Research

CLINICAL INVESTIGATION

Propofol Promotes Endotoxin Tolerance by Upregulating *MicroRNA-let-7e*

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Abstract

Background: Endotoxin Tolerance (ET) refers to the hypo-responsiveness to repeated endotoxin stimulation. During ET, the production of Lipopolysaccharide (LPS)-triggered cytokines is reduced after pre-exposure to LPS, and macrophages polarize toward M2-phenotype. ET protects against hyperactive inflammation, but leads to immunosuppression. MicroRNA-let-7e (*miR-let-7e*) mitigates toll-like receptor 4 signaling, leading to ET development. Propofol, a widely used sedative agent for critical patients, upregulates *miR-let-7e* expression. Effects of propofol on ET remain unstudied.

Methods: To induce ET, J774.1 cells were stimulated with LPS (200 ng/ml, 24 h) after pre-exposured to LPS (200 ng/ml, 24 h). Propofol (10 μg/ml, 24 h) was administered before pre-exposure to LPS. Levels of Tumor Necrosis Factor-α (TNF-α), Interleukin-6 (IL-6), Nuclear Factor-κB (NF-κB) p65, and arginase1 (Arg1, an M2-associated enzyme) were measured with or without inhibiting *miR-let-7e*.

Results: During ET, propofol mitigated NF-κB-triggered TNF-α and IL-6, and increased Arg1 expression, suggesting the enhancing effect of propofol on ET. Furthermore, propofol upregulated *miR-let-7e*. Effects of propofol on cytokine levels, Arg1 expression, and NF-κB activity were counteracted by inhibiting *miR-let-7e*.

Conclusions: propofol promoted ET by upregulating *miR-let-7e*. The immunosuppressive effect of propofol through enhancing ET in critical patients should be considered.

Keywords: Endotoxin tolerance • Propofol • Nuclear factor-κB • Microrna • Lipopolysaccharide

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Introduction

Endotoxin tolerance is defined as a reduced responsiveness to repeated stimulation of endotoxin, a component of the outer wall of Gram-negative bacteria [1]. The development of endotoxin tolerance is demonstrated by downregulation of Lipopolysaccharide (LPS)-triggered pro-inflammatory cytokines, such as Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6), following pre-exposure to LPS [1, 2]. After LPS binding to Toll-Like Receptor 4 (TLR4), activation of Nuclear Factor-κB (NF-κB) pathway leads to inflammatory cascades. Mitigation of NF-κB activation is critical for the development of endotoxin tolerance [1]. Furthermore, endotoxin-tolerant macrophages polarize to M2 (anti-inflammatory or alternative) phenotype [2-4]. The expression of M2-associated markers, such as arginase-1 (Arg1), is upregulated during endotoxin tolerance [5]. Endotoxin tolerance protects against hyperactive inflammation. On the other hand, endotoxin tolerance may also lead to an immunosuppressive status, which worsens the outcomes of diseases if it persists. In clinical, endotoxin tolerance is accompanied by many inflammatory pathologies such as sepsis [6, 7]. Various microRNAs are involved in the development of endotoxin tolerance, including microRNA-let-7e (*miR-let-7e*) which is one member of let-7 family [8, 9]. A previous report showed that *miR-let-7e* mitigates TLR4 signaling and leads to the development of endotoxin tolerance [10].

Propofol (2,6-diisopropylphenol), a γ-aminobutyric acid receptor agonist, is an intravenous anesthetic drug to facilitate the conduction of general anesthesia [11]. Propofol is also used as a sedative agent in critical patients in the Intensive Care Unit (ICU). According to a

metaanalysis, propofol increases the mortality rate in critically ill patients, compared to other sedative agents [12]. Till now, there has been no conclusive explanation to the worse outcomes of patients sedated with propofol. Propofol also possesses immunomodulatory effects [13]. Propofol regulates LPS-induced inflammatory signals, and modulates macrophage polarization [14-16]. A microarray analysis showed that propofol increases the expression of miR-let-7e, a mediator of endotoxin tolerance. Today, the effect of propofol on endotoxin tolerance remains unstudied [17].

Methods

Cell culture

J774.1 cells (a murine macrophage cell line, American Type Culture Collection) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, MA, USA) containing 10% Fasting Blood Sugar (FBS) (Thermo Fisher Scientific, MA, USA) and 1% *penicillin/streptomycin* (Life Technologies, CA, USA), incubated at 37°C in a mixture of 95% air and 5% CO_2. Cells of fewer than 15 passages were employed in this experimnt.

