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Research Article
Andrographolide had Positive Effects on Anti-inflammatory and Protected Against LPS-induced DIC in Rabbits

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Abstract

Background: The occurrence of Disseminated Intravascular Coagulation (DIC) could lead to multiple organ failure, promoting the development of the critically ill patient's condition. Recently pharmacological studies had shown that andrographolide had effects on anti-inflammatory and preventing thrombosis. **Materials and Methods:** In order to monitor, if andrographolide has protective effects on DIC rabbits, the DIC models were established by infusing lipopolysaccharide (LPS) in 60 mL of saline solution at a rate of 10 mL/kg/h through the rabbits' marginal ear vein over a period of 6 h. The DIC rabbits were divided into 5 groups; low (1.0 mg kg^{-1}), medium (2.0 mg kg^{-1}), high (5.0 mg kg^{-1}) andrographolide groups, LPS (saline solution) group, heparin (500 IU kg^{-1}) group. The normal control group treated with saline solution, which was given neither LPS nor andrographolide before. Survival rate was monitored by recording the deaths within 6 and 24 h after drug treatment and renal function was detected, including Blood Urine Nitrogen (BUN) and tissue section. Moreover, the concentrations of TNF-α and IL-1β were determined and the activities of ATIII, protein C, PAI-1 and t-PA in serum level were measured, which was collected at 2 and 6 h post-infusion. All data was presented as Mean±Standard Deviation. Survival rate was analyzed by chi-square test. Differences between two groups were analyzed by one way ANOVA analysis. **Results:** Treatment of andrographolide significantly increased survival rate and reduced renal damage and increased the activities of protein C, the levels of ATIII and t-PA. But the levels of FDP and PAI-1 were decreased and the suppression of TNF-α and IL-1β secretion could be found as well. **Conclusion:** All the data suggested that had positive effects in intervention in DIC rabbits and prevented excessive inflammation via inhibiting the level of TNF-α and IL-1β in serum. Andrographolide could be a potential drug for DIC treatment.

Key words: Andrographolide, LPS, DIC, inflammatory, renal damage, survival rate

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lipopolysaccharide (LPS) is one of the most powerful toxin constituents of Gram negative bacteria cell walls[1]. When it entered human or experimental animals' bodies, it would cause many pathophysiologic and immune responses. For example, the macrophages would be activated and lead to series of reactions[2]. Including secretion of pro-inflammatory cytokines, expression of adhesion molecules and coagulation factors[3]. These uncontrollable changes could eventually lead to Disseminated Intravascular Coagulation (DIC), which would turn into multiple organ dysfunction or even death[4].

The DIC is a life threatening syndrome arising from many kinds of causes, including LPS-induced endotoxemia[5,6]. It is a thrombosis hemorrhagic disorder and occurs as a secondary complication in many diseases[7]. As an acquired syndrome, it can not only active the subsequent intravascular fibrin formation and intravascular coagulation, but also be accompanied with bleeding tendency or deficient fibrinolysis[8,9]. As a result, there would be diffuse activation of blood clotting, as well as increment of fibrin formation.

Moreover, there are lots of pro-inflammatory cytokines released in DIC, such as TNF-α and IL-1β and TNF-α is quite important one[10]. The level of TNF-α is potentially involved in tissue damage[11] and it was reported that protective effects can be gained while suppressing level in DIC animal models[12,13]. The IL-1β is another cytokine, which can stimulate cell proliferation and differentiation in immunoreactions[14-16]. Moreover, excessive release of IL-1β *in vivo* would also result in hyperalgesia and mediator neuroinflammation[17,18]. Therefore, if a reagent could suppress TNF-α and IL-1β levels in the early phase of DIC, it indicated that it had protective effects against DIC.

In recent years, treatments of DIC have focused on how to decrease the over production of inflammatory cytokine, inhibit coagulation processes and promote fibrinolysis and it was reported that some effective drugs could protect against DIC via direct degradation of thrombosis and activation of protein C[19]. *Andrographis paniculata* is a traditional herbal medicine and it had been approved as a botanical drug in clinical application for many years in China. Its main constituent andrographolide, has been shown to have positive effects on anti-inflammatory activity and preventing the formation of thrombus in clinical efficacy[20]. Therefore, andrographolide had been used on DIC models in this study.

