Level and Effect of Adrenomedullin on Endotoxin Induced Disseminated Intravascular Coagulation Model in Rabbits

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ABSTRACT

Adrenomedullin (ADM) is a regulatory peptide having a variety of pharmacological properties secreted from vascular endothelial cells and adrenal medulla. There are only a few studies about the effects of ADM on coagulation and platelets which is secreted from endothelial cells. The aim of this study is to assay ADM levels in rabbits with endotoxin induced disseminated intravascular coagulation (DIC) model and to investigate the probable effects of ADM on coagulation parameters in this model. Four groups (Control, DIC, Heparin, ADM) each consisting of eight New Zeland rabbits were formed randomly. A DIC model was developed by infusion of endotoxin from Escherichia coli. Then the effect of standard dose heparin and 0.05µg/kg/min ADM infusion were evaluated. Administration of endotoxin resulted in severe changes of coagulation parameters as shown by the significant prolongation of the prothrombin time and activated partial thromboplastin time, decrease in platelet count, plasma fibrinogen level, antithrombin, and protein C activity. These results were in accordance with DIC findings and were significantly different from baseline and control group. In DIC group, the baseline, second, and sixth hour mean ADM levels were 7.09±1.63, 8.10±1.55, 9.35±0.76 ng/dl, respectively. ADM levels on sixth hour were significantly higher than baseline and control group. Heparin and ADM were used in the DIC model but both of them were not effective. In conclusion, ADM levels were found elevated during the progress of endotoxin induced DIC model. ADM were not effective in overt DIC model but it might be useful in the early phase of the DIC.

Keywords: Disseminated Intravascular Coagulation, Adrenomedullin, Heparin, Endotoxins

ÖZET

Dissemine İntravasküler Koagülasyon Geliştirilen Tavşanlarda Adrenomedullin Düzeyleri ve Tedavi Etkinliği

Adrenomedullin (ADM) 52 aminoasitten oluşan, adrenal medulla ve endotel başta olmak üzere pek çok organdan salgılanan bir peptiddir. Endotelden salgılanan bir peptid olarak ADM'nin koagülasyon üzerine etkileri çok az çalışmada incelenmiştir. Dissemine intravasküler koagülasyon (DİK) patofizyolojisindeki rolü ise bilinmemektedir. Bu çalışmanın amacı DİK geliştirilen tavşanlarda ADM düzeylerini belirlemek ve ADM'i tedavide kullanarak koagülasyon sistemi üzerine olası etkilerini araştırmaktır. Çalışma için her biri 8 erkek Yeni Zelanda tavşanından oluşan 4 grup (Kontrol, DİK, Heparin ve ADM) oluşturuldu. DİK grubunda E. Coli endotoksini infüzyon şeklinde verilerek standart bir DİK modeli geliştirildi. Bu modelde standart dozda heparin infüzyonu ile 0.05 µg/kg/dk dozunda ADM infüzyonu yapılarak tedavi sonuçları karşılaştırıldı. DİK geliştirilen tavşanlarda altıncı saat sonunda ortalama protrombin zamanı (PT) ve parsiyel tromboplastin zamanı (aPTT) değerlerinde belirgin uzama ile ortalama platelet sayısı, fibrinojen düzeyi, antitrombin (AT) ve protein C aktivitesinde belirgin düşme izlendi. Bu sonuçlar DİK bulguları ile uyumlu, kontrol grubu ve başlangıç değerleri ile karşılaştırıldığında anlamlı farklılık tespit edildi.

DİK grubunda başlangıç, ikinci ve altıncı saat ortalama ADM değerleri sırası ile 7.09±1.63, 8.10±1.55, 9.35± 0.76 ng/dl olup kontrol grubuna ve başlangıç değerlerine göre altıncı saat ADM değerleri istatistiksel olarak anlamlı yüksek bulundu. Heparin ve ADM bu DİK modelinde tedavide denendi ancak ikisi de etkili bulunmadı. Sonuç olarak, DİK geliştirilen tavşanlarda ADM değerlerinin yükseldiği tespit edildi. ADM aşikar DİK tedavisinde etkili bulunmadı. Ancak erken fazlarda etkili olabileceği düşünülebilir.