Induction of endotoxin tolerance

To determine the temporary sequence of endotoxin tolerance development, cells were firstly stimulated with LPS (200 ng/ml) for indicated period of time (0 h, 4 h, 8 h and 24 h). Then, re-stimulation with LPS (200 ng/ml) for 0 h, 4 h, 8 h and 24 h after replacement with fresh medium was administered subsequently in cells which had been treated with LPS for 24 h. The protocol of stimulation and re-stimulation of LPS was shown in (Figure 1).

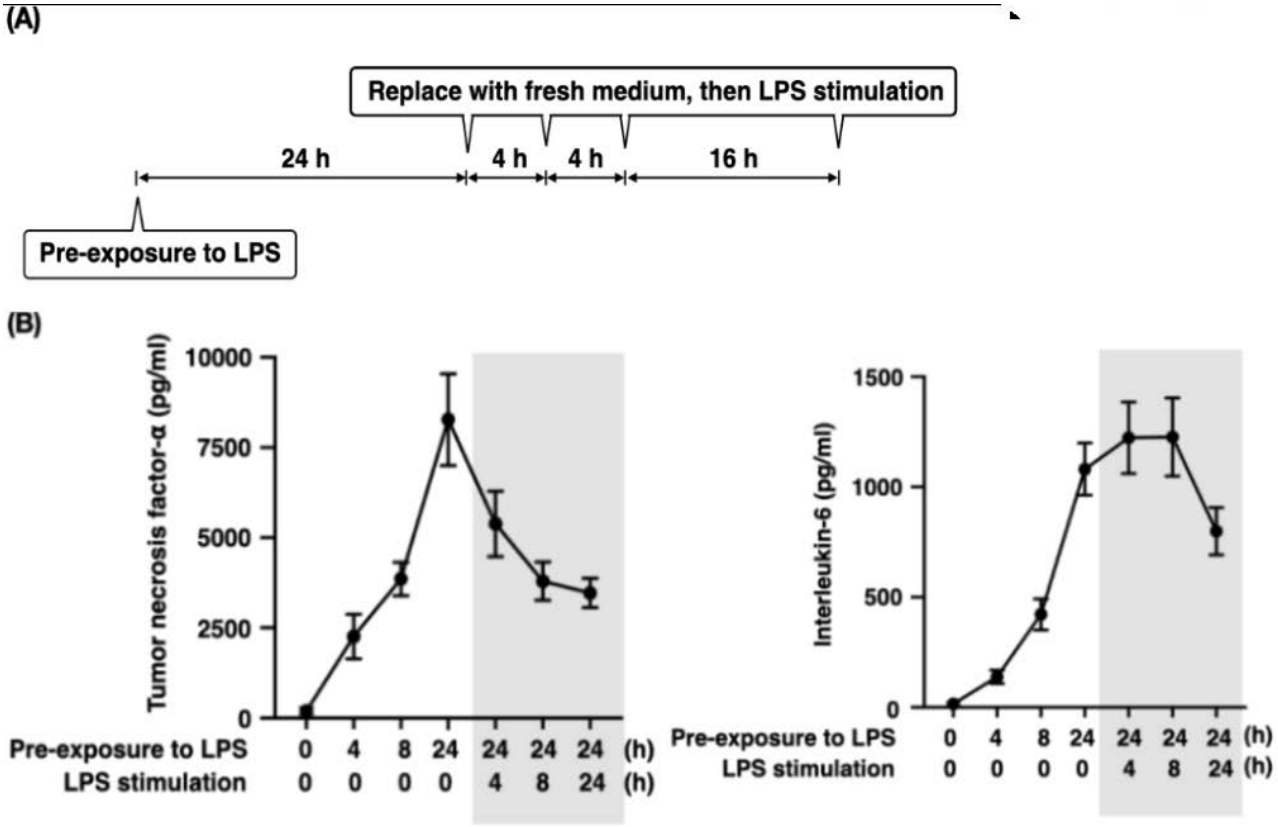

Figure 1: Changes of cytokine release with or without pre-exposure to Lipopolysaccharide (LPS). (A) To determine the onset of endotoxin tolerance, J774.1 cells were stimulated with LPS (200 ng/ml) for 0, 4, 8 or 24 h, with or without pre-exposure to LPS (200 ng/ml, 24 h). (B) Levels of Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6) were measured using enzyme-linked immunosorbent assay. Data were derived from four independent experiments and expressed as means ± standard deviations