To study, if andrographolide had protective effects against DIC, an LPS induced DIC model in rabbits was used, both survival rate and organ function were detected, including blood urine nitrogen (BUN, the marker of renal injury) and tissue section and then, pro-inflammatory cytokines level, including TNF-α and IL-1β, was determined to evaluate its effects on anti-inflammatory activity. Moreover, to detect coagulation and fibrinolytic system in plasma, ATIII, protein C, PAI-1 and t-PA activity was measured according to the reagent pack instruction based on chromogenic substrates. This study had demonstrated that andrographolide had positive effects on LPS-conduced DIC via improving fibrinolytic system and anticoagulative system and inhibiting over-expression of inflammatory cytokines in rabbits.

MATERIALS AND METHODS

Experimental animals: Adult male New Zealand white rabbits (weight 2-3 kg), were supplied by Medical Experimental Animal Center of Guangdong Province, China. All rabbits were kept in cages before experiment with water and food *ad libitum*. All animal experiments were conducted according to the ethical guidelines of National Guide for the Care and Use of Laboratory Animals and approved by Jinan University Animal Care and Use Committee (Guangzhou, China).

Materials: Andrographolide injection (50 mg/2 mL Cat. No. 20081226) was purchased from Jiangxi Qingfeng Pharmaceutical Co. Ltd., China. Heparin (500 IU kg^{-1}) was gained from Shandong Bausch and Lomb Freda Co. Ltd., China. The fibrinogen concentration determination reagent pack (Clauss method) and the reagent packs for the activity assays of antithrombin III (ATIII), protein C, plasminogen, PAI-1 and t-PA were obtained from Sun Biotechnology Company (Shanghai, China). The t-PA ELISA kit was purchased from ASSAYPRO (USA). The PAI-1 ELISA kit, the recombined TNF-a, IL-β and their antibodies and the t-PA antibody were purchased from R and D systems, Inc (USA). The protein C was purchased from abcam (USA), all other reagents were analytical grade from commercial sources.

Survival rate experiment: To determine the protective effects of andrographolide, different groups of rabbits were monitored and the amounts of dead rabbits of each group were recorded. Six groups were established: Three andrographolide treatment groups (low, medium, high-dose), a normal control group, an LPS group and a heparin group and in andrographolide treatment groups, animals were given 1.0 mg kg^{-1} in low-dose group, 2.0 mg kg^{-1} in medium-dose group and 5.0 mg kg^{-1} in high-dose group according by intraperitoneal injection and the heparin group was given 100 U kg^{-1}. All these treatments were administered

simultaneously with LPS. Meanwhile, the normal control group received no treatment. At 6 and 24 h after the andrographolide treatment, the dead rabbits were recorded to analyze survival rate.

Animal models and drug treatments: Firstly rabbits were anesthetized via an intramuscular injection of 30 mg kg^{-1} ketamine hydrochloride and intramuscular supplements of ketamine hydrochloride were given throughout the experiment. To established DIC experimental models, rabbits were induced by infusing LPS in 60 mL of saline solution at a rate of 10 mL/kg/h through the rabbits' marginal ear vein over a period of 6 h.

Treatments started simultaneously with LPS infusion through the contralateral marginal ear vein of rabbits. Rabbits were randomly divided into six groups: Three andrographolide treatment groups, LPS group, heparin group and normal control group, each group contained 10 animals. In andrographolide treatment groups (low, medium and high dose andrographolide) were totally given 1.0, 2.0 and 5.0 mg kg^{-1}. The LPS group was injected with saline solution. Heparin group was infused with 500 IU kg^{-1} heparin. The last group, normal control group, which was given neither LPS nor andrographolide, was given saline solution through both marginal ear veins of the rabbits.

Blood sample collection: After LPS infusion began, blood samples were collected at 2 and 6 h through using a catheter inserted into a femoral artery. Subsequently, Blood was collected in 3.8% sodium citrate (1:10 vol/vol citrate/blood) and centrifuged at 2000 g for 15 min at 4°C and then stored at -70°C until assayed.

Blood sample detection: Serum samples were collected at 2 and 6 h after the treatment of andrographolide and then stored at -70°C.

The assay of serum levels of BUN was performed by a 7170A automatic analyzer (HITACHI, Japan).

According to the ELISA reagent pack instruction based on chromogenic substrates, ATIII, protein C, PAI-1 and t-PA activity in serum was measured.

