression by Attenuating Intracellular Calcium Elevation in Rat Peripheral Mononuclear Cells**

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### **KEYWORDS**

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Mononuclear cells  
Intracellular calcium

**Abstract** Immune response in sepsis divided into two phases, hyper-inflammation (cytokine storm) and hypo-inflammation (immunosuppression). The mechanism of immunosuppression appears to involve apoptosis of immune cells, particularly mononuclear cells and it predisposes to secondary infection that is believed as the predominant driving force for mortality. We aimed to determine the effect of ketamine on mononuclear cells count related to intracellular calcium contained within these cells during the immunosuppression phase of sepsis.

This *in vivo* study was performed in *Rattus Novergicus* with Fecal Induced Peritonitis (FIP) procedure to induce polymicrobial sepsis. Rats were treated with each respective dose of ketamine (2.5, 5, and 10 mg/kg) 1 hour after sepsis induction. Murine Sepsis Score was measured at 1 and 24 hours post-FIP. After 24 hours, animals were sacrificed, and the percentage of intracellular calcium inside CD4, and CD8 T cells, B cells and monocytes, along with these cells counts were determined with flowcytometry.

There is significant elevation of intracellular calcium in CD4 T-cells, B-cells and monocytes after FIP-induction and ketamine treatment suppressed this FIP-induced elevation. Measurement of mononuclear cells count showed a relevant result, in which FIP induced mononuclear cells massive loss and ketamine could inhibit the loss. MSS data showed ketamine 5 mg/kg could improve 24 hours MSS with 100% survivability.

These findings suggest that ketamine have an inhibitory effect in mononuclear-cells apoptosis mechanism through attenuating intracellular calcium elevation in polymicrobial sepsis. These inhibitory effects of ketamine might correlate with a better survival and clinical outcome.

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### **Introduction**

Sepsis, a syndrome of physiologic, pathologic, and biochemical abnormalities induced by infection, is a major health problem and is considered as the leading cause of death in intensive care units (ICU) (Singer et al., 2016; Unsinger, McDonough, Shultz, Ferguson, & Hotchkiss, 2009). Many patients in ICU cannot

recover because there is an ongoing infection. Despite broad-spectrum antibiotics and aggressive source control measures, many patients do not eradicate their infections, and develop secondary hospital acquired infections instead. Therefore, therapy that boosts immune competence could affect outcomes by leading to more rapid resolution of the primary infection

and prevention of lethal secondary infections (Hotchkiss, Monneret, & Payen, 2013b).

Sepsis can evolve into two phases: the first being hyper inflammation (cytokine storm) and the second being hypo inflammation (immune paralysis or immune dysfunction) (Boomer, Green, & Hotchkiss, 2014). Immune dysfunction predisposes septic patients to secondary infection that can delay recovery. In addition, immune dysfunction may delay restitution of the necessary milieu crucial to healing following severe sepsis and can potentially contribute to ongoing multi organ dysfunction (Sundar & Sires, 2013). Prolonged sepsis patient can also undergo reactivation of latent viruses, similar to those occurring in transplant patients on immunosuppressive therapy (Walton et al., 2014). The mechanism for this immune dysfunction appears to involve apoptosis of immune cells, in particular lymphocytes (Lang & Matute-Bello, 2009).

CD4<sup>+</sup> T cells along with CD8<sup>+</sup> T cells make up the majority of T-lymphocytes (Luckheeram, Zhou, Verma, & Xia, 2012). CD4<sup>+</sup> T cells are typically classified as helper T cells and control cells of the adaptive system. CD8<sup>+</sup> T cells are classified as cytotoxic (CTL) and kill targeted cells such as virally infected or tumor cells, recently CD8<sup>+</sup> T cells also found to participate in innate immunity function through IFN- $\gamma$  production (Lauvau & Goriely, 2016). Antibody production, termed humoral immunity, by B cells also requires T-cell help (Boomer et al., 2014).

Hotchkiss et al. (2013) did rapid tissue harvesting at the bedsides of patients dying of sepsis and showed that patients had striking apoptosis-induced loss of cells of the innate and adaptive immune system including CD4<sup>+</sup> and CD8<sup>+</sup> T cells, B-cells and monocytes (Hotchkiss et al., 2013b). The loss in CD4<sup>+</sup> T cells in patients with sepsis is frequently as severe as occurs in patients with AIDS (Hotchkiss, Monneret, & Payen, 2013a). Whereas, CD4<sup>+</sup> T cells are crucial regulators of monocyte and macrophage

function. Therefore, given the profound loss and dysfunction of CD4<sup>+</sup> T cells in sepsis, envisioning how many innate immune cells (i.e., monocytes or macrophages) could have sustained hyper activation is difficult (Hotchkiss et al., 2013b). Moreover, the CD4<sup>+</sup> T cells carry out multiple functions, ranging from activation of the cells of the innate immune system, B-lymphocytes, cytotoxic T cells, as well as non-immune cells (Luckheeram et al., 2012). Condotta et al. (2013) also report that polymicrobial sepsis model induce CD8<sup>+</sup> T cells loss, thus this loss didn't specified only to dominant activated immune system, whereas CD8<sup>+</sup> T cells dominantly activated in intracellular and viral infection, furthermore this loss could persisted for months after induction (Condotta, Rai, James, Griffith, & Badovinac, 2013), thus there is a need for immunomodulatory therapy which soften the hyper inflammatory yet sustained immunity in hypo inflammatory state.