The experimental protocols and groups

Cells were stimulated with LPS (200 ng/ml; from Escherichia coli O111: B4, Sigma-Aldrich, MO, USA) for 24 h, following pre-exposure to LPS (200 ng/ml, 24 h) and subsequently replaced with fresh medium. In the groups with treatment of propofol, propofol (10 μg/ml; Sigma-Aldrich, MO, USA) was added 24 h before the pre-exposure to LPS. The dose of propofol was based on previous reports [18, 19]. J774.1 cells were randomlly allocated to six groups, denoted as the Ctrl, LPS, ET, p, p+LPS and p+ET group. Experimental treatments for each group were shown in (Figure 2). Cells in the groups without

propofol were added with dimethyl sulfoxide (the solvent of propofol) of equal volume to propofol. Endotoxin tolerance was induced in both ET and p+ET groups. The Ctrl and p group served as the control groups.

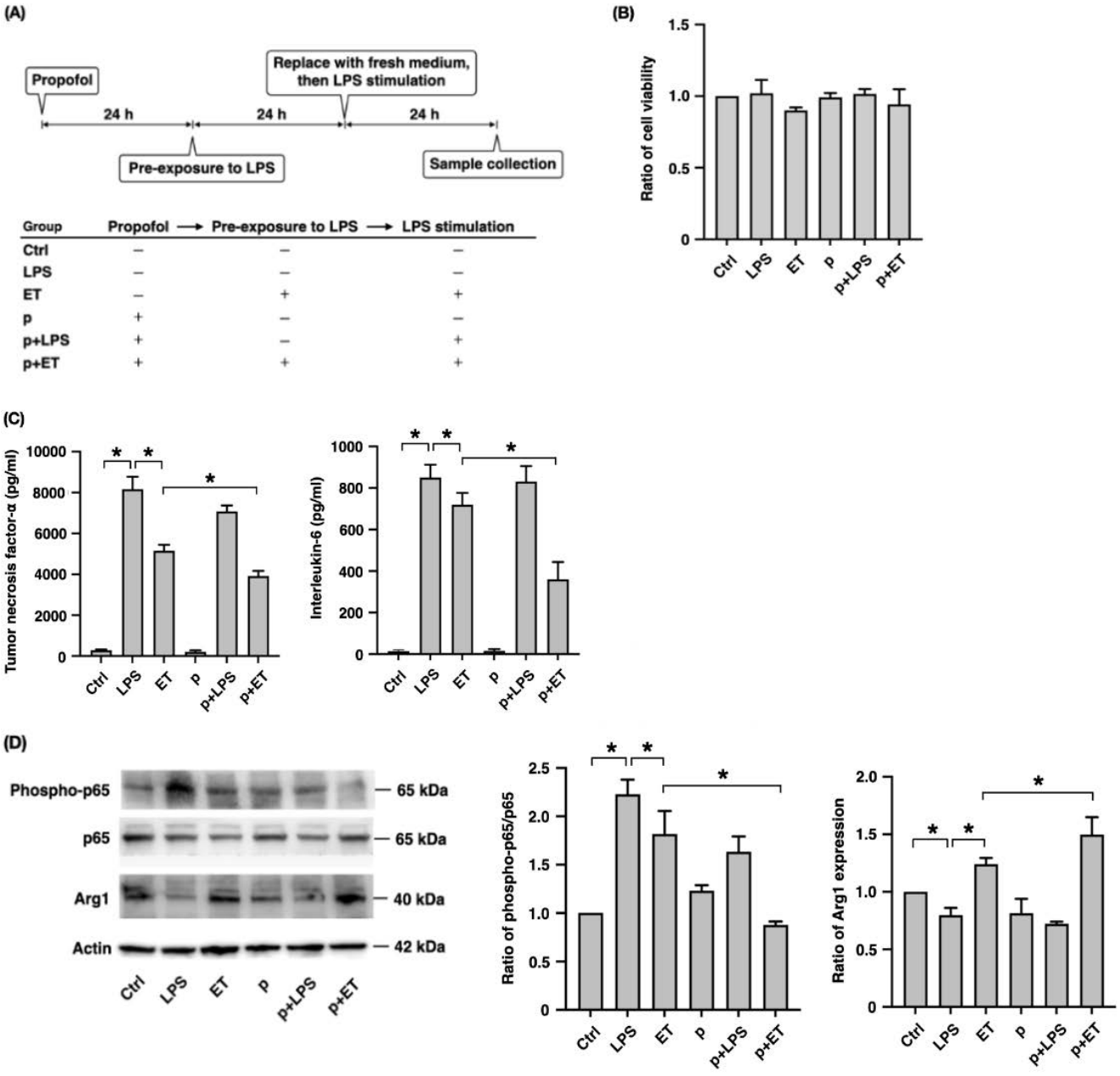

Figure 2: Effects of propofol on cytokine release, Arginase-1 (Arg1) expression and nuclear factor-κB (NF-κB) activation during endotoxin tolerance. (A) To induce endotoxin tolerance, J774.1 cells were stimulated with lipopolysaccharide (LPS) (200 ng/ml, 24 h) after pre-exposure to LPS (200 ng/ml, 24 h). In the groups with propofol, propofol (10 μg/ml) was administered 24 h before the pre-exposure to LPS. Cells were randomly allocated into six groups (Ctrl, LPS, ET, p, p+LPS and p+ET group). (B)The cell viability was measured using the MTT assay. (C) Levels of Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6) were measured using enzyme-linked immunosorbent assay. (D)The protein expression of NF-κB p65, Phospho-p65 (p-p65), and Arg1 were measured by immunoblotting. Actin was the internal control. The level of NF-κB activation was reported as the ratio of p-p65/p65 to actin, relative to the Ctrl group, and the level of Arg1 was reported as the ratio of Arg1 to actin, relative to the Ctrl group. Data were derived from four independent experiments and expressed as means ± standard deviations. *p<0.05

Cell viability

The cell viability was determined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay kit (Sigma-Aldrich, USA). Cells in each group were added with MTT and incubated at 37°C for 4 h. Then, reduced MTT was measured using a plate reader

