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Atractylenolide Ⅰ protects against lipopolysaccharide-induced disseminated intravascular coagulation by anti-inflammatory and anticoagulation effect

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ABSTRACT

Objective: To investigate whether atractylenolide Ⅰ (ATL-Ⅰ) has protective effect on lipopolysaccharide (LPS)-induced disseminated intravascular coagulation (DIC) *in vivo* and *in vitro*, and explore whether NF-κB signaling pathway is involved in ATL-Ⅰ treatment.

Methods: New Zealand white rabbits were injected with LPS through marginal ear vein over a period of 6 h at a rate of 600 μg/kg (10 mL/h). Similarly, in the treatment groups, 1.0, 2.0, or 5.0 mg/kg ATL-Ⅰ were given. Both survival rate and organ function were tested, including the level of alanine aminotransferase (ALT), blood urine nitrogen (BUN), and TNF-α were examined by ELISA. Also hemostatic and fibrinolytic parameters in serum were measured. RAW 264.7 macrophage cells were administered with control, LPS, LPS + ATL-Ⅰ and ATL-Ⅰ alone, and TNF-α, phosphorylation (P)-IκBα, phosphorylation (P)-NF-κB (P65) and NF-κB (P65) were determined by Western blot.

Results: The administration of LPS resulted in 73.3% mortality rate, and the increase of serum TNF-α, BUN and ALT levels. When ATL-Ⅰ treatment significantly increased the survival rate of LPS-induced DIC model, also improved the function of blood coagulation. And protein analysis indicated that ATL-I remarkably protected liver and renal as decreasing TNF-α expression. *In vitro*, ATL-I obviously decreased LPS-induced TNF-α production and the expression of P-NF-κB (P65), with the decrease of P-IκBα.

Conclusions: ATL-Ⅰ has protective effect on LPS-induced DIC, which can elevate the survival rate, reduce organ damage, improve the function of blood coagulation and suppress TNF-α expression by inhibiting the activation of NF-κB signaling pathway.

1. Introduction

Disseminated intravascular coagulation (DIC) is an acute acquired syndrome characterized by intravascular coagulation and widespread inflammatory response. With 30–40% mortality rate, DIC may occur secondary to a variety of diseases such as infection, cancer, *etc*. [1]. As individuals are infected by gram-negative bacteria, LPS can stimulate the activation of macrophages, leading to various inflammatory cytokines secreted, and endotoxemia such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1). TNF-α is one of the most dominant early releasing cytokines in endotoxemia. With the accumulation of TNF-α, exacerbation of acute inflammation response ensues from up-regulation of cell adhesion molecule and reactive oxygen species. It may conduce to endothelial cell injury and intravascular coagulation, finally result in DIC within a short time [2–7]. Thus, inflammation played a vital role in the progress of DIC.

Rhizoma Atractylodis Macrocephalae (‘Baizhu’ in Chinese) is an important Chinese traditional medicine used to treat inflammatory diseases and digestive disorders [8,9]. ATL-Ⅰ is one of the major bioactive components separated from Baizhu and

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it has been widely used for anti-inflammation and has showed protective effect on lung edema induced by LPS [10]. In addition, previous studies indicated that ATL-I can significantly depress the level of pro-inflammatory cytokines such as serum IL-1, TNF-α [11]. And recent researches also showed that ATL-Ⅰ can alleviate inflammation reaction to offer protective effect on the mice model of sepsis [12]. Therefore, we assumed that ATL-I has protective effect on LPS-induced DIC.

Furthermore, NF-κB pathway is an important signaling cascade in regulating inflammatory responses [13,14]. LPS can induce inflammatory response by increasing translocation of NF-κB from cytoplasm into nucleus as well as increasing phosphorylating IκBα in the cytoplasm, which can generate the accumulation of TNF-α and exacerbation of acute inflammation response, finally leads to DIC [15,16]. Thus, those studies indicated that ATL-Ⅰ can inhibit the translocation of NF-κB induced by LPS.

Given all that, we highly hypothesize that ATL-Ⅰ may have the protective effect on DIC. Thus, we investigated the potential role of ATL-I in DIC models and tried to clarify the mechanism *in vitro* and *in vivo*. Both survival rate and organ function were tested, including the level of ALT, BUN, and TNF-α were examined by ELISA. Also hemostatic and fibrinolytic parameters in serum were measured. Moreover, TNF-α, phosphorylation (P)-IκBα, IκBα, phosphorylation (P)-NF-κB (P65) and NF-κB (P65) were determined by Western blot to investigate whether NF-κB pathway was involved in ATL-Ⅰ treatment.