The objective of the current study was to perform in vivo study of ketamine HCl treatment in sepsis. In particular, the effect of ketamine HCl on CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, B lymphocytes, and monocytes counts related to intracellular calcium contained within these cells during the hypo inflammatory phase of sepsis.

## Material and methods

### Animals

Rats (*Rattus Novergicus*) were obtained from the Rattus Breeding Centre (Malang, East Java, Indonesia). All Fecal Induced Peritonitis (FIP) and Murine Sepsis Score (MSS) measurement procedures were performed according to "A robust scoring system to evaluate sepsis severity in an animal model" (Shrum et al., 2014) with some modification, supervised and monitored by veterinarian from Brawijaya University - Biosains Laboratory and approved by the Brawijaya University Ethical Committee (Malang, East Java, Indonesia) with

legalized document No. 448/EC/KEPK/12/2016 (Malang, East Java, Indonesia).

#### *Antibodies and reagents*

Antibodies were purchased from eBioscience (San Diego, CA, USA), Biolegend (San Diego, CA, USA), and ThermoFisher (Waltham, MA, USA). The following rat antibodies were used for phenotypic analysis of human immune effector cells: eBioscience—CD4 PE (Cat. #12-0040) for rat CD4 T cells, CD8 PE (Cat. #12-0084) for rat CD8 T cells, CD11b PE (Cat. #12-0110) for rat monocytes; Biolegend (San Diego, CA, USA) - CD45RA PE (Cat. #20-2307) for rat B cells. The staining for intracellular calcium was performed using the fluo3-AM (Cat. #F14218) purchased from ThermoFisher (Waltham, MA, USA). Ketamine-HCl (Ketalar®) was purchased from PT Pfizer, Indonesia.

#### *Sepsis model*

##### ● **Fecal solution**

Fecal solution used to cause FIP was made by the following procedure: fresh feces were collected from rats, weighed, and mixed with a saline solution with 100 mg/mL concentrations. To ensure reproducibility, the procedure was standardized using feces prepared from rats living in the same conditions as the experimental animals. The solution was filtered in order to remove large particle matter.

##### ● **Sepsis induction**

Each rat was given an intraperitoneal (i.p.) injection of 0.5 mL per 1 kg body weight of the fecal solution using a syringe and 27G needle (Shrum et al., 2014). The Ethical Committee at Brawijaya University School of Medicine approved the animal experimentation.

#### *Ketamine HCl treatment*

Ketamine HCl (Ketalar) was diluted with normal saline to obtain 1 mg/ml concentration. Ketamine solution then introduced to animals via intraperitoneal. Treated animals then injected

respectively according to their weight and groups, 2.5 mg per kg (treatment A); 5 mg per kg (treatment B) and 10 mg per kg (treatment C).

#### *Rat monitoring*

Monitoring of the health of the rats was conducted at 1 hour after the induction of sepsis and then 24 hours thereafter, the investigators (veterinarian) was blinded to the treatment so as to test for the reproducibility of the MSS. Mice were evaluated while they were still in their cages (with the lids removed for better visualization).

#### *Blood and organ harvest*

Rats were sacrificed and organs harvested 24 hours after FIP. Splenocytes and thymocytes were prepared by gently pressing the organs through a 70- filter. Bone marrow was flushed out of tibias and femurs. Blood was harvested by cardiac puncture then collected in Vacutainer™ tubes containing EDTA (Becton Dickinson). Collected blood was diluted 1:1 in 0.9% NaCl, then PBMC was collected as instructed using Lymphoprep™.

#### *Flow cytometry staining*

Collected PBMC was diluted 1:10 in PBS + Ca<sup>2+</sup> containing 1 mM sodium pyruvate. Intracellular calcium was stained using Fluo3-AM with 5 μM concentration and were incubated for 30 minutes in the dark at room temperature (RT). Fluo3-AM contains an acetoxymethyl ester (AM) which masks the Ca<sup>2+</sup> binding region and makes the fluo3-molecule lipid soluble thus enable Fluo3-AM cross the cell membrane. Cytoplasmic Ca<sup>2+</sup> ions then bind the fluo3 creating complex which emits increased fluorescence following excitation, with an intensity that is proportional to Ca<sup>2+</sup> ions. This fluorescence then detected by flow cytometry to measure the intracellular calcium percentage (Schepers, Glorieux, Dhondt, Leybaert, & Vanholder, 2009).

CD4 and CD8 T cells, B cells and monocytes population were identified by direct immunostaining for their respective antigen CD4,

CD8, CD45RA and CD11b after 15 minutes incubation in dark room at RT. Cells then washed in PBS, resuspended in PBS+Ca<sup>2+</sup> buffer containing 1 mM sodium pyruvate and 25 mM HEPES to imitate in vivo condition that allows release of Ca<sup>2+</sup> from the intracellular stores and entry Ca<sup>2+</sup> from extracellular into cytosol.