(Bio-Rad, Hercules, CA, USA) at a wavelength of 570 nm and a reference wavelength of 630 nm. The cell viability in each group was reported as the ratio to the Ctrl gorup.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cells were seeded in the 6-well plates at a density of 10^6 cells/ml. After treatment as previous description, the supernatant in each group was collected for the assay of TNF-α and IL-6. The concentration of TNF-α and IL-6 were quantified using the commercial ELISA kit of TNF-α (R&D System, MN, USA) and IL-6 (R&D System, MN, USA), MN, USA respectively, in accordance with the manufacturer's protocol.

Immunoblotting

The protein expression of NF-κB p65, Phospho-p65 (p-p65), and Arg1 were determined by the immunoblotting assay. In brief, the cell lysates were prepared using the lysis buffer (Cell Signaling Technology, USA). The protein levels of cell lysates in each group were quantified using the bicinchoninic acid assay kit (Thermo Scientific, USA). The proteins in each sample were separated on 10% SDS-PAGE gel, and transferred onto polyvinylidene fluoride membranes (Bio-Rad, USA). The membranes were blocked with 5% bovine serum albumin and then were incubated with primary antibody against p65 (1:1000; Cell Signaling Technology, USA), p-p65 (1:1000; Cell Signaling Technology, USA), Arg1 (1:500; Genetex, USA), and actin (1:10000; Sigma-Aldrich, MO, USA), followed by incubation with secondary antibody (1:10000; horseradish peroxidase-conjugated anti-mouse IgG antibody; Amersham Pharmacia Biotech, Inc., NJ, USA). We employed the Chemiluminescence (ECL plus kit; Amersham Pharmacia Biotech, Inc., NJ, USA) to visualize the protein bands on the membranes. The software ImageJ was used to quantify the density of the protein bands. The level of NF-κB activation was reported as the ratio of p-p65/p65 to actin, relative to the Ctrl group. The expression of Arg1 was reported as the ratio of Arg1 to actin, relative to the Ctrl group.

Real-Time Polymerase Chain Reaction (PCR)

The transcriptional expression of *miR-let-7e* was measured using real-time PCR. Cells were seeded at a density of 10^6/ml, and collected for isolation and purification of total miRNA using NucleoSpin miRNA kit (Macherey-Nagel, USA) according to the manufactures' protocol. Then the samples were reverse-transcribed to cDNA using miScript II RT kit (Qiagen, German). For real-time PCR analysis, iTaq Universal SYBR Green Supermix from Bio-Rad Laboratories (Hercules, CA, USA) and a CFX96 real-time PCR instrument (Bio-Rad) were employed. U6 was used as an internal control. The relative expression levels of *miR-let-7e* were normalized against U6 by using the $2^{-\Delta\Delta}$ Ct method. The primers used in this experiment are listed in (Table 1).

Table 1: Lists of oligonucleotide sequences used in this experiment.