2. Materials and methods

2.1. Animals

New Zealand white rabbits (male, weight 2.0–2.5 kg, Grade Ⅱ) were purchased from the Medical Experimental Animal Center of Guangzhou Province, China. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures utilized in this investigation were in accordance with institutional guidelines for animal research. This study also conformed to the Guide for Using and Caring Laboratory Animals approved by the National Institutes of Health. This article does not contain any studies with human participants performed by any of the authors.

2.2. Materials

ATL-Ⅰ (purity > 98%) was purchased from PureOne Biotechnology (Shanghai, China). LPS was from Sigma (St Louis, USA). Fatal bovine serum (FBS) and dulbeccos modified Eagles medium (DMEM) were purchased from BD Bioscience (Franklin Lakes, N.J. Heparin was purchased from Beijing Tobishi Pharmaceutical company. TNF-α ELISA kit was obtained from Rapid Bio Lab (Calabasas, CA). Reagents used for testing the activity of antithrombin Ⅲ (ATⅢ) and protein C were purchased from Sun Biotechnology Company (Shanghai, China). All other reagents were supplied by commercial sources.

2.3. Models establishment and drugs treatment

All procedures were conducted in accordance with the ethical guidelines approved by Laboratory Animals Care and Use Committee of Sun Yat-sen University (Guangzhou, China). All animals were anesthetized with pentobarbital (30 mg/kg) injected through the marginal ear vein. After that, to maintain animal anesthesia, 30 mg sodium pentobarbital was intraperitoneally injected for every 2 h, 4 h and 6 h. DIC models were performed by injecting LPS (in 60 mL of saline solution) through marginal ear vein over a period of 6 h at a rate of 600 μg/kg (10 mL/h).

With LPS infusion, treatments were conducted concurrently through the contralateral marginal ear vein. Twelve different groups were established, 6 of which (15 animals in each) were for the measurement of survival rate, another 6 (10 animals in each) were for the measurement of biochemical indexes and TNF- α. The 6 groups were as follows. Normal control group were only given 60 mL of saline solution at a rate of 10 mL/h through marginal ear veins. The LPS-induced group were injected LPS with saline solution over a period of 6 h at a rate of 600 μg/kg (10 mL/h). The heparin control group were injected with heparin in 60 mL of saline solution over a period of 6 h at a rate of 600 IU/kg (10 mL/h). While the treatment groups (low-, medium-, or high-dose ATL-Ⅰ) were respectively given 1.0, 2.0, or 5.0 mg/kg in 60 mL of saline solution over a period of 6 h at a rate of 10 mL/h.

2.4. Determination of survival rate

The 6 groups which contained 15 animals in each were given different ATL-Ⅰ treatments. Subsequently, the survival rates of different groups were recorded for 24 h. All live animals after all the experimental procedures were injected over dose pentobarbital.

2.5. Sample analyses

Before LPS infusion and at 2 h and 6 h after the infusion, blood specimens (1 mL) were taken from all animals. Based on chromogenic substrates, protein C and ATⅢ were tested according to the reagent pack instructions. The prothrombin time (PT), activated partial thromboplastin time (APTT), fibrin fibrinogen degradation products (FDP), fibrinogen (FIB) and platelets level were measured by the automatic analyzer (Sysmex SE-9500, Sysmex CA 1500, Japan). Moreover, the blood urine nitrogen (BUN) and alanine aminotransferase (ALT) were determined by 7170A automatic analyzer (Hitachi, Japan).

2.6. Elisa kit

We collected 1 mL plasma from each rabbit of all animals and stored it at −20 °C until assayed. ELISA kit was utilized to respectively detect the concentrations of TNF-α in plasma samples.

2.7. Cell culture

The RAW 264.7 macrophage cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated FBS, and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin), and were maintained at 37 °C in 5% CO_2 incubators.