The concentrations of TNF-α and IL-1β were determined by using quantitative sandwich enzyme linked immunosorbent assay (ELISA) kits (RapidBio Lab, Calabasas, CA) using commercial reagents following the manufactory's instruction.

Histopathology examination of kidney: In order to determine the extent of renal tissue injury in LPS-induced DIC and the effects of andrographolide, histopathology examination was accomplished. When the above experiments were finished, rabbits were killed and then kidneys were taken from the chest cavities and fixed in 4% paraformaldehyde overnight, dehydrated, embedded in paraffin and then sliced into 5 μm thick sections. After deparaffinization with different concentration gradient of alcohol, slices were stained with phosphotungstic acid-hematoxylin stain and examined for the presence of fibrin microthrombi by a pathologist.

Statistical analysis: All data was presented as Mean±Standard Deviation. Survival rate was analyzed by Chi-square test. Differences between two groups were analyzed by one way ANOVA analysis. Data in Table 1 at 2 and 6 h were converted to percentages with a value of 100% assumed for basal data. Statistical analysis of survival rate of LPS-induced DIC were done using SigmaPlot software. A result with a p-value of <0.05 was considered statistically significant.

RESULTS

Andrographolide decreased the mortality of LPS-induced DIC rabbits: In the survival rate experiment, the protective effects of androgrphlide on LPS induced DIC rabbits were monitored (Table 1). In normal control group, all rabbits (n = 10) were survived within 24 h. In the LPS group,

Table 1: Survival rate at within 6 and 24 h in normal, LPS, low, medium, high-dose of andrographolide and heparin groups

		Groups					
			Andrographolide				
	Time (h)	LPS (%)	Low (%)	Medium (%)	High (%)	Heparin (%)	Normal (%)
Survival rate	6	60	80	100*	100*	100*	100
	24	40	60	80	80	70	100

All these treatments were administered simultaneously with LPS. The data was analyzed using Chi-squared test. *p<0.05 vs the LPS group

Table 2: Haemostatic and inflationary parameters 2 h and 6 h after LPS infusion into rabbits in normal, LPS-treated andrographolide-treated and heparin-treated groups

Group	Time (h)	APTT(s)	PT(s)	Platelets ($\times 10^9$ L^{-1})	Fibrinogen (g L^{-1})	FDP (μg L^{-1})	Protein C (%)	AT (%)	t-PA (%)	PAI-1 (%)
Normal	2	9.87±1.02**	5.14±0.82**	387.24±37.02**	3.44±0.52	<0.05**	97.78±3.23**	97.21±4.53**	96.37±7.02**	101.87±4.79**
	6	8.01±0.89**	6.13±0.59**	410.25±40.92**	3.76±0.73**	<0.05**	99.32±4.50**	98.36±5.71**	103.29±5 54**	99.32±3.75**
LPS-control	2	27.64±5.49	13.87±3.38	346.75±28.65	3.64±0.74	54.05±13.11	58.71±18.29	79.80±12.26	73.58±15.19	268.94±38.91
	6	42.36±7.32	21.15±4.61	205.16±33.16	1.28±0.45	83.73±20.62	33.26±12.58	48.74±15.68	39.94±14.35	384.25±52.14
Andrographolide										
Low-dose (1.0 mg kg^{-1})	2	19.40±2.27**	10.83±1.65*	368.36±46.57	3.19±0.49	30.16±11.57**	84.18±11.76*	83.32±16.95	87.01±16.74	184.39±31.40**
	6	37.67±7.91	14.55±4.41*	302.19±27.73**	2.84±0.76**	32.43±17.98**	70.65±16.09**	84.98±16.21**	98.39±5.42**	242.87±34.01**
Medium-dose	2	18.53±4.86**	6.37±1.16**	376.24±37.09	3.97±1.09	33.98±13.47*	86.92±19.98*	88.06±7.55	89.15±11.81*	179.12±22.64**
(2.0 mg kg^{-1})	6	29.96±6.38**	11.91±2.29**	385.98±23.50**	3.81±0.92**	26.45±9.64**	76.14±17.63**	85.62±8.26**	95.76±6.38**	189.06±19.83**
High-dose	2	12.45±2.62**	6.42±2.18**	392.27±37.16*	3.42±0.61	39.25±8.64*	85.31±19.06*	89.87±9.16	93.14±11.67**	110.92±12.13**
(5.0 mg kg^{-1})	6	17.99±3.17**	8.63±1.46**	383.81±24.62**	3.89±0.78**	23.31±13.43**	83.41±17.57**	92.83±4.72**	94.82±7.75**	148.46±26.82**
Heparin-control	2	18.57±3.41**	8.87±2.17**	372.73±27.08	3.57±1.01	34.18±10.57*	80.19±15.74*	76.35±12.40	82.43±19.26	138.75±26.62**
	6	28.68±7.06**	10.58±2.06**	385.36±39.27**	2.73±0.65**	39.76±12.68**	75.37±16.68**	78.38±13.19**	88.14±15.89	163.42±21.19**