#### *Determination of intracellular calcium level*

Flowcytometry analysis for intracellular calcium level was performed according to "Flow cytometric calcium flux assay: Evaluation of cytoplasmic calcium kinetics in whole blood leukocytes"(Schepers et al., 2009) with some modification. Samples were run on the FACScalibur® flow cytometer (Becton Dickinson). The mean fluorescence intensity of intracellular calcium was analyzed using CellQuest Pro™ software. Changes in the fluorescence (FL) intensity of the fluo3–Ca<sup>2+</sup>-complex were measured on the FL-1 channel (voltage 600).

#### *Determination of CD4 and CD8 T-cells, B-cells and monocytes count*

CD4 and CD8 (T lymphocytes), CD45RA (B lymphocytes) and CD11b (monocytes) cell percentage were analyzed by FACScalibur® flow cytometer (Becton Dickinson) and using CellQuest Pro™ software. The PE-fluorescence, a measure for the CD4-, CD8-, CD45RA- and CD11b-expression, was evaluated on the FL-2 channel (voltage 593). On the light scatter dot blot the three types of leukocytes were gated and analyzed separately based on their size (forward scatter, FSC) and their granularity (side scatter, SSC). CD4 and CD8 T cell percentage then multiplied with absolute lymphocyte number from complete blood count measurement by Clinical Pathology Laboratory, Brawijaya University (Malang, East Java, Indonesia).

#### *Statistical analysis*

Data are presented as Mean ± SEM. Data were analyzed with statistical software SPSS 18. Nonparametric Wilcoxon paired-test were used to analyze 1 and 24 hours MSS data, non-

parametric Kruskal Wallis test followed by Mann Whitney were used to compare 24 hours MSS data between groups, One-way ANOVA test continued with post-hoc Tukey test, and Pearson correlation were used to analyze cell counts and intracellular calcium levels of mononuclear cells. P values of less than 5% (p<0.05) were considered statistically significant. The graph was made using GraphPad Prism 7.

## **Results and discussion**

### *Murine Sepsis Score Improvement in Ketamine HCl- treated groups*

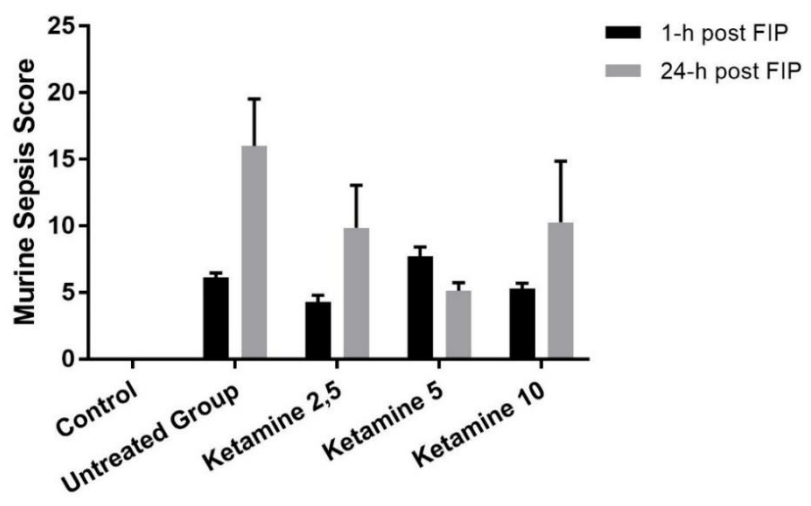
Murine sepsis score (MSS) was measured 1-hour post-FIP to confirm the septic state of rats and 24 hours post-FIP to evaluate the progressivity of sepsis clinical condition. Measurement of MSS was performed by veterinarian from Biosains Laboratory, Brawijaya University. The murine sepsis score is presented in Figure 1. A MSS of 3 (Youden score of 0.61) was selected as the cut-off point for animals that progressed to severe sepsis post-FIP induction: the sensitivity (±95% C.I.) and specificity (±95% C.I.) of this score was 57% (47-67%) (Shrum et al., 2014).

At an hour post-FIP, we observed that MS Score of all rats induced with fecal solution were higher than 3. This result showed that all those rats were already in a septic state at 1-hour post-FIP. After 24 hours post-FIP, we reassessed the MSS to evaluate sepsis progressivity. The 24 hours survival rate of rats from control and treatment B group was 100%, while in untreated group we found only 67% survivors, 86% survivors from treatment A group, and 71% survivors from treatment C group (Figure 2). This suggest that ketamine HCl treatment increase 24h survival rate of septic rats with the dose of 5 mg/kg related with the highest survival rate.