2.8. Cell viability assay and cytotoxicity assay

The cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma–Aldrich, St. Louis, MO) assay. Briefly, RAW 264.7 macrophage

cells were seeded in 96-well plates at the density of 2000 cells/well. The control group and the four ATL-Ⅰ treatment groups (low, medium, high dose) were set and reagents were added accordingly: cultured medium, 0, 10, 20, 50, 100 μM ATL-Ⅰ. A total of 10 mL MTT solution (5 mg/mL in PBS) was added to each well after culturing for 24 h, and incubating for 4 h to induce the production of formazan crystals. After that 100 mL DMSO was added to achieve solubilization of the formazan crystal, and the optical density (OD) being determined at 490 nm using an EXL 800 microimmunoanalyzer (Bio-Tek Instruments, Burlington, VT).

2.9. Established LPS-induced endotoxemia model in vitro

To investigate the effect of ATL-I on inflammatory cytokines from LPS-induced DIC cell model, RAW 264.7 macrophage cells were cultured in the 6-well plates at the density of 5×10^5 a well. A control group, an LPS-induced group, an LPS + ATL-Ⅰ group and a ATL-Ⅰ treatment group were set and cultured medium, 1 mg/mL LPS, 1 mg/mL LPS + 50 μM ATL-Ⅰ, 50 μM ATL-Ⅰ were added respectively. Then all groups were cultured with 2 mL cultured medium. After culturing for 6 h, cells were collected for Western blot assays.

2.10. TNF-α assay in vitro

To measure TNF-α level *in vitro*, the concentration of TNF-α in the collected cell-free supernatants was measured by commercial ELISA kit (Rapid Bio Lab, Calabasas, CA) following the manufactory's instruction.

2.11. Western blot

The protein levels of P-NF-κB (P65), NF-κB (P65), P-IκBα, IκBα, TNF-α and β-actin were measured by Western blot, using specific antibodies against the corresponding proteins. Moreover, NF-κB (P65) and β-actin were chosen as loading controls. Briefly, the culture medium in each well was removed and the dishes were washed for three times with phosphate buffer saline (PBS) after 8 h *in vitro* experiment. After that, 80 mL of RIPA (cell lysis buffer, 150 mM NaCl, 0.1% SDS, 2 mg/mL aprotinin, 2 mg/mL leupeptin, 1% NP-40) was respectively added to each well, cell extractions were removed and only the supernatants were collected after centrifugation. 8–10% SDS–PAGE was used to segregate the cell extractions and electro-transferred to PVDF membranes. The membranes were blocked using 5% skim milk in TBST (Trisbase with 0.1% Tween-20) for an hour, washed by TBST and incubated with primary antibodies of P-NF-κB (P65), NF-κB (P65), TNF-α and β-actin (diluted 1/1000 in 5% skim milk with TBST) at 4 °C over night. Then the membranes were incubated with anti-rabbits IgG antibodies for an hour. Finally, they were exposed in darkness to show the immunoreactive bands. The intensity of total protein bands per lane was evaluated by densitometry. Negligible loading/transfer variation was noted between samples.

2.12. Data analyses

Differences between group data were evaluated for significance, using either a non-para-metric test (the Kruskal–Wallis H test) or two-way repeated measures. The repeated measures analysis of variance was used for the multivariate analyses. All experiments were repeated at least three times, and the data were presented as the mean ± SD unless otherwise noted. Data of the activities of ATⅢ and protein C and the concentration of ALT and BUN at 2 h and 6 h were converted to percentages, with a value of 100% assumed for basal data. Differences with P values of less than 0.05 were considered to be statistically significant.

3. Result

3.1. ATL-I improved the survival rate on LPS-induced DIC in vivo

To appraise the protective effect of ATL-I on LPS-induced DIC, a 24 h survival experiment was performed. New Zealand white rabbits were injected LPS (in 60 mL of saline solution) through marginal ear vein over a period of 6 h at a rate of 600 μg/kg (10 mL/h) LPS with ATLⅠ treatment. As shown in Figure 1, in the LPS-control group, only 4 rabbits survived in fifteen rabbits (26.7%). In ATL-I treatment groups, the survival rate had improvement of various degree. According to ATL-Ⅰ treatment groups, the survival rate of low (1 mg/kg), medium (2 mg/kg), high-dose (5 mg/kg), specifically was 33.3% (5/15), 46.7% (7/15), 66.7% (10/15).

3.2. Serum ALT and BUN level decreased in LPS-induced DIC with ATL-Ⅰ treatment in vivo

To investigate whether ATL-Ⅰ could protect organ and reduce damage against LPS-induced DIC, serum samples were obtained to test the ALT and BUN level to assess the protection of ATL-I in liver and kidney. As the results showed, compared with the control group, the ALT and BUN was increased significantly in the LPS-induced group.