APTT: Activated partial thromboplastin time, PT: Prothrombin time, FDP: Fibrin and degradation product. Plasma levels of protein C, AT-III, t-PA and PAI-1 were converted to percentages with a value of 100% assumed for basal data, *p<0.05 vs the LPS group, **p<0.01 vs the LPS group, Data was presented as the Mean±SD

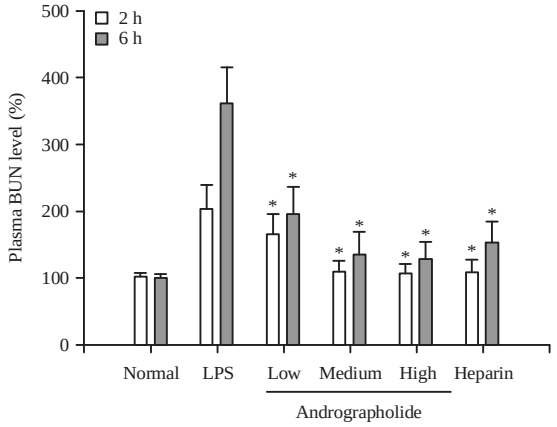

Fig. 1: Andrographolide attenuated the renal injury in LPS-induced DIC rabbits. Values were expressed as the Mean±SD (n = 10) percent of the initial value before LPS infusion. *p<0.05 and **p< 0.01 as compared to the LPS group. Plasma levels of BUN at 2 and 6 h were converted to percentages assuming a value of 100% for basal data

the survival rate was 60% in 6 h and 40% in 24 h. Survival rate was 70% within 24 h in heparin group, but no rabbits died in the early 6 h. In 6 h, the specific survival rates were 80, 100 and 100% and in 24 h were 60, 80 and 80% in the androgrphlide group according to low, medium, high-dose androgrphlide group. It's obvious that the survival rates in the low, medium and high-dose andrographolide groups were higher than in the LPS group at both 6 and 24 h, which showed an obvious increasing trend. Chi-square test had been used and it proved that androgrpholide treatment effectively increased the survival rates in medium-dose group at 6 h, in high-dose group at 6 h and in heparin group at 6 h compared to LPS group (p<0.05). These data indicated that andrographolide had protective effects on LPS-induced DIC.

Andrographolide reduced the BUN level on serum: When giving LPS into rabbits by intravenous injecting (100 mg kg^{-1} h^{-1}) in 2 and 6 h, it caused an obvious increase in plasma levels of BUN (an indicator of renal injury) compared with the saline group (p<0.01, Fig. 1). After infusion of a low, medium and high-dose of andrographolide, the level of BUN was significantly decreased (p<0.01) and the decrease could also be detected in heparin treatment group (p<0.01). These findings indicated that andrographolide was able to ameliorated renal injury after injection of LPS.

Andrographolide improved the hemostatic parameters in LPS-induced DIC rabbits: As Table 2 showed, at 2 h after intravenous injection of LPS (100 mg kg^{-1} h^{-1}) into rabbits, the activities of protein C, the concentration of ATIII and t-PA had a significant decrease (p<0.01, compared with the saline solution group). On the contrary, under similar experimental conditions, PAI-1 activity and FDP level increased (p<0.01). At 6 h, fibrinogen, ATIII and t-PA concentration and protein C activity decreased (p<0.01), while PAI-1 and FDP levels increased (p<0.01). This data was consistent with the data obtained at 2 h. It suggested that LPS infusion destroy the fibrinolytic system and blood coagulation system.