Name	Oligonucleotide sequence (5' to 3')
miR-let-7e primer	CCAGCTGGGTGAGGTAGGAGGTTGT
U6 primer	CTCGCTTCGGCAGCACA
miR-let-7e inhibitor	ACTATACAACCTCCTACCTC
miR-let-7e negative control	CTACAATCACTACCCTACCT

Transfection of *miR-let-7e* inhibitors

Chemically modified Locked Nucleic Acid (LNA) (Exiqon, CA, USA) was used for *miR-let-7e* inhibition. J774.1 cells were seeded at a density of 4×10^5/ml, and were transfected with *miR-let-7e* inhibitors using TransIT-X2® Dynamic Delivery System according to the manufacture's protocol. In brief, TransIT-X2 Transfection Reagent and siRNA for *miR-let-7e* (10 μM) were mixed in serum-free DMEM (Gibco), and rested at room temperature for 10 min to induce the formation of the TransIT-X2:siRNA complexes. The TransIT-X2:siRNA complexes were added dropwise to cells at a final concentration of 20 nM. After incubated at 37°C for 24 h, cells were collected to facilitate the investigation. Another set of cells were added with a scrambled sequence and used as the negative control. To determine the efficiency of transfection, we measured the transcriptional expression of *miR-let-7e*. The oligonucleotide sequences used in this experiment are listed in (Table 1).

Statistical analysis

Statistical analysis was performed using a commercial software package (SigmaStat for Windows; SPSS Science, Chicago, IL, USA). All data were presented as mean ± standard deviations, and analyzed by one-way analysis of variance in conjunction with Tukey's post hoc test. A p-value < 0.05 was considered statistically significant.

Results

Repeated stimulation of LPS downregulated TNF-α and IL-6

Levels of TNF-α and IL-6 after LPS stimulation for 0 h, 4 h, 8 h and 24 h were measured using ELISA and shown in (Figure 1). Without the pre-exposure to LPS, concentrations of TNF-α and IL-6 increased as the time of LPS stimulation increased. The peak of TNF-α concentration was noted after LPS stimulation for 24 h. Of note, pre-exposure to LPS changed the LPS-triggered upregulation of TNF-α downward. Following pre-exposure to LPS, TNF-α expression after re-stimulation of LPS was obviously low. IL-6 expression also reduced after the pre-exposure to LPS, though the onset of IL-6 turning downward was later than TNF-α. The grey squares in Figure 1 indicated the development of endotoxin tolerance.

Propofol reduced NF-κB-triggered TNF-α and IL-6 during endotoxin tolerance

In the following experiment, we chose to use the experimental protocol shown in (Figure 2). There was no significant difference in cell viability between groups figure 2, n=4. Endotoxin tolerance was induced by stimulation with LPS 24 h after pre-exposure to LPS 24 h. The levels of TNF-α and IL-6 in the Ctrl group were low figure 2, n=4. LPS stimulation upregulated the levels of TNF-α and IL-6 (both $p<0.0001$, LPS group versus Ctrl group). Development of endotoxin tolerance significantly reduced LPS-triggered TNF-α and IL-6 ($p<0.0001$ and =0.0435, ET group versus LPS group). Propofol downregulated TNF-α and IL-6 released by endotoxin-tolerant cells ($p=0.0005$ and <0.0001, p+ET group versus ET group).

Activation of NF-κB pathway triggers the production of TNF-α and IL-6. NF-κB p65 and p-p65 were measured using immunoblotting and shown in figure 2, n=4. The ratio of p-p65/p65 in LPS group was significantly higher than in the Ctrl group ($p<0.0001$), indicating that LPS stimulation activated NF-κB pathway. During endotoxin tolerance, LPS-triggered NF-κB activation was mitigated ($p=0.0404$, ET group versus LPS group). Propofol further reduced the ratio of p-p65/p65 in endotoxin-tolerant cells ($p<0.0001$, p+ET group versus ET group). These data suggested that propofol enhanced endotoxin tolerance through mitigating NF-κB-triggered cytokine release.

Propofol increased arg1 expression in endotoxin-tolerant cells

The protein expression of Arg1, an M2-associated marker, was assayed using immunoblotting (Figure 2D, n=4). Arg1 expression in the LPS group was significantly lower than that in the Ctrl group ($p=0.0292$). The LPS-triggered downregulation of Arg1 increased after the development of endotoxin tolerance ($p<0.0001$, ET group versus LPS group). Notably, propofol upregulated Arg1 expression in endotoxin-tolerant cells ($p=0.0035$, p+ET group versus ET group).