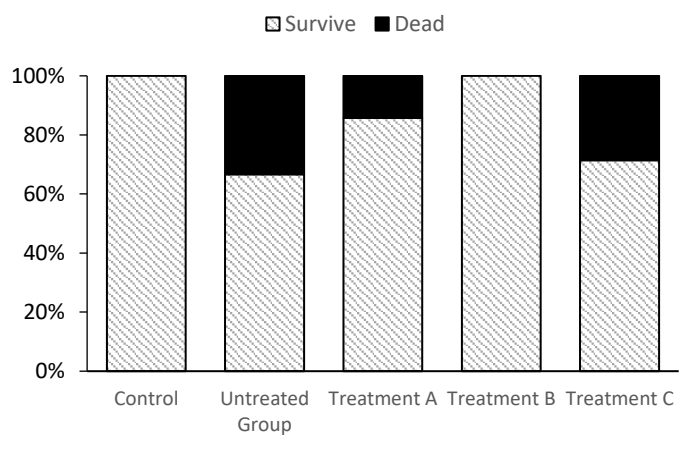
From the MSS data 24 hours post-FIP, we observed a significant difference between untreated and treatment B group (p = 0.004). Non-parametric Wilcoxon paired test for murine

sepsis score at 1 hour and 24 hours post-FIP showed significant increase in untreated group ( $p = 0.028$ ) and treatment A ( $p = 0.046$ ), but not in treatment C. While from non-parametric Mann Whitney test, a significant decrease of MSS 24h post-FIP were observed in treatment B group

( $p = 0.027$ ) (Figure 1). Taken altogether, this result suggests that ketamine HCl treatment suppress the increase of MSS 24 hours after FIP induction thus might provide a better clinical outcome and survival rate for late sepsis.



**Figure 1.** Murine Sepsis Score of Sepsis Rat Model Treated with various dose of ketamine compared to control: without ketamine (untreated group); ketamine 2,5 mg/kg (treatment A); 5 mg/kg (treatment B); 10 mg/kg (treatment C).



**Figure 2.** Survival Rate of Rats from Each Groups at 24 hours post-FIP. The 24 hours survival rate of rats from control and treatment B group was 100%, while in untreated group we found only 67% survivors, 86% survivors from treatment A group, and 71% survivors from treatment C group.

*Elevation of Intracellular Calcium of Mononuclear cells in Polymicrobial Sepsis Model and Attenuation by Ketamine*

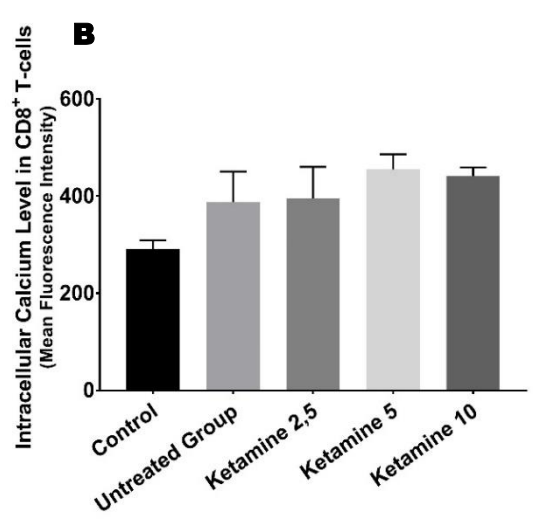
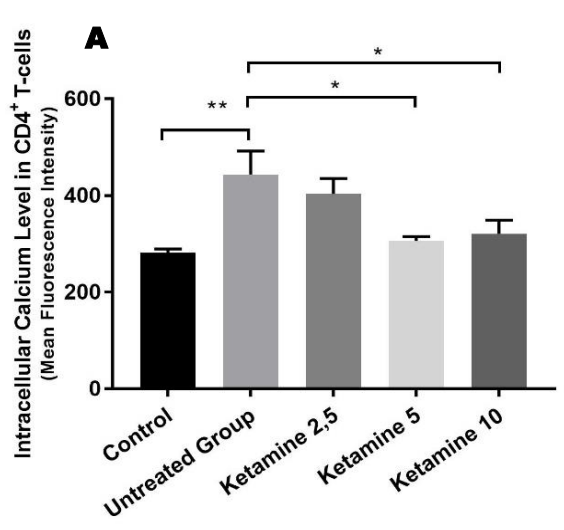
The levels of intracellular calcium in rat peripheral CD4<sup>+</sup> T-cells were elevated after FIP, compared to control with p-value 0.0034 ( $p < 0.05$ ). Ketamine at the doses of 2.5 mg/kg, 5 mg/kg and 10 mg/kg