Significantly, the serum ALT and BUN level in the LPS-induced group increased compared with the control group. And compared to the LPS-induced group, ATL-I treatment groups notably decreased the serum ALT level ($P < 0.05$, Table 1).

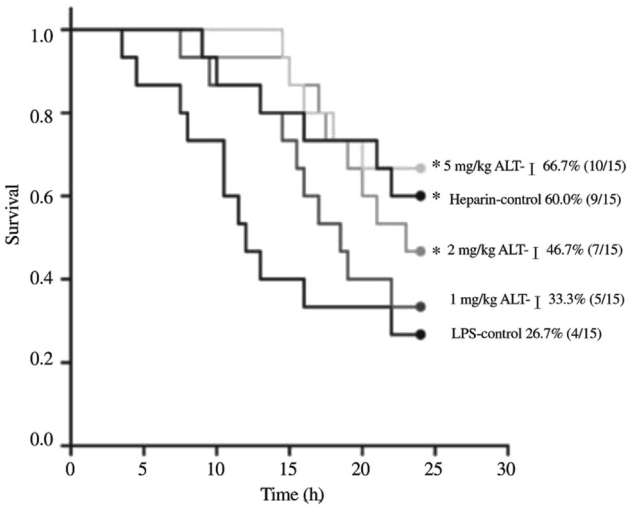

Figure 1. ATL-Ⅰ improved the survival rate on LPS-induced DIC rabbit model.
DIC was induced by 600 μg/kg LPS. 1, 2, 5 mg/kg ATL-1 and 600 IU/kg heparin were simultaneously intravenous injected with LPS. Survival of each group was monitored on over 24 h. $^*P < 0.05$ compared with the LPS-control group ($n = 15$).

Table 1
Effect of ATL-I on the level (%) of plasma ALT and BUN in LPS-induced DIC group *in vivo* and protein C (%) and ATIII (%) 2 h and 6 h after LPS infusion into rabbits in different treatment groups (n = 10).

Group	Plasma ALT level		Plasma BUN level		Protein C		ATIII	
	2 h	6 h	2 h	6 h	2 h	6 h	2 h	6 h
Normal control	105.19 ± 2.12	103.23 ± 9.11	116.48 ± 3.34	112.25 ± 6.29	99.16 ± 0.98	98.17 ± 1.36	100.18 ± 1.79	99.27 ± 1.43
LPS	197.55 ± 23.28$^{\#}$	319.86 ± 40.42$^{\#}$	209.11 ± 20.21$^{\#}$	415.39 ± 27.91$^{\#}$	68.58 ± 15.72	51.25 ± 13.92	86.78 ± 5.47	60.81 ± 12.03
1 mg/kg ATL-I	169.74 ± 20.89*	249.33 ± 29.12*	154.74 ± 17.46*	272.42 ± 23.13*	79.16 ± 15.69	76.21 ± 20.33	92.48 ± 12.65*	80.04 ± 16.84*
2 mg/kg ATL-I	134.75 ± 18.26*	199.36 ± 38.81*	151.41 ± 15.91*	201.54 ± 28.13*	87.02 ± 16.74*	89.42 ± 19.37	93.64 ± 11.92**	85.21 ± 14.47**
5 mg/kg ATL-I	113.20 ± 16.76*	147.62 ± 30.51*	145.89 ± 18.12*	157.29 ± 20.12*	90.33 ± 10.74*	84.59 ± 14.52	91.06 ± 7.93**	91.89 ± 4.85**
Heparin-control	115.81 ± 19.19*	147.75 ± 34.33*	140.98 ± 25.62*	178.05 ± 25.89*	80.31 ± 16.17*	80.93 ± 13.48	86.43 ± 12.55**	89.98 ± 9.62**

Before LPS infusion and 2 h, 6 h after the start of the infusion, blood samples were collected immediately. An automatic analyzer detected the level of ALT and BUN separately. Result of ALT and BUN at 2 h and 6 h were converted to percentages assuming a value of 100% of basal data. Values were expressed as the mean ± SD percent of the initial value before LPS infusion for ALT and BUN. Protein C and ATIII data were expressed as the mean ± SD. $^{\#}P < 0.05$, compared with the control group; $^{*}P < 0.05$, compared with the LPS-induced group; $^{**}P < 0.01$, compared with the LPS-induced group.