Propofol upregulated *miR-let-7e* expression during endotoxin tolerance

The transcriptional expression of *miR-let-7e* was measured by real-time PCR after LPS stimulation for 0 h, 4 h, 8 h figure 3, n=4. LPS stimulation did not significantly alter *miR-let-7e* expression. After repeated stimulation with LPS or the development of endotoxin tolerance, *miR-let-7e* levels gradually increased. The levels of *miR-let-7e* in each group after LPS stimulation for 8 h were compared as shown in figure 3, n=4. The *miR-let-7e* level in the ET group was higher than that in the LPS group ($p=0.0025$). Propofol significantly increased the *miR-let-7e* expression triggered by the development of endotoxin tolerance ($p=0.0003$, p+ET group versus ET group).

miR-let-7e mediated the effect of propofol on endotoxin tolerance

To investigate the role of *miR-let-7e* in the effect of propofol on endotoxin tolerance, we conducted the transfection of *miR-let-7e* inhibitors (Figure 4). The success of transfection was confirmed by measuring *miR-let-7e* expression using real-time PCR. Cells in Negative Control (NC) group was transfected with a scrambled sequence, and cells in the Inhibitor group was transfected with *miR-let-7e* inhibitors. *miR-let-7e* expression was reported as the ratio to the Wild-Type (WT) group figure 4, n=4. The levels of *miR-let-7e* in the Inhibitor group was significantly lower than that in the WT and NC group (both $p<0.0001$), indicating the successful inhibition of *miR-let-7e* after transfection.

The expression of TNF-α and IL-6 with or without inhibiting *miR-let-7e* were measured and shown in figure 4, n=4. In contrast to the data in WT cells, propofol did not significantly downregulate levels of TNF-α or IL-6 in cells transfected with *miR-let-7e* inhibitors. In the p+ET group, inhibiting *miR-let-7e* significantly altered the levels of TNF-α ($p=0.025$) and IL-6 ($p=0.0328$), compared to WT cells. Collectively, the results suggested that the enhancing effect of propofol on endotoxin tolerance was counteracted by inhibiting *miR-let-7e*. Furthermore, the protein expression of p-p65, p65, and Arg1 were measured

using immunoblotting figure 4, n=4. In the p+ET group, the p-p65/p65 ratio and Arg1 expression in cells transfected with *miR-let-7e* inhibitors were significantly different from that in WT cells figure 4, p<0.0001 and =0.0001 respectively. After inhibiting *miR-let-7e*, neither p-p65/p65 nor Arg1 expression was altered by propofol. Consistent to the cytokine data, the effects of propofol on NF-κB activation and Arg1 expression in endotoxin tolerant-cells were mediated by *miR-let-7e*.

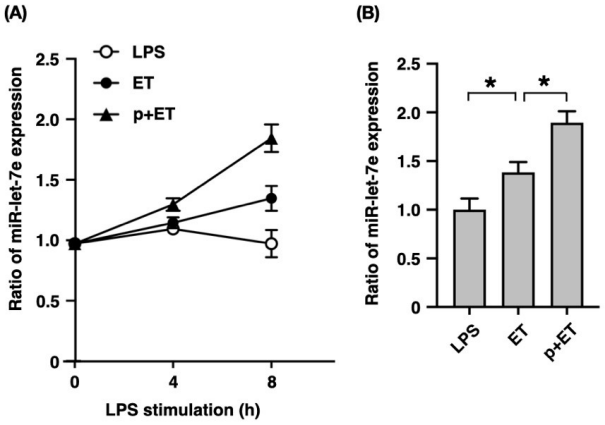

Figure 3: Effects of propofol on microRNA-let-7e (*miR-let-7e*) expression. (A)The transcriptional expression of *miR-let-7e* after stimulation with LPS for 0 h, 4 h or 8 h was measured using real-time Polymerase Chain Reaction (PCR). In each group, levels of *miR-let-7e* were reported as the ratio to the data at LPS 0 h which equaled to 1. (B) Levels of *miR-let-7e* after stimulation with LPS 8 h were compared. Cells were randomly allocated to six groups shown in Figure 2. Data were derived from four independent experiments and expressed as means ± standard deviations. *p<0.05.