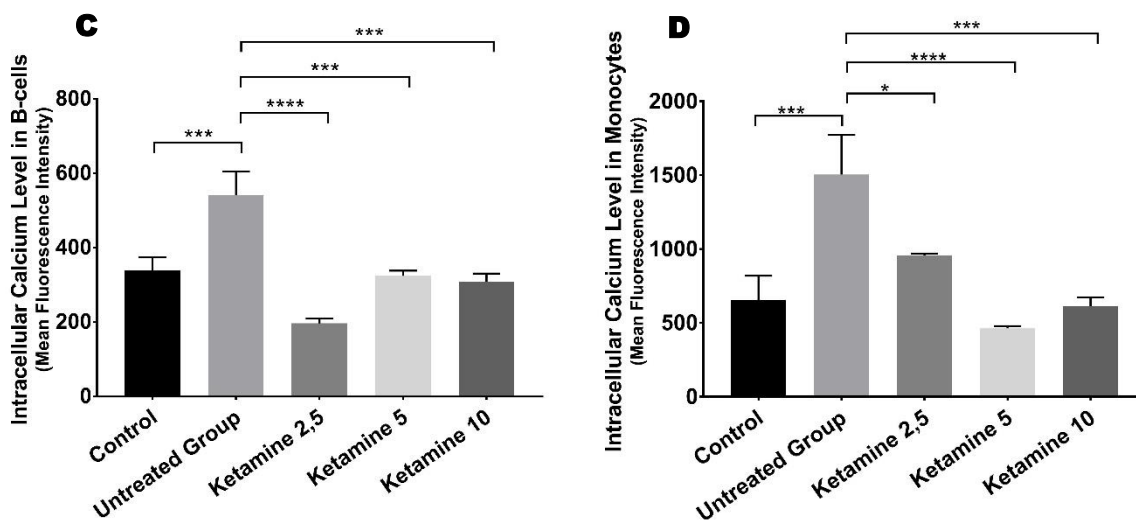
suppressed calcium elevation in CD4<sup>+</sup> cells after experimental FIP, but the significant difference only observed between untreated group with treatment B and C (p = 0.014 and p = 0,0306 respectively). There is no significant difference between untreated group with treatment A (p = 0.815) (Figure. 3a). This suggest that intracellular calcium level in CD4<sup>+</sup> T-cells increased significantly in a homeostatic manner after septic condition and ketamine at the dose 5 and 10 mg/kg could attenuate this elevation.

After FIP induction, there is a slight elevation in the levels of intracellular calcium in rat peripheral CD8<sup>+</sup> T-cells, compared to control, but no statistically significant difference observed with p-value 0.397 (p < 0.05). Ketamine at the doses of 2.5 mg/kg suppressed calcium elevation after experimental FIP, but no significant difference statistically (p = 0.99). The levels of intracellular calcium in rat peripheral CD8<sup>+</sup> T-cells in treatment B and C even show some slight elevation compared with untreated groups (Figure. 3b). This suggest that intracellular calcium level in CD8<sup>+</sup> T cells may be also increased in a homeostatic manner after septic condition. Calcium is possibly not the dominant pathway involved in CD8<sup>+</sup> T cells apoptotic mechanism, and ketamine didn't alter the calcium dynamics in CD8 T cells.

In B lymphocytes, we found the increase of intracellular calcium in polymicrobial sepsis compared to negative control significantly (p = 0.0009). We also obtained that ketamine can reduce intracellular calcium levels of B lymphocytes significantly in all treatment group (A, B, C) compared to untreated groups (p < 0.000, p = 0.0003, p = 0.0003, respectively) (Figure. 3c). Uniquely we also found significant differences between treatment A compared to treatment B (p = 0.047) and negative control compared to treatment A (p = 0.028). This suggest that the elevation of intracellular calcium level in B lymphocytes caused by stimulation in septic condition, and ketamine could attenuate this elevation significantly in a dose-dependent manner.

The levels of intracellular calcium in rat peripheral monocytes were elevated after FIP, compared to control with p-value 0.000 (p < 0.05). We also obtained that ketamine can reduce intracellular calcium levels of monocytes significantly at the doses of 2,5; 5; and 10 mg/kg (A, B, C) compared to untreated group (p = 0,031, p < 0.000, p = 0.000, respectively) (Figure 3d). This suggest that intracellular calcium level in monocytes increased significantly in septic condition and ketamine could attenuate this elevation (Figure 3d).





**Figure 3.** Intracellular calcium level (MFI) in (A) CD4<sup>+</sup> T-cells, (B) CD8<sup>+</sup> T-cells, (C) B-lymphocytes, and (D) monocytes of Sepsis Rat Model Treated with various dose of ketamine compared to control. Intracellular calcium level evaluated by flowcytometry. One-way ANNOVA and post hoc Tukey were used to evaluate differences within all groups.

Calcium is a secondary messenger which took crucial role in the activation and proliferation of T cell during an infection (Feske, 2007). Unfortunately, in sepsis, uncontrolled calcium elevation also cause apoptosis and cellular dysfunction of immune cell via ER-stress pathway, both of which caused hypo-inflammatory state, secondary infection, and ultimately, death (Hotchkiss et al., 2013a). This elevation of calcium suspected to be moderated by TRPM2 which expression is upregulated after TCR stimulation (Parenti, De Logu, Geppetti, & Benemei, 2016). TRPM2 is a multifunctional Ca<sup>2+</sup> permeable, non- selective cation channel in which its activation brings a sizeable increase of Ca<sup>2+</sup> intracellular concentration (Ru & Yao, 2014). TRPM2 channels are strongly expressed on the immune cell surface, primarily PMNs, monocytes/macrophage and also T and B cells, with the exception of dendritic cell, where they are localized in the lysosomal membranes as a Ca<sup>2+</sup> release channel (Mortadza, Wang, Li, & Jiang, 2015; Parenti et al., 2016).