Anahtar Kelimeler: Dissemine intravasküler koagülasyon, Adrenomedullin, Heparin, Endotoksin

INTRODUCTION

Disseminated intravascular coagulation (DIC) is an acquired hemostasis dysfunction, characterized by systemic intravascular activation of coagulation, leading to widespread fibrin deposition in the circulation. The highly complex and variable pathophysiology of DIC often results in a lack of uniformity in clinical manifestations, a lack of consensus in the specific appropriate laboratory criteria of diagnosis, and a lack of specific therapeutic modalities. DIC patients are extremely heterogeneous depending on the underlying disease. Severe cases are managed in intensive care units and the treatment of DIC is extremely complex and difficult.[1-3] In addition, there continues to be a lack of well-controlled studies regarding various methods of management in relatively homogeneous groups of patients. Therefore treatment continues to be controversial and probably will remain so in the near future. It needs new treatment modalities and drugs. Experimental models of DIC have provided substantial insight into the pathogenesis of this disorder, which may ultimately result in improved treatment.

Adrenomedullin (ADM) is a peptide consisting of 52 amino acids and is secreted from vascular endothelial cells and adrenal medulla.[4] Earlier studies have shown that plasma ADM level is increased in patients with cardiovascular diseases, especially in congestive heart failure.[5-7] ADM has multifunctional biological activities, such as vasorelaxation, platelet cAMP increase, diuretic action, inhibition of aldosteron production, etc.[8-11], however, few reports have addressed its effect on hemostasis.[12,13] The inhibitory effect of ADM on the expressions of tissue factor and plasminogen activator inhibitor-1 (PAI-1) was mainly mediated by the cAMP-dependent signal transduction.[12] These findings suggest that ADM may play an important role in the regulation of homeostasis as a paracrine or autocrine factor as well as a circulating hormone by vascular endothelial cells mainly via the cAMP pathway.

There are only a few studies about the effects of ADM on coagulation and platelets even though it's secreted from endothelial cells.[12-15] The aim of this study was to develop an experimental animal model for DIC, to assay ADM levels in the DIC model, to investigate the possible effects of ADM on coagulation parameters in rabbits injected with endotoxin.

MATERIAL AND METHODS

The model described below was approved by the local ethical committee of Erciyes University and all experiments conformed to the NIH guidelines for animal research. Thirty-two male New Zealand white rabbits (weight, 2.5 to 3.5 kg) were used in the study. All rabbits were kept the same environmental condition and fed with commercial rabbit diet for at least 10 days before the experiment.

Experimental Model

Animals were anaesthetized by an intramuscular injection of 30 mg/kg ketamin hydrochloride (10% Ketasol, Richter Pharma AG) and 5 mg/kg xylazin (2% Rompun, Bayer) followed by intramuscular boosts of ketamin hydrochloride (10 to 30 mg/kg/hour) throughout the experiment. Except in the control group, DIC was induced in all rabbits by intravenous infusion of 100 μg/kg/h lipopolysaccharide-endotoxin (LPS, Escherichia coli serotype 0111:B4-Sigma) in 120 ml (20 ml/h) saline and 5% dextrose solution (40 ml saline + 80 ml 5% dextrose) through the marginal ear vein for 6 hours.

Four groups (Control, DIC, Heparin, ADM) each consisting of 8 New Zeland rabbits were formed randomly.

Control Group: Rabbits were infused with saline and 5% dextrose solution 20 ml/h for 6 hours through one ear vein, as control group. At the second hour of the infusion 40 ml saline was added simul-

taneously with infusion through the contralateral marginal ear vein as placebo.

DIC Group: Rabbits were infused with 100 μg/kg/h LPS in 120 ml saline and 5% dextrose solution for 6 hours and at the second hour of the infusion 40 ml saline was added.

Heparin Group: Rabbits were infused with 100 μg/kg/h LPS in saline and 5% dextrose solution 20 ml/h for 6 hours. At the second hour of the infusion, 100 IU/kg/dose heparin was given with bolus injection and then 20 IU/kg/h heparin with 40 ml saline was infused through the contralateral marginal ear vein.