After infusion of the low, medium and high-dose of andrographolide, it increased the level of protein C (p<0.05) and decreased the concentration of FDP and PAI-1 (p<0.05) at 2 h compared with LPS group in all doses of andrographolide groups. Compared to LPS group at 6 h, measurements were made at all doses of infused andrographolide and it showed a significant increase in the activities of t-PA, ATIII and protein C (p<0.01) and a decrease in the concentration of FDP and PAI-1 (p<0.01). All the data indicated that andrographolide could positively improve the disorder of

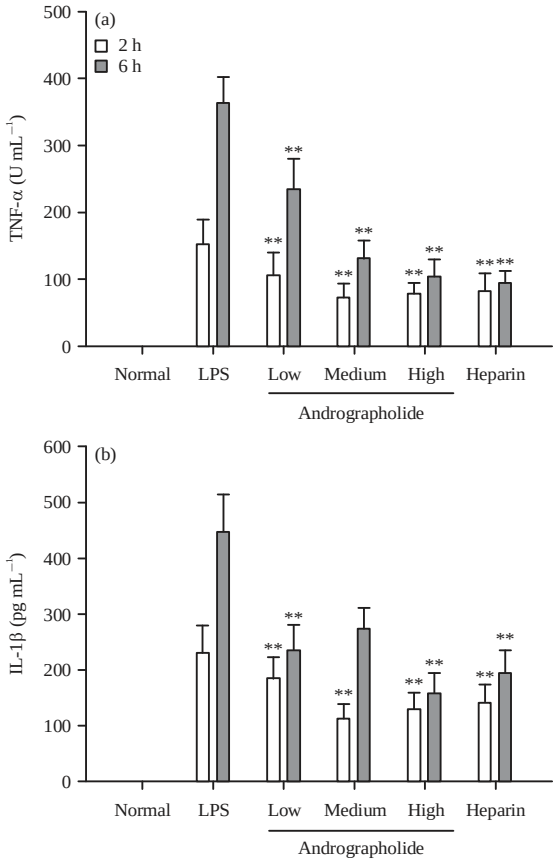

Fig. 2(a-b): Secretion of TNF-α and IL-1β in serum level of 6 groups in rabbits models at 2 h and 6 h after andrographolide and heparin treatment. Data was expressed as Mean±SD (n = 10), analyzed by one way ANOVA. *p<0.05 and **p<0.01 vs LPS group

fibrinolytic system and blood coagulation system, which caused by LPS and ameliorate the hemostatic parameters in DIC rabbits.

Andrographolid decreased the level of the inflammatory cytokines: To investigate the andrographolide inhibition effect of serum inflammatory cytokines production on LPS-induced DIC rabbits, TNF-α and IL-1β were also detected (Fig. 2a and b). After LPS infusion, TNF-α and IL-1β levels in serum remarkably elevated at 2 h while the control group was maintained in a low and stable level ($p<0.01$). After infusion of andrographolide, TNF-α and IL-1β in all the doses of andrographolide treatment groups had a significant decrease compared with the LPS-induced group both in 2 and 6 h ($p<0.01$). This showed that TNF-α and IL-1β levels could be effectively inhibited by andrographolide.

Andrographolide reduced the renal damage in LPS-induced DIC rabbits: To determine the protective effects of andrographolide on renal damage, tissue sections of kidney in DIC rabbits were monitored. Intense fibrin deposition was detected in kidney in most LPS treated rabbits compared with the saline control group (Fig. 3a-b). A high level of fibrin deposition was detected in most of the low-dose andrographolide group, whereas, a lower level of fibrin deposition was observed in most of the medium-dose andrographolide group. No fibrin deposition was detected in most of the high-dose andrographolide group and little fibrin deposition was found in most of the treated heparin group (Fig. 3c-f).

DISCUSSION

The DIC can lead to the injury of tissue and organ, which is a life-threatening syndrome with high mortality[21]. The situation is severe that its clinical prognosis is poor. Now there is still no effective therapy for DIC but injecting heparin, which had been recognized as a valuable anticoagulant and antithrombotic[22]. Although, heparin could reduce the high mortality of DIC, which was about 50-60% in clinical treatment[21], it would cause many side effects in clinical. Therefore, it is extremely urgent to find another therapy with better impact and fewer side effects.

Wang *et al.*[23] found that *Andrographis paniculata* had demonstrated an increase of blood-clotting time and Thisoda *et al.*[24] reported could inhibit Platelet-Activating Factor (PAF) induced platelet aggregation. In addition, Handa and Sharma[25] rendered that andrographolide was extremely nontoxic, even at high dose and the side effects were common. Consequently, it could be tentatively put forward that andrographolide could be a better choice of therapy for the patients with DIC.