Recent study showed that TRPM2, acting in concert with NMDARs, may provide the basis for

a positive feedback loop in which Ca<sup>2+</sup> influx is facilitated through a pathway involving aberrant NMDAR activation and the formation of ROS all of which leads to the activation of TRPM2 in neuron cells (Xie, MacDonald, & Jackson, 2010). More recent study showed NMDA regulated calcium influx is mediated in large part by TRPM2 (A. Zeiler, 2015).

We thus postulated that ketamine, NMDA antagonist, could attenuate the elevation of calcium influx through TRPM2 channel thus inhibit lymphocyte and monocyte apoptosis mechanism via Ca-induced ER-stress pathway in sepsis, so it represents an innovative therapy for septic patients. Therefore, at the end it could prevent septic patient to develop immune paralysis phase in late-sepsis.

Moreover, previous study has showed ketamine could suppress NF-kappa B activation and calcium elevation in monocytes exposed to endotoxin (Sun, Zhou, Lv, Li, & Xu, 2004). Ketamine also found to reduce IL-10 production which contribute to immune suppression in hypoinflammatory state (Taniguchi, Kanakura, Takemoto, Kidani, & Yamamoto, 2003). Reduce



of cytokines production like TNF- $\alpha$  caused by ketamine may prevent apoptosis in sepsis, but further research is still needed. In vitro studies showed that ketamine reduce intracellular calcium elevations and caspase-3 in HUVEC cultured of sepsis model (Asmoro et al., 2015). The inflammatory response is a physiological and necessary phenomenon which is an active actor of homeostasis. Ketamine attenuate exacerbation of inflammation without blunting the local processes. By this, ketamine has to be considered as a immunomodulatory agent that optimizes inflammation in order to restore homeostasis (Loix, Hospitalier, & Chwapi, 2011).

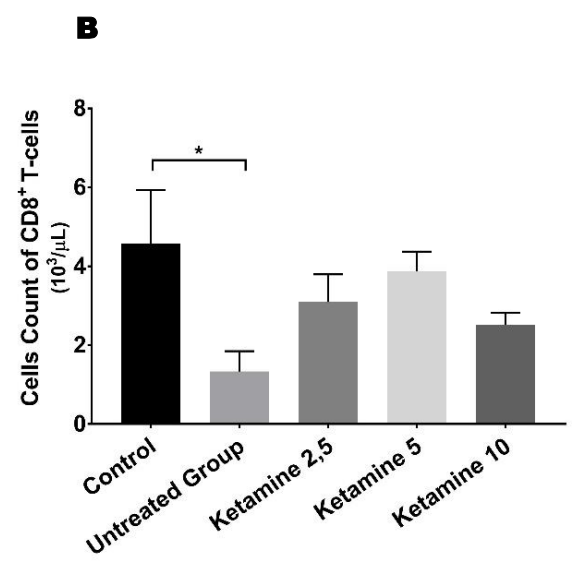
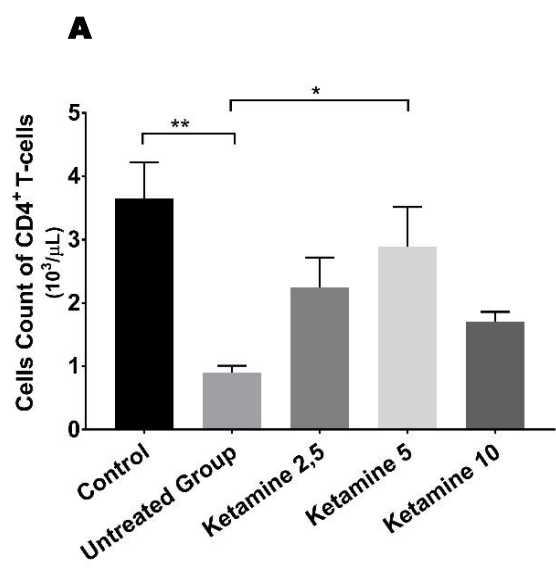
*Extensive Mononuclear Cells loss in Polymicrobial Sepsis Model and Improvement by Ketamine*

FIP induced a significant decrease in peripheral CD4<sup>+</sup> T-cells count (p = 0.0011, p < 0.05). At the dose of 2.5 mg/kg, 5 mg/kg and 10 mg/kg, ketamine showed inhibitory effect at the loss of CD4<sup>+</sup> T-cells after FIP but only at the dose of 5 mg/kg (treatment B) showed a statistically difference with untreated septic rats group (p = 0.0184) (Figure. 4a). No significant differences were observed between untreated group with treatment A and C (p = 0.163 and p = 0.613 respectively). This suggest that extensive CD4+