ADM Group: Rabbits were infused with 100 μgr/kg/h LPS in saline and 5% dextrose solution 20 ml/h for 6 hours. At the second hour of the infusion, 0,05 μg/kg/minute ADM with 40 ml saline was infused through the contralateral marginal ear vein.[16,17]

ADM (1-52)(Phoenix Pharmaceutical, Inc. Belmont, USA.) 100 μg white powder peptide was dissolved with 10 ml saline and this solution was used in the same day.

Surviving rabbits were slaughtered 15 hours after the start of LPS infusion by ketamin overdose. Died rabbit counts were noticed at 6th, 9th, and 15th hours for surviving rate. Renal and hepatic tissue sections were prepared, fixed in formalin, embedded in paraffin, stained with hematoxylin-eosin and examined for histopathological changes.

Laboratory Methods

Blood samples were taken through a catheter inserted into the femoral artery immediately before the infusion, and 2 and 6 hours after the start.

Blood for white blood cell (WBC), hemoglobin and platelet counts were collected into tubes containing K3-EDTA and these tests were determined in an automatic analyzer (Sysmex XT-2000i, Sysmex Corporation, Chuo-ku, Kobe, JAPAN).

Coagulation parameters such as prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, antithrombin (AT), and protein C tests were collected into tubes containing 3.2% citrate. PT (Simplastin HTF), aPTT (MDA Platelin LS), fibrinogen (Fibriquik), antithrombin (bioMerieux, Antithrombin III), and protein C (bioMerieux, Protein C) determination were measured using commercially available kits in an automated coagulometer (bioMerieux Inc. Durham, North Carolina, USA).

Adrenomedullin Assay

Blood for the ADM analysis was collected into the vacutainer tubes containing K3-EDTA. And it was immediately transferred into the chilled glass tubes, containing 100 μg aprotinin and gently rocked for several times to inhibit the activity of proteinases. Platelet-poor plasma was obtained by centrifugation of blood samples at 1600 x g for 15 min at 4^0C and stored at -80^0C until assay. Before the measurement of plasma ADM concentration by enzyme immunoassay, plasma samples were extracted through the Sep-pak C-18 column and dried with an overnight-freeze drying method using a lyophilizier. For assay, the lyophilized material was dissolved and the solution was submitted to enzyme immunoassay (Phoenix Pharmaceuticals Inc. Harbor Boulevard, Belmont, California 94002) as been reported previously.[18]

Statistical Analysis

Descriptive summary statistics (N, mean, median, SD) were computed for treatment group. Comparison of variables between groups were made by using the One Way Analysis of Variance (ANOVA). Post-hoc comparison on variables were performed using the Tukey Procedure. Comparison of baseline values of the haemostatic variables with values at second and at 6th hour within the groups were made by using the One Way Repeated Measures Analysis of Variance (Multiple Comparison Procedures: Bonferroni). Differences in survival rate within 15 hours were assessed by the Ki-kare test. All analyses were performed with the statistical package for scientist (SIGMASTAT) Windows version 3.10. A value of $p< 0.05$ was considered as significant.

RESULTS

In the control group of rabbits without endotoxin, no change in the analyzed parameters was observed during the experiment (data not shown).

Table 1 shows the plasma levels of hemostatic parameters during the experiment in DIC group. Infusion of endotoxin caused significant changes with respect to the control group in platelets and coagulation parameters at second and 6th hour of the experiment. These results were in accordance with DIC findings and were significantly different from the findings of control group at second and 6th hour.[19,20] Also administration of LPS induced decreases of WBC count. No changes in the hemoglobin levels were observed during the experiment.

Table 2 shows the ADM levels at baseline, second and 6th hour in Control and DIC group. ADM levels on sixth hour were significantly higher than baseline and Control group. ADM levels were found elevated during the progress of DIC model in rabbits.

Table 3 shows the plasma levels of hemostatic parameters in treatment groups. Infusion of endotoxin into rabbits caused significant changes in platelets and coagulation parameters at second hour of the experiment. After the second hour, Heparin and ADM infusion were started for the treatment. But no significant difference was observed in platelets and coagulation parameters at 6th hour of the experiment in both treatment groups compared with DIC group. When all groups compare with each other, no significant difference was seen on the laboratory parameters. Neither heparin nor ADM had an effect on the laboratory parameters after LPS injection in DIC model. All changes in the groups were showed in Figure 1.