This study explored the effect of andrographolide on survival rate and organ injuries by detecting the BUN level and tissue section in DIC rabbits. In order to have a better understanding about the therapeutic efficiency of andrographolide, the content of protein C, ATIII, t-PA, PAI-1, FDP, the concentration of fibrinogen and the inflammatory factors, TNF-α and IL-1β were detected. The data suggested that andrographolide had a therapeutic potential in DIC.

The LPS is the toxic component of endotoxin and it could result in DIC. In this study LPS had been used to establish DIC models on rabbits and the DIC group mortality could reach a relatively high level (60%) within 6 h, which was similar to

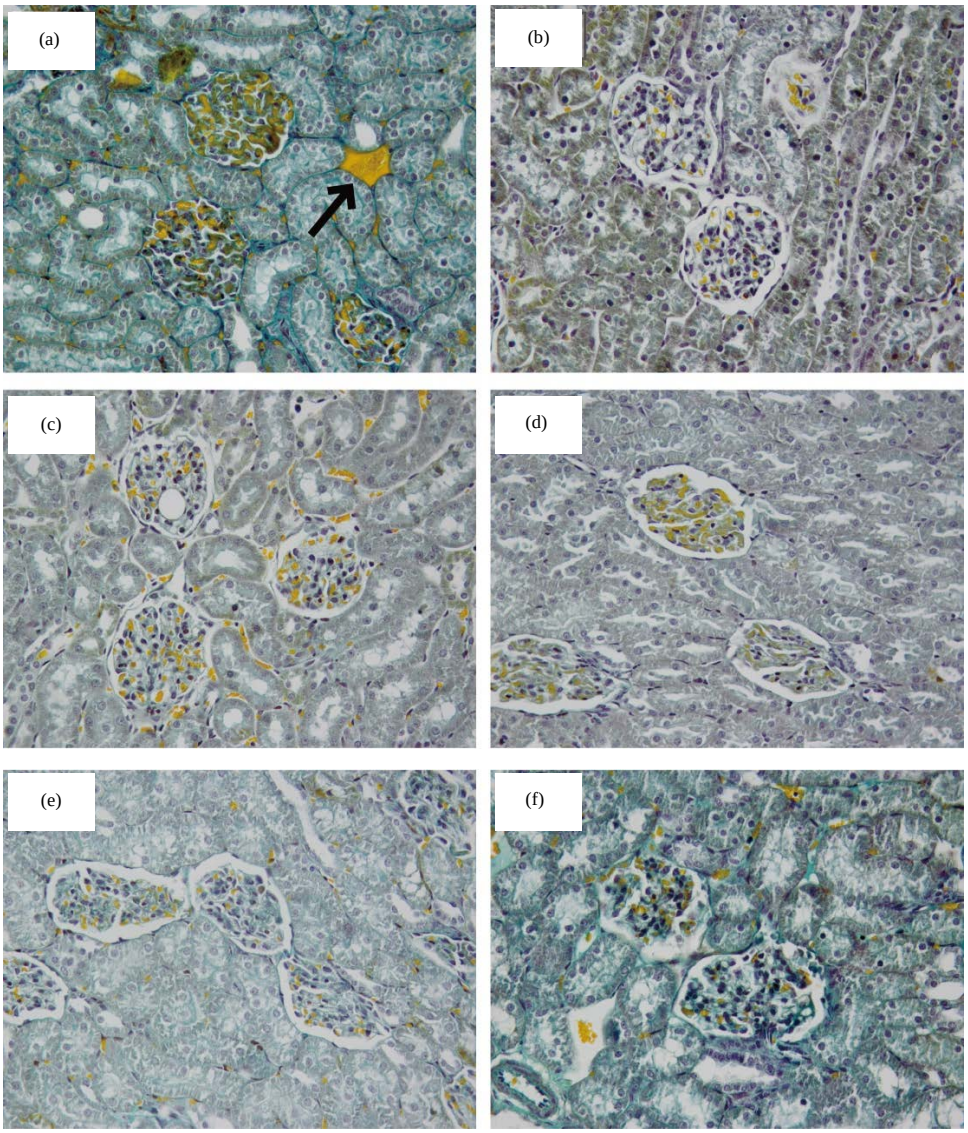

Fig. 3(a-f): Andrographolide attenuated the renal injury in LPS-induced DIC rabbits, (a) Intense fibrin deposition, which was highlighted by black arrow, was detected in most LPS-treated rabbits in kidney, (b) Little fibrin deposition was monitored in most of the heparin group, (c) No fibrin deposition was observed in most normal control group, (d) A high level of fibrin deposition was detected in most of the low-dose andrographolide group, (e) A lower layer of fibrin deposition was detected in most of the medium-dose andrographolide group and (f) No fibrin deposition was detected in most of the high-dose andrographolide group