Similarly, the serum BUN level showed change as the serum ALT level, significantly decreased in ATL-I treatment groups, compared with the LPS-induced group ($P < 0.05$, Table 1).

3.3. ATL-I prevented thrombosis and embolism in LPS-induced DIC in vivo

Base on above results, it could be highly hypothesized that ATL-I could prevent thrombosis and decrease the possibility of blood clots so that blood vessels could keep pristine and improve organ perfusion. To verify this hypothesis, determination of hematological parameters, which included the plasma APTT, PT, platelet counts, fibrinogen levels, FDP levels, activities of protein C and ATIII, was performed for all groups. Compared with the normal group, the value of LPS-induced group indicated in enhancement of APTT, PT, FDP, and descent of platelet, fibrinogen, activities of protein C and ATIII (Table 1).

After the infusion of both ATL-I and heparin, the levels of APTT (s), PT (s) and FDP (μg/L) decreased, and the levels of platelet ($\times 10^9$/L), fibrinogen (g/L) increased. The activities of protein C and ATIII were also alleviated significantly (Table 2).

3.4. ATL-I reduced TNF-α expression in vivo

TNF-α was one of major factor involved in the acute inflammation, which generally showed higher expression in the early period of DIC. To investigate whether ATL-I could reduce the expression of TNF-α, and ameliorate pathological injury in main organs in LPS-induced DIC, the variances of TNF-α in 12 h-observation were traced in different groups *in vivo* (Figure 2). The expression of TNF-α significantly increased at 1, 4, 8 and 12 h after the infusion of LPS, and the highest point appeared at 8 h, compared to the normal group.

In ATL-I treatment groups, the expression of TNF-α declined to varying degrees according to low-dose, middle-dose and high-dose. Although the low-dose group (1 mg/kg) did not obviously restrain the high expression of TNF-α at 1 h, as time advance, lower level of TNF-α than LPS-induced group were observed in 4, 8 and 12 h. Furthermore, middle-dose (2 mg/kg) and high-dose (5 mg/kg) ATL-I significantly decreased the level of TNF-α at 1, 4, 8, and 12 h, which showed similar results in the heparin group.

3.5. ATL-I impacted RAW 264.7 macrophage cells on cell viability and cytotoxicity

To assess the effect of ATL-I on cell viability and cytotoxicity, the MTT test was performed. There were no significant differences among the 10, 20, 50, 100 μM ATL-I treatment groups and the control group. According to ATL-I treatment groups, the optical density of 10, 20, 50, 100 μM ATL-I, specifically was 1 ± 0.11, 0.95 ± 0.07, 0.92 ± 0.05, 0.90 ± 0.08, 0.86 ± 0.06. However, compared with the control group, optical density of the 100 μM ATL-I group was slightly decreased, which indicated that the concentration of ATL-I was less than 100 μM, it would not result in considerable cell apoptosis.

3.6. ATL-I suppressed TNF-α expression in RAW 264.7 macrophage cells

We hypothesized that ATL-I suppressed TNF-α level in RAW 264.7 cell, and alleviated the inflammation reaction. To verify this hypothesis, inflammatory cell model was constructed by LPS acting RAW264.7 macrophage cell line and the expression of TNF-α in different groups were measured by Western blot (Table 3). As the results revealed, there was a decided difference in different groups that the amount of TNF-α in the ATL-I treatment group was significantly decreased compared with LPS-induced group. And both in the control group and the ATL-I group, the level of TNF-α was too low to be observed.

Table 2
Hemostatic and fibrinolytic parameters 2 h and 6 h after LPS infusion into rabbits in different treatment groups (n = 10).