Survival Rate

Four of 8 rabbits (50%) died in the first 6 hours following LPS infusion in the DIC group, whereas all rabbits infused with saline survived in the Control group. 2/8 (25%) rabbits which were infused with LPS without treatment had died during the first 9 hours and all of the rabbits had died 15 hours later the end of experiment.

Both heparin and ADM treatments did not remarkably increase the survival rate. In the heparin and ADM treatment groups, the survival rates were 3/8 (38%) and 7/8 (88%) at the 6th hour, and 1/8 (18%) and 4/8 (50%) at the 9th hour of the experiment respectively (Figure 2). Also all rabbits had died at the end of experiment.

The survival rates were higher in rabbits treated with ADM at the 6th and 9th hour and difference was significant when compared with the heparin group ($p= 0.02$ and $p= 0.002$ respectively). But there was no significant difference in the survival rate between treatment groups and DIC group at the end of experiment.

Histological Examination

Excluding the control group, the DIC induced histopathological changes in the kidneys and in the liver were not significantly different among the groups.

Table 1. Laboratory variables at baseline, second and 6th hour after the start of the LPS infusion in the DIC group (n = 8).

Variables	Baseline	2. hour	6. hour
WBC (/mm^3)	8607±2248	3700±1869***	4222±1575***
Hemoglobin (mg/dl)	13.2±1.7	13.4±2.0	13.5±2.1
Platelets (x10^9/l)	440±128	191±94***	101±66***
PT (sec.)	10.5±1.1	14.9±1.5***	19.8±2.1***
aPTT (sec.)	85.9±9.2	125.9±26.5**	236.9±40.3***
Fibrinogen (mg/dl)	377.0±54.6	283.8±58.7***	158.0±83.9***
AT (%)	125.5±13.8	103.8±12.7***	90.0±8.3***
Protein C (%)	101.8±15.7	63.2±34.9***	28.8±17.9***

*p< 0.05, ** P< 0.01, *** p< 0.001 when compared with baseline values

Table 2. ADM levels at baseline, second and 6th hour in Control and DIC group.

Groups	n	Adrenomedullin (ADM) (ng/ml) (mean.± SD)p Baseline	2. hour	6. hour	
Control	8	5.63±2.38	6.38±1.88	6.49±2.32	p> 0.05
DIC	8	7.09±1.63	8.10±1.55	9.35±0.76**†	p< 0.01

*p< 0.05, ** P< 0.01, *** p< 0.001 when compared with baseline values.

† p< 0.05 when compared with Control group.

DISCUSSION

DIC is an extremely complex pathophysiologic process. DIC is characterized primarily by excessive thrombin generation, hypercoagulability with intravascular fibrin formation, and platelet aggregation, and secondarily by increased bleeding tendency.[1,3] A variety of clinical conditions may cause systemic activation of coagulation. Sepsis is the most common cause of acute DIC.[21,22] Endotoxin, which is considered to be a major pathogenic factor contributing to sepsis, initiates DIC in human and animals.[2,21,22] The injection of endotoxin into rabbits causes a decompensated activation of the hemostatic system in the DIC model.[23-26] DIC is a continuously progressing process. Systemic generation of thrombin in animal models of DIC was shown to be mediated by extrinsic pathway, involving tissue factor and activated factor VII.[3,27] Significant prolongation of aPTT and PT, and decrease in platelet count, plasma fibrinogen, AT, and protein C activity could be found.[1,3,19,20] In this experiment we studied ADM levels on endotoxin induced DIC model in rabbits and investigated the effect of ADM administration on coagulation parameters in rabbits injected with LPS. Administration of endotoxin in our model resulted in severe changes on coagulati-

Table 3. Laboratory variables at baseline, second and 6th hour after the start of the LPS infusion in the DIC and treatment groups (n = 8).