Qi *et al.*[26]. It was said that DIC models were established successfully. After andrographolide infusion within 24 h, all the treatment groups showed that the mortality was decreased directly and it also demonstrated that andrographolide could effectively increase the survival rate by Chi-square test analysis. The data inferred that andrographolide had overall protective effects on DIC rabbits.

Kidney can excrete metabolic waste, which produced by our bodies. When it fails, the stability of electrolyte and acid-base balance will be destroyed. Recently, study suggested that 27 million people have chronic kidney disease and a 30% increase in the past 10 years in America[27]. However, kidney is the first organ to fail when multiple organ failure syndrome occurred[28] and it is thought to be especially prone

to fibrin formation in DIC[29]. According to the observation of renal section in LPS-induced DIC models, microvascular thrombosis could be detected and the protective effects could be gained in this study. In present study andrographolide could remarkably reduce the fibrin deposition, which indirectly improved the low survival rate caused by LPS.

The recent study also showed us that andrographolide can inhibit the expression of cytokines, such as TNF-α and IL-1β[30]. According to Fig. 2, the LPS-induced DIC group showed that the levels of TNF-α and IL-1β were increased compared to the saline control group and the accumulation of pro-inflammatory cytokines could lead to inflammatory cascades and thus overwhelming inflammation responses would appear. Therefore, many of them, such as TNF-α and IL-1β have been chosen as therapeutic targets for DIC. According to the data andrographolide showed its anti-inflammatory effect, which weakened the excessive inflammation. The result implied it was similar to the result of Iruretagoyena *et al.*[31] to some extent. This explained that andrographolide could improve the renal injury and reduce the mortality via suppressing the over production of TNF-α and IL-1β.

Antithrombin III (AT III) is the major plasma inhibitor of coagulation proteases. It is found to inhibit factor VIIa, which is a Tissue Factor (TF)-dependent blood coagulation plasma factor[19]. According to Qi *et al.*[26], tissue-type and urokinase-like plasminogen activators (t-PA and u-PA) combined together to be plasminogen, which is a precursor of plasmin. Plasmin can dissolve fibrin to degradation products and eliminate soluble fibrin from the circulation and solubilizing existing clots. Plasminogen Activator Inhibitor (PAI-1) is the principal inhibitor of plasminogen activation and it had been proved that it is the most involved DIC and increased PAI-1 had been associated with a predisposition to thrombosis, which is a specific inhibitor of t-PA. Protein C is a plasma, vitamin K-dependent zymogen of a serine protease[32] and the protein C anticoagulant pathway is a major mechanism in controlling microvascular thrombosis and it was said that activated protein C could be beneficial for DIC therapy[26]. In this study, the data showed andrographolide could increase the activities of protein C and ATIII in DIC rabbits and this explained that andrographolide had particular prognostic significance in the clinical management of DIC[29]. The decrease of PAI-1 showed the protective effects of andrographolide on thrombosis. That's to say andrographolide could protect against DIC via improving the anticoagulant activity and fibrinolytic activity. The result was the same as in previous study, which reported that andrographolide might have a high therapeutic potential to treat thromboembolic disorders[33].

It should be noted that this study has examined only *in vivo* and the experiment *in vitro* was not completed. Furthermore, it was pity that some limitations were existed in the determination of the survival rate. Not withstanding their limitations, this study suggest the increased expression of protein C, t-PA and u-PA and the inhibition of cytokines, induced PAI-1 production, which symbolized the positive effects in preventing continuing intravascular coagulation and thrombosis. Andrographolide could be a potential therapeutic drug and it will be achieved in the following exploration.

CONCLUSION

Andrographolide was able to protect significantly against LPS-induced DIC and DIC-induced renal dysfunction. Moreover, andrographolide significantly decreased pro-inflammatory cytokines TNF-α and IL-1β levels, which suggested that it could be a potential reagent for DIC therapy.

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