Group	APTT		PT		Platelets		Fibrinogen		FDP	
	2 h	6 h	2 h	6 h	2 h	6 h	2 h	6 h	2 h	6 h
Normal control	9.14 ± 0.87	8.67 ± 0.72	6.92 ± 1.07	7.23 ± 0.84	428.17 ± 26.94	406.63 ± 27.04	4.65 ± 0.67	4.80 ± 0.85	–	–
LPS-induced	15.65 ± 2.09	24.86 ± 3.53	10.17 ± 1.61	18.39 ± 2.45	314.58 ± 30.61	176.75 ± 21.74	3.06 ± 0.94	1.92 ± 0.53	68.39 ± 15.02	96.94 ± 20.36
1 mg/kg ATL-I	13.45 ± 1.92*	15.14 ± 2.81*	8.02 ± 1.92**	9.54 ± 0.86**	365.33 ± 29.78**	254.29 ± 20.87**	3.83 ± 0.66*	3.04 ± 0.57**	37.85 ± 15.27**	60.39 ± 21.38
2 mg/kg ATL-I	11.24 ± 1.47**	12.62 ± 1.73**	7.13 ± 1.06**	8.34 ± 0.87**	360.63 ± 29.46**	354.82 ± 31.58**	4.13 ± 0.62*	4.06 ± 0.80**	30.34 ± 14.87**	43.62 ± 13.04*
5 mg/kg ATL-I	10.76 ± 0.94**	10.59 ± 1.51**	6.95 ± 1.14**	7.63 ± 0.89**	375.26 ± 30.01**	355.53 ± 24.97**	4.33 ± 0.94*	3.92 ± 0.78**	32.36 ± 8.14**	40.77 ± 9.58*
Heparin-control	13.27 ± 1.18**	16.29 ± 2.26*	7.35 ± 0.92**	8.77 ± 1.10**	320.14 ± 34.65	302.22 ± 27.47	4.15 ± 1.18*	3.67 ± 0.84**	35.16 ± 10.78**	44.95 ± 11.93*

$^{*}P < 0.05$, compared with the LPS-induced group, $^{**}P < 0.01$, compared with the LPS-induced group.

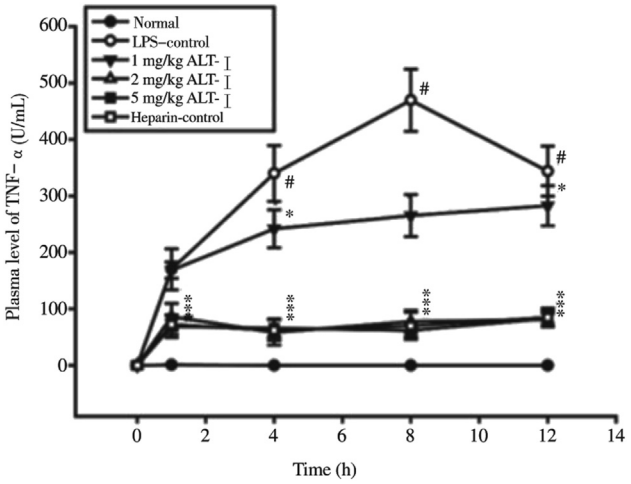

Figure 2. Effect of ATL-I on the plasma levels of TNF-α in LPS-induced DIC rabbits.

Values were expressed as the mean ± SD percent of the initial value before LPS infusion. $n = 10$. $^{\#}P < 0.05$, compared with the control group; $^{*}P < 0.05$, compared with the LPS induced group.

Table 3

Western blot analyses on LPS-induced DIC macrophage model.

Group	TNF-α/β-actin	P-NF-/β/β-actin	P-I4ct/β-actin
Control	0.23 ± 0.08	0.38 ± 0.10	0.17 ± 0.05
LPS	1.10 ± 0.35$^{\#}$	1.76 ± 0.36$^{\#}$	1.46 ± 0.26$^{\#}$
LPS + ATL-I	0.35 ± 0.23*	1.01 ± 0.16*	1.21 ± 0.22*
ATL-I	0.18 ± 0.17*	0.44 ± 0.1*	0.42 ± 0.09*

Data were expressed as mean ± SD, analyzed by one way ANOVA. $^{*}P < 0.05$, compared with LPS group; $^{\#}P < 0.05$, compared with the control group; $n = 3$. Control: the control group; LPS: the LPS-induced group, LPS + ATL-I: LPS + 50 μM ATL-I; ATL-I: 50 μM ATL-I.

3.7. ATL-I inhibited TNF-α level by regulating NF-κB (P65) signaling in RAW 264.7 macrophage cells

To further investigate whether the NF-κB (P65) signaling pathway was involved in ATL-I inhibiting the TNF-α level, inflammatory cell model was constructed again by LPS acting RAW264.7 macrophage cell line for the exploration of mechanism. Both the expression of phosphorylation (P)-IκBα and phosphorylation (P)-NF-κB(P65) decreased in the ATL-I treatment group, compared to the LPS-induced group.