Groups	Time	Platelets ($x10^9$/L)	PT (Sec)	aPTT (Sec)	Fibrinogen (mg/dl)	AT (%)	Protein C (%)
DIC	Base	440±128	10.5±1.1	85.9±9.2	377.0±54.6	125.5±13.8	101.8±15.7
	2. h	191±94***	14.9±1.5***	125.9±26.5**	283.8±58.7***	103.8±12.7***	63.2±34.9**
	6. h	101±66***	19.8±2.1***	236.9±40.3***	158.0±83.9***	90.0±8.3***	28.8±17.9***
Heparin	Base	478±117	11.1±1.3	80.5±7.5	431.1±25.5	120.8±6.3	104.6±13.9
	2. h	296±175**	15.9±3.3	175.7±65.5**	388.6±63.2	106.9±17.0*	66.5±24.5***
	6. h	131±59***	19.9±3.4***	282.3±32.8***	211.5±123.1**	86.7±16.3***	24.2±13.4***
ADM	Base	341±55	11.5±0.9	94.6±11.1	346.5±94.3	110.5±12.3	96.3±12.5
	2. h	138±50***	13.3±1.4	114.7±16.3	286.6±94.7	96.2±14.2**	53.9±14.9***
	6. h	68±40***	17.4±2.8**	240.2±56.4***	192.1±83.8**	86.4±16.1***	27.2±10.2***

*p< 0.05, ** p< 0.01, *** p< 0.001 when compared with baseline values.

† p< 0.05 when compared with DIC group values.