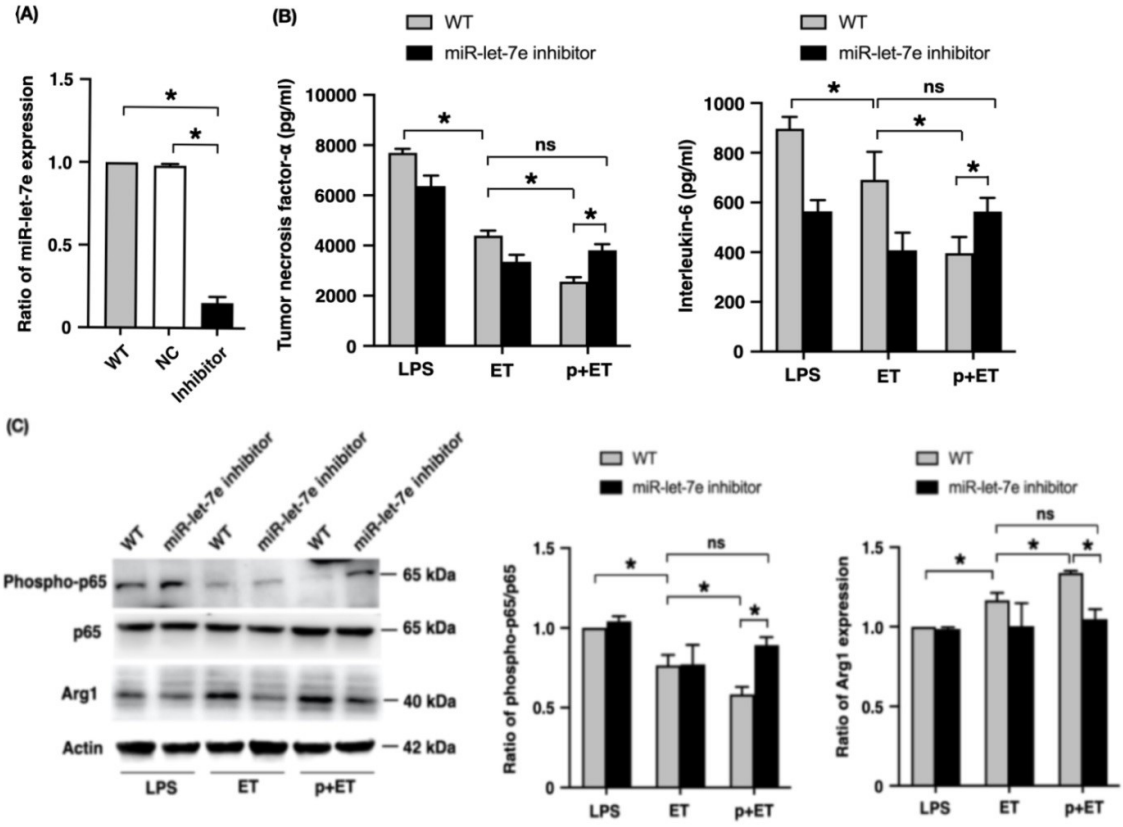

Figure 4: microRNA-let-7e (*miR-let-7e*) mediated effects of propofol on endotoxin Tolerance. (A) The transcriptional expression of *miR-let-7e* was measured using real-time Polymerase Chain Reaction (PCR) after transfection of *miR-let-7e* inhibitors to J774.1 cells. Levels of *miR-let-7e* were reported as the ratio to the Wild-Type (WT) group. (B)The expression of Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6) were measured using enzyme-linked immunosorbent assay. (C) The expression of Nuclear Factor-κB (NF-κB) p65, Phospho-p65 (p-p65), and Arginase-1 (Arg1) were measured by immunoblotting. Actin was the internal control. The level of NF-κB activation was reported as the ratio of p-p65/p65 to actin, relative to Lipopolysaccharide (LPS) WT group. The level of Arg1 was reported as the ratio of Arg1 to actin,

relative to LPS WT group. Cells with or without transfection of *miR-let-7e* inhibitors were randomly allocated to six groups shown in Figure 2. Data were derived from four independent experiments and expressed as means ± standard deviations. NC: negative control. ns: not significant. *p<0.05.

Discussion

In this study, we found that propofol strengthens endotoxin tolerance through inhibiting the release of TNF-α and IL-6. Previous reports demonstrated that propofol attenuates LPS-triggered inflammation by blocking TLR4 signaling or inhibiting Nod-like receptor protein 3 inflammasome [20, 21]. Here we revealed a novel mechanism underlying the anti-inflammatory effect of propofol. We also found that the development of endotoxin tolerance led to the upregulation of Arg1, which was supported by previous reports showing polarization of endotoxin-tolerant macrophages toward M2-phenotype [2-4]. In addition, our results that propofol increased Arg-1 expression during endotoxin tolerance may infer that propofol shifted endotoxin tolerant-macrophages toward M2 phenotype (Figure 5). However, it is hard to determine macrophage polarization through single gene expression [22]. The validity of cell markers indicating the effect of propofol on macrophage M2-polarization remains to be determined.