4. Discussion

DIC is an acute acquired syndrome characterized by intravascular coagulation, and micro-thrombi formation, which leads to acute systemic inflammatory response and hypoperfusion-induced organ damage [17,18]. And the rate of complications of DIC generally increases in proportion to occurrence of systemic inflammatory response syndrome (SIRS) [19,20]. In addition, septicemia, especially those caused by gram-negative bacteria, is thought to be an important cause of DIC. LPS is the most frequent material used to establish DIC model, which is a major constituent of outer membrane of gram-negative bacteria. As we all know, LPS-induced DIC mainly involves two aspects of mechanism: one is evoking systemic inflammatory response, the other one is motivating coagulation and anti-coagulation disturbance [21,22]. Thus, APTT, PT, FDP, platelet, fibrinogen, activities of protein C and ATIII were selected as the indicators for estimating severity of microcirculation disturbance. ALT and BUN were observed for estimating severity of organ damage. And TNF-α was observed for estimating severity of acute inflammation. In this study, LPS-induced DIC model appeared representative change, characterized by the increase of APTT, PT, FDP and decrease of platelet, fibrinogen, activities of protein C and ATIII. And also the increase of plasma levels of TNF-α, ALT and BUN were observed.

In LPS-induced DIC model, after evoking inflammatory response and coagulation disorders, multiple organs failure appears constantly, possibly leading to fatal damage [23–25]. In this study, we found that ATL-I showed the protective effect on LPS-induced DIC, which elevated the survival rate as the dose increase. Combined with the decrease of plasma levels of ALT and BUN, it indicated ATL-I reduced LPS-induced damage and improved organs function, resulted in the development of survival rate. Thus, based on those result, we highly hypothesized that coagulation and anti-coagulation disturbance was improved as the organ function elevated under the ATL-I treatment.

As we know, coagulation and anti-coagulation disturbance is the important characteristic of DIC, characterized by intravascular coagulation and the consumption of clotting factors. When microthrombosis forms in organs, hypoperfusion directly results in resultant cellular dysfunction [26]. APTT and PT are respectively indicators of intrinsic and extrinsic coagulation pathway. From the results can be seen that both ATL-I and heparin infusion significantly attenuated the increase of APTT and PT. Also platelet counts, fibrinogen concentrations, FDP were measured. The decrease of platelet and FDP, increase of fibrinogen concentrations, which mean ATL-I can address severe coagulopathy caused by the reduction of the consumption of coagulation factors. Thus, the activation of protein C and ATIII further suggested that ATL-I can improve prognosis. Taken together, ATL-I prevented thrombosis and decreased the possibility of blood clots, improved organ perfusion and function and lowered the incidence of multiple organ dysfunction.

Moreover, there is a wealth of evidence to suggest that inflammation and thrombosis form a vicious circle. Serious inflammation reactions can motivate coagulation disorders, as coagulation disorder progresses, inflammation respond intensifies [22]. In our study, the level of TNF-α *in vivo* and *in vitro* were detected respectively by ELISA and western blot. The analysis indicates that the level of TNF-α *in vivo* and *vitro* were both down-regulated after the ATL-I treatment, compared with the LPS-induced group. Thus, we found the increasing evidence showed that ATL-I has an anti-inflammatory effect through inhibited the expression of inflammatory cytokine, such as TNF-α.

Furthermore, the most well-known anti-inflammatory molecular mechanism is NF-κB signaling pathway. As NF-κB signaling pathway was inhibited, inflammation cytokine secretion decrease. To investigate that whether the reduction of TNF-α expression is regulated by NF-κB signaling pathway in ATL-I treatment, western blot analysis was processed. And the result indicated that the level of TNF-α decreased, while both P-NF-κB and P-IκBα decreased in the ATL-I treatment group. The reduction of P-IκBα decreased the phosphorylation of NF-κB, and it would inhibit the activation of downstream signal and reduce the accumulation of TNF-α. It suggested that ATL-I

inhibited TNF-α expression by regulating NF-κB (P65) signaling.

Given all above result, our study indicated that ATL-Ⅰ had protective effect on LPS-induced DIC, which improved the function of blood coagulation and suppressed inflammation response, such as decreasing TNF-α expression by inhibiting the activation of NF-κB signaling pathway. Thus, organ damage reduced under the ATL-Ⅰ treatment, as the survival rate rose. And over other potential ATL-Ⅰ treatment needs more clinical trial to prove.

Conflict of interest statement

We declare that we have no conflict of interest.

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