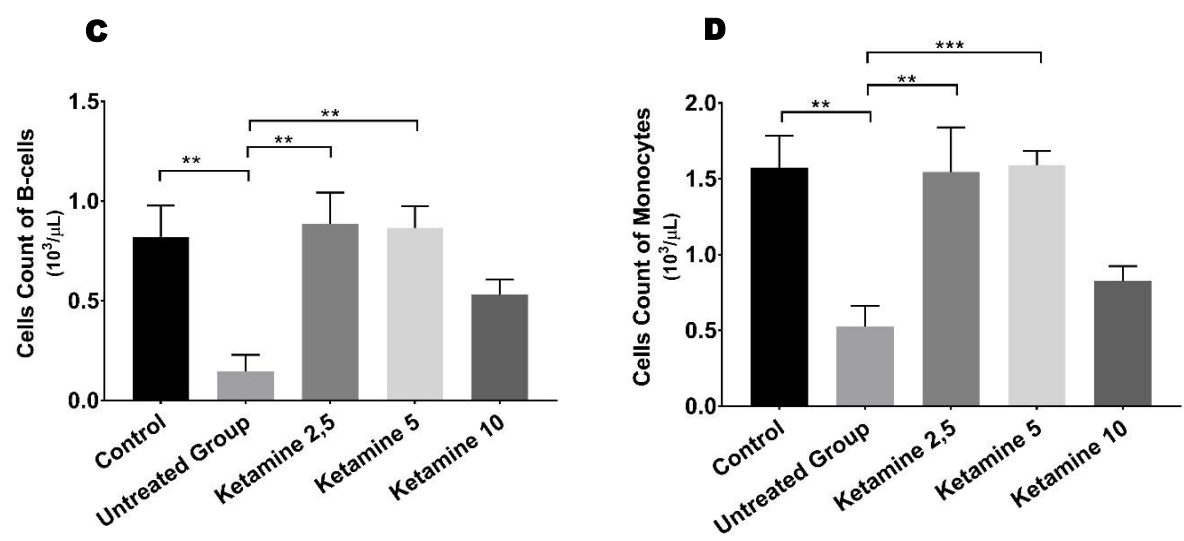
T-cells loss in sepsis is improved by ketamine, with 5 mg/kg as an optimum inhibiting dose.

Compared to control group, FIP induced a significant decrease in untreated group's peripheral CD8<sup>+</sup> T-cells count (p = 0.042, p < 0.05). Treatment with ketamine in all of doses, attenuate this loss until there is no significant difference with control group with p = 0.427 (treatment A), p = 0.994 (treatment B) and p = 0.180 (treatment C) (Figure 4b). The highest cell counts between CD8+ T cells found in 5 mg/kg dose. This result suggests that extensive CD8+ T-cells loss in sepsis is attenuated by ketamine, with 5 mg/kg as an optimum inhibiting dose.

Compared to control group, FIP induced decrease in peripheral B lymphocyte counts significantly (p = 0.0072). Ketamine at the dose of 2.5 mg/kgBW, and 5 mg/kgBW inhibited the loss of B lymphocytes after FIP significantly (p = 0.001 and p=0,001, respectively). No significant differences were also observed between control and all treated groups with p = 0.448 (treatment A), p = 0.480 (treatment B), and p = 1.000 (treatment C) (Figure.4c). This suggest that extensive B cells loss in sepsis is improved by ketamine, with 2.5 mg/kgBW and 5 mg/kgBW as recommended dose.







**Figure 4.** Number of peripheral cells count (10<sup>3</sup>/µL) of (A) CD4<sup>+</sup> T-cells, (B) CD8<sup>+</sup> T-cells, (C) B-lymphocytes, and (D) monocytes evaluated by flowcytometry.

FIP induced a significant decrease in peripheral monocytes count ( $p = 0.002, p < 0.05$ ). Ketamine at the dose of 2.5 and 5 mg/kgBW inhibited the loss of monocytes after FIP significantly ( $p = 0.002, p = 0.0006$ , respectively). No significant differences were observed between control and all treated groups (Figure.4d). This suggest that extensive monocytes cells loss in sepsis is improved by ketamine, with 2.5 mg/kgBW and 5 mg/kgBW as recommended dose.

Immune response of sepsis follows a biphasic pattern, with an initial ‘hyperinflammatory’ phase characterized by high levels of pro-inflammatory cytokines (cytokine storm), and a second phase characterized by decreased responsiveness of immune cells to inflammatory stimuli - the ‘immunoparalysis’ phase. The mechanism for this immune paralysis appears to involve apoptosis of immune cells, in particular lymphocytes and monocytes (Lang & Matute-Bello, 2009).

The lymphocyte population is mainly made up of the thymus-derived lymphocytes (T-lymphocytes), bone marrow derived (B-

lymphocytes), and the natural killer cells (NK cells). T-lymphocytes mediating the cellular immunity, along with B lymphocytes mediating humoral immunity, provide adaptive immunity, which work in close collaboration with the innate immune system (Luckheeram et al., 2012).