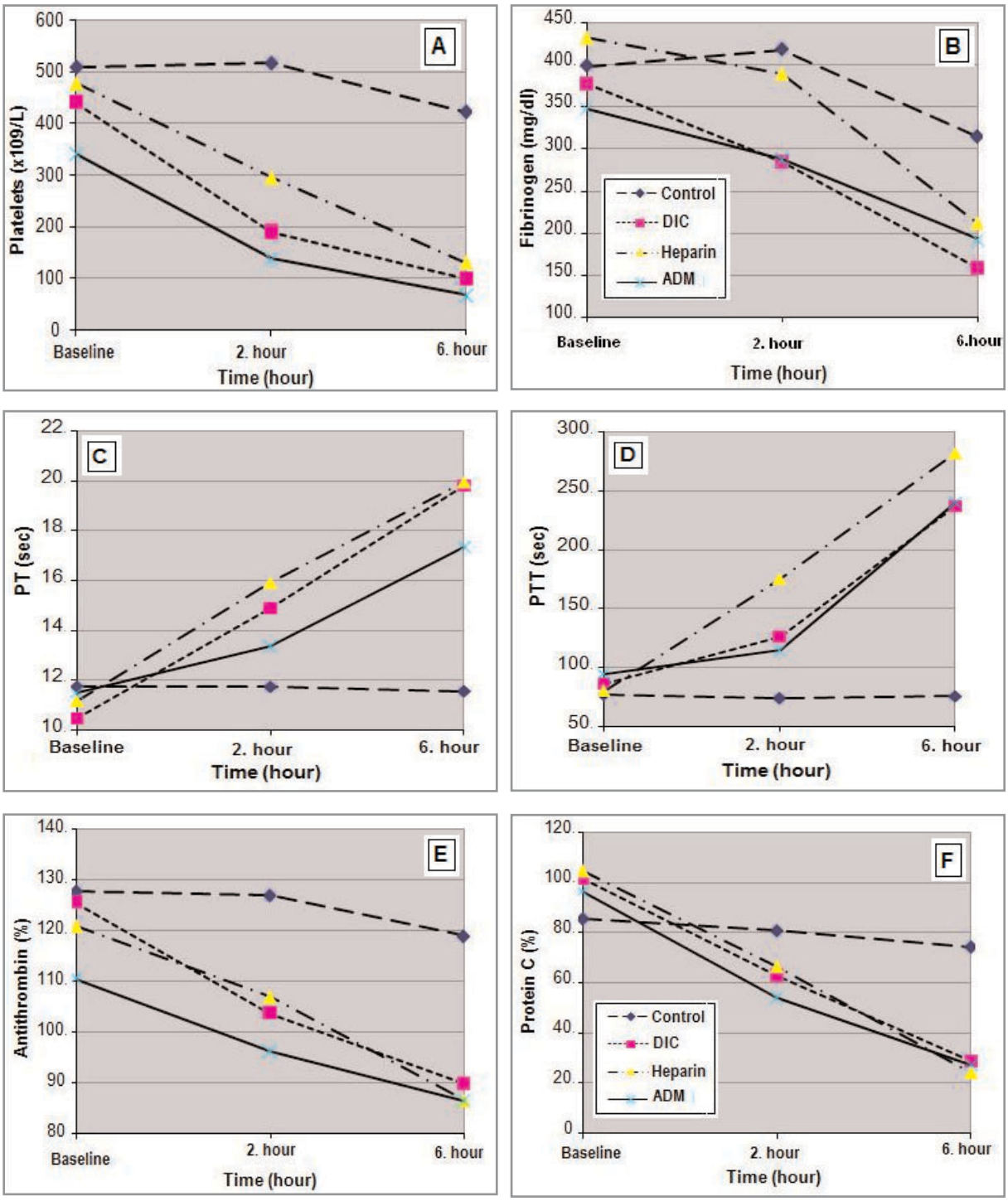

Figure 1. Hemostatic parameters which are platelets **(A)**, fibrinogen **(B)**, prothrombin time **(C)**, activated partial thromboplastin time **(D)**, antithrombin **(E)**, and protein C **(F)** at baseline, second and 6th hour after the start of the LPS infusion in the DIC and treatment groups with Control group.

on parameters as shown by the marked decrease in platelets, fibrinogen, AT, and protein C, which is in agreement with previous clinical and experimental studies. Moreover, very high mortality rate was assessed as a result of the endotoxin infusion. This model was previously performed by Munoz and Hermida.[24,25]

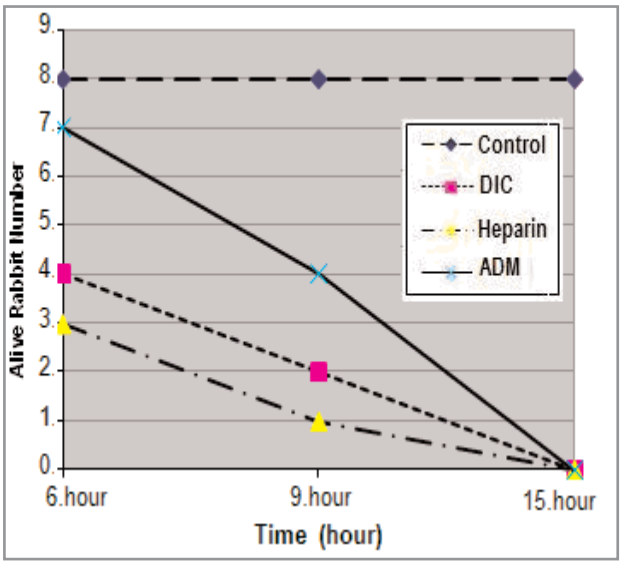

Figure 2. Alive Rabbit Number in the groups

ADM is a vasorelaxing peptide that was originally isolated from human pheochromocytoma tissue.[4] The peptide consists of 52 amino acids and is widely distributed in a variety of tissues and organs, especially in cardiovascular and endocrine tissues.[10,11] Endothelial cells, smooth muscle cells, and cardiac myocytes are considered to be the main sources of ADM.[28-30] ADM has multifunctional biological activities and the concentration of ADM in plasma is increased in patients with hypertension and heart failure.[5,9,11] Also recent clinical studies have shown that the plasma levels of ADM is increased in thrombotic diseases, such as acute myocardial infarction, sepsis, septic shock, atherothrombotic ischemic stroke, and thrombosis associated with advanced cancers.[5,13,17,31,32] However, there are only a few studies about the effects of ADM on coagulation and platelets which is secreted from endothelial cells.[12-15] Therefore, we determined the ADM levels in rabbits injected with LPS in this study. The plasma ADM concentration in DIC group increased slowly in two hours after LPS infusion. But rising in ADM level was not significant when compared with the baseline values. Thereafter, ADM levels went on increasing till the sixth hour of the LPS infusion and difference was significant according to the baseline ADM levels.