Propofol regulates a variety of microRNAs that target LPS signaling, including miRNA-155 and miRNA-216a-5p [23, 24]. Based on our data, propofol promoted endotoxin tolerance mediated by *miR-let-7e*. Upregulation of miR-let-7e leads to the inhibition of TLR4 expression,which may mediate the enhancing effect of propofol on endotoxin tlerance [10]. Figure 5 shows our hypothetical illustration of how propofol acts on endotoxin tolerance. It is not surprising that *miR-let-7e* plays a key role in the effect of propofol on endotoxin tolerance, since the members of let-7 family modulate NF-κB pathway and modulate immunologic responses [25]. Notably, let-7 family microRNAs were also demonstrated to regulate opioid tolerance targeting the μ-opioid receptor [26].

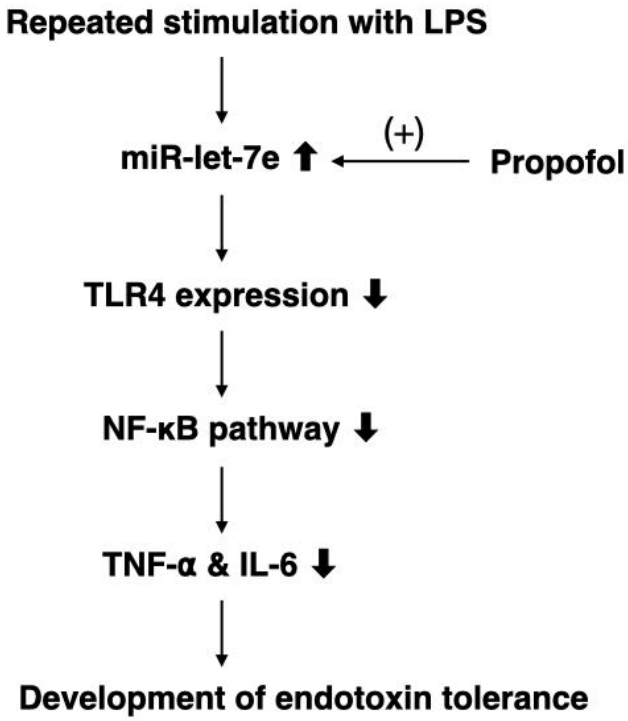

Figure 5: The hypothetical illustration of how propofol acts on endotoxin tolerance.

Our study had some limitations. First, we only investigated propofol at a single dose which may exert an effect different from that of the accumulated dose. Second, how propofol upregulates *miR-let-7e* remains unstudied. Despite these limitations, our findings have clinical impacts in the future. In clinical, propofol is widely used as a sedative agent in critical patients [12, 27]. Some studies show that sedation with propofol increases length of ICU stay, compared to sedation with other sedative agents [12, 28, 29]. A clinical study revealed that propofol infusion for septic patients prolongs duration of mechanical ventilation [27]. Furthermore, in a rat model of sepsis, propofol increased morbidity and mortality [30]. As endotoxin tolerance is often accompanied by sepsis, the impact of propofol on

the outcomes of septic patients should be considered. Based on our findings, we speculate that continuous propofol infusion strengthens endotoxin tolerance, which may lead to persistent immunosuppression and high susceptibility to secondary infection. This study may provide a novel perspective for searching for an explanation for the previously published worse outcomes from propofol [31].

Conclusion

In conclusion, propofol enhances endotoxin tolerance by upregulating *miR-let-7e*. From this perspective, propofol infusion may also prolong the immunosuppressive status when used continuously as a sedative for critical patients. The immunomodulatory effect of propofol may determine the outcomes of inflammatory pathologies accompanied by endotoxin tolerance.

Statement of ethics

This study protocol was reviewed and approved by Far-Eastern Memorial Hospital Biosafety Committee (MOST 109-2314-B-418-015-MY2).

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Author contributions

The authors confirm contribution to the paper as follows: study conception and design: YYC and CWL; data collection: YYC, YHC and WHJ; analysis and interpretation of results: YYC, and TYL; draft manuscript preparation: YYC, TYL and CWL. All authors reviewed the results and approved the final version of the manuscript.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

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