CD4<sup>+</sup>T cells along with CD8<sup>+</sup>T cells make up the majority of T-lymphocytes (Luckheeram et al., 2012). CD4 T cells are typically classified as helper T cells and control cells of the adaptive system. CD8 T cells are classified as cytotoxic (CTL) and kill targeted cells such as virally infected or tumor cells and help orchestrate innate immunity (Lauvau & Goriely, 2016). Antibody production, termed humoral immunity, by B cells requires T-cell help (Boomer et al., 2014). B lymphocytes also known had good ability to detect foreign antigen through TLR and presented to T helper lymphocytes. Adaptive responses of B cells in producing antibodies intensify pathogen eradication via toxin inactivation, microbial blockage, opsonization and complement activation (de Pablo, Monserrat, Prieto, & Alvarez-Mon, 2014; Monserrat et al., 2013).

Lymphocyte apoptosis has been increasingly recognized as an important step in the pathogenesis of sepsis, by inducing a state of 'immune paralysis' that renders the host vulnerable to invading pathogens (Lang & Matute-Bello, 2009). Multiple independent laboratories have demonstrated, through the use of various strategies including transgenic and knockout mice, anti-apoptotic cytokines, caspase inhibitors and death receptor blockers that preventing lymphocyte apoptosis improves survival in sepsis (Hotchkiss et al., 2013a).

In the other hand, monocytes are leukocytes that also play key roles in inflammation, pathogen challenge and homeostasis. They originate from progenitors in the bone marrow and travel through the blood stream to peripheral tissues. At the site of infection or injury in tissues, they differentiate into dendritic cells or macrophages that mediate both innate and adaptive immune responses to disease (Review, 2010). Macrophages were required as cells to induce activation of both T and B lymphocytes (Eibl & Mannhalter, 1982). Thus, apoptosis of this monocyte cells also contribute to induce a state of immune dysfunction in sepsis (Sundar & Sires, 2013).

*Elevation of intracellular calcium was correlated with mononuclear cells loss in polymicrobial sepsis*

We found that intracellular calcium has significant negatively medium correlation towards CD4<sup>+</sup> T cells count ( $p = 0.007$ ;  $r = -0.570$ ;  $R^2 = 0.325$ ), B lymphocytes count ( $p = 0.001$ ;  $r = -0.553$ ;  $R^2 = 0.306$ ) and monocytes count ( $p = 0.023$ ;  $r = -0.379$ ;  $R^2 = 0.144$ ). But there is no significant correlation observed between intracellular calcium level of CD8<sup>+</sup> T Cells and the cell counts ( $p = 0.090$ ;  $r = -0.370$ ). This result is relevant since there is no significant intracellular calcium elevation observed in CD8 T-cell of septic rats.

Correlation of intracellular calcium towards CD4<sup>+</sup> T Cells, B cells and monocytes count in this

study showed significant negatively medium correlation. It means that elevation of intracellular calcium related to decrease of CD4<sup>+</sup> T cells, B cells and monocytes, vice versa. It showed that apoptosis of mononuclear cells in sepsis not only influenced by intracellular calcium levels, but also others like pathogen virulence, genetic predisposition, comorbid, proinflammatory cytokines, and oxidative stress caused by ROS which produced by inflammation (Ayala et al., 2008; Mortadza et al., 2015).

This study proved that ketamine has a role in regulate intracellular calcium of mononuclear cells beside of reducing NF- $\kappa$ B activation and proinflammatory cytokine in sepsis. Its regulation might be as upstream pathways in reducing of NF $\kappa$ B and proinflammatory cytokines considering the close association of this effect with calcium signal. Intracellular calcium regulation by ketamine may further related to apoptosis in late phases of sepsis. Influence of ketamine in reducing NF- $\kappa$ B and cytokines production in sepsis need further research to find the relations with intracellular calcium which in turn also in prevent apoptosis of immune cells.

Ketamine effects in reducing intracellular calcium levels and improving mononuclear cells count in this study were made of points and new paradigm about pathogenesis of sepsis. Pathogenesis of sepsis itself was complex and yet known clearly. Correlation of intracellular calcium towards apoptosis from this study could be starting point for further research to reveal ketamine used in sepsis. Ketamine used in sepsis treatment still need to be studied, especially about pathways through by ketamine in inflammatory regulation and also pharmacologic profile in precisely used for sepsis. Ketamine used as supportive therapy in purpose to modulate immune respons instead of sepsis treatment algorithm including oxygen supplementation, fluid resuscitation, and also antibiotics (Dellinger et al., 2013).

## Conclusions

Ketamine treatment in this study showed many favorable results, attenuate the elevation of CD4 T-cells, B cells and monocytes intracellular calcium, increase the number of all mononuclear cells count, including CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, B cells and monocytes, yet it also inhibits sepsis clinical condition worsening and improves survival in sepsis. Interestingly, ketamine treatment at the dose of 10 mg/kg shows attenuation of this effect, thus we suggest that ketamine effect is dose dependent and needed further study to determine the optimum dose for inhibiting the apoptosis mechanism.

Overall, these findings suggest that ketamine have an inhibitory effect in mononuclear cells apoptosis mechanisms through attenuating intracellular calcium elevation in polymicrobial sepsis. These inhibitory effects of ketamine might correlate with a better survival and clinical outcome.

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