The present study demonstrated an increased plasma ADM level during DIC model in rabbits. The plasma concentration of ADM has been reported to increase markedly in both endotoxic animals and septic patients.[31,33,34] But it has been a subject of controversy whether or not ADM participates as a vasodilator in the blood pressure regulation in endotoxin shock. Matsui et al. showed that ADM concentration did not increase in rats when an obvious reduction of mean blood pressure was observed after LPS injection.[31] Plasma ADM levels increased to higher than baseline value at 4th hour after the LPS injection, which time point the mean blood pressure returned to the basal levels.[31] Similarly, ADM levels increased at the late part of the experiment in our study. Thereafter our hypothesis is that if we use ADM in the early part of DIC model we can prevent progression of DIC.

Vascular endothelial cells exhibit a particularly high abundance of ADM mRNA and represent the major source of circulating ADM. The endothelial derived peptide also acts in an auto/paracrine fashion and has been shown to stimulate nitric oxide synthesis in endothelial cells.[14,15] The interaction between platelets and the vascular wall plays a crucial role in hemostasis and thrombosis, and ADM may be an important mediator of this.[14] Finally, ADM is a biologically active peptide released from the vascular wall, which increases blood flow through its vasorelaxant effects and prevents platelet activation by stimulation of nitric oxide synthesis.[14] Lang-Rollin et al. did not demonstrate the direct effect of ADM on thrombin-induced platelet aggregation but they said that ADM is very likely to inhibit platelet aggregation in vivo as it stimulates nitric oxide release by endothelial cells, and nitric oxide is a very potent blocker of platelet aggregation.[14,15]

Theoretically, interruption of coagulation should be benefit in patients with DIC. Indeed, experimental studies have shown that heparin can partially inhibit the activation of coagulation in cases that are related to sepsis or other causes. A logical therapeutic intervention would be directed against tissue-factor activity. Novel, antithrombin or independent inhibitors of thrombin might be more effective than heparin, and experimental studies have had promising results.[3,27] Sugano et al. reported that the inhibitory effect of ADM on the expressions of tissue factor and PAI-1 was mainly mediated by the cAMP-de-

pendent signal transduction.[12] Furthermore, the inhibitory effect of ADM on tissue factor and PAI-1 expression was partly attenuated by a nitric oxide synthase inhibitor. ADM is shown to contribute to the regulation of blood coagulation and fibrinolysis by vascular endothelial cells mainly via the cAMP pathway.[12] After this report, Marutsuka et al. demonstrated ADM augmented tissue factor pathway inhibitor (TFPI) release and production in a dose-dependent manner. They have also reported that ADM reduced tissue factor expression of endothelial cells associated with apoptosis.[13] Taken together, we know that ADM inhibits TF and PAI-1 expression and increases TFPI release on the coagulation pathway.[12,13] Also ADM which is secreted from endothelial cells prevents platelet activation and aggregation.[14,15] These effects are aimed in the DIC treatment. ADM could play an important role in the anticoagulant mechanism. For this reason, we used ADM for treatment of DIC model.

Yang and Gonzalez-Rey et al. recently reported that the therapeutic effect of ADM was mediated by decreasing the local and systemic levels of a wide spectrum of inflammatory mediators, including cytokines, chemokines, and the acute phase protein.[35,36] Importantly, ADM treatment was therapeutically effective in established endotoxemia. Human sepsis and endotoxemia causes excessive inflammatory cytokine production.[35,36] These findings indicate that ADM might play an important role in the endotoxin induced DIC and the present work proposed a new therapy for endotoxemia based on DIC in rabbits.

ADM has an inhibitor effects on platelet aggregation, tissue factor and PAI-1 expression, production of inflammatory mediators and moreover exhibitor effects on TFPI release.[12-15,35,36] ADM can be used in DIC management with these effects. Therefore, we investigated the effect of ADM in endotoxin induced DIC treatment in rabbits. ADM infusion treatment was not effective in the present DIC model. But ADM was used at the second hour of the LPS infusion in this model. We think that ADM infusion could be effective in the beginning of the DIC model for the inhibitor effect on tissue factor and PAI-1 expression and production of inflammatory mediators. Also it can be infused with high dosage.

In summary, a marked increase of plasma ADM levels was observed 6 hours after LPS infusion in the DIC model. ADM was not effective in overt DIC therapy but it might be useful in the beginning of the DIC model. New treatment strategies with ADM should be designed in future studies.

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