

NOVEL FUNCTION OF ISOAMYLAMINE IMPROVES SURVIVAL IN ENDOTOXEMIC MICE BY AMELIORATING COAGULOPATHY AND ATTENUATING MMP-9 EXPRESSION THROUGH P-ERK/P-P38 SIGNALING AT EARLY STAGE

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ABSTRACT—When a host suffers endotoxemic shock or septic shock, it results in many symptoms including disseminated intravascular coagulation (DIC). Septic shock (SS) causes coagulation time to decrease and then gradually increase, finally becoming prolonged and giving rise to DIC. Isoamylamine (IA) is one of the main components of grape products and can improve the survival rate of endotoxin lipopolysaccharide (LPS)-induced endotoxemic shock. The aim of this study was to elucidate if IA ameliorates coagulopathy in the early phase of LPS-induced damage. We studied the effects of IA on the coagulation system of extrinsic (prothrombin time [PT]) and intrinsic (activated partial thromboplastin time [aPTT]) pathways. PT and aPTT were tested in plasma drawn from mice following intraperitoneal (IP) injection of 1 mL of 1,000 ppm IA after LPS administration. Shortened PT was ameliorated by 1,000 ppm IA 1 h after LPS administration, but there was no effect on aPTT. In conclusion, IA 1,000 ppm partially intervenes in the early phase of LPS-induced damage, shortening plasma PT 1 h after LPS treatment in mice. Furthermore, we found 1,000 ppm IA also could attenuate MMP-9 expression through p-ERK/p-p38 signaling in mice hepatocyte extracts. This study focused on the effects of IA on blood coagulation function and inflammatory proteins. In the current situation of absence of effective treatment for SS, IA can increase survival rate and may offer another choice of patient avoiding causing death during endotoxemic shock.

KEYWORDS—Activated partial thromboplastin time (aPTT), disseminated intravascular coagulation (DIC), high mobility group box B1 (HMGB1) protein, isoamylamine (IA), matrix metalloproteinases (MMPs), prothrombin time (PT)

INTRODUCTION

Septic shock (SS) refers to the risk of severe sepsis. The clinical symptoms of SS include hypotension with fluid infusion and boosted vasopressor agent, resulting in vital organ perfusion insufficiency, disseminated intravascular coagulation (DIC), multiple organ failure, and other conditions. Septic shock is mainly induced by endotoxin lipopolysaccharide (LPS) (1, 2) of gram-negative bacteria and results in a mortality rate of more than 40% (3). From a review of the literature, there is currently no effective treatment or specific medication for SS.

In this study, we focused on early coagulopathy of DIC. DIC is a serious phenomenon caused by up-regulation of procoagulant molecules, primarily tissue factor (TF); impairment of

physiological anticoagulant pathways, for example, antithrombin, protein C pathway, and inhibitor for tissue factor pathway; and suppression of fibrinolysis (1). DIC can cause coagulation time to decrease and then gradually increase, becoming prolonged and giving rise to SS (4, 5).

Isoamylamine (IA), or 3-methyl-1-butanamine or isopentylamine, is an organic chemical compound of colorless liquid, with an analog structure to arginine, based on our screening process (US patent number 6,011,066). It is found in concentrations up to 1.23 mg/L in red wine and 1.168 mg/L in grape juice (6, 7). In this study, we found that IA improves mortality rate of mice suffering from endotoxemic shock. From the results of coagulation experiments, 1,000 ppm IA can ameliorate LPS-induced damage in extrinsic (prothrombin time [PT]) pathways, but not intrinsic (activated partial thromboplastin time [aPTT]) pathways, 1 h following LPS administration.

Hemostasis (8, 9) is mainly divided into three phases. The first is vascular phase with vascular contraction. The second is coagulation phase with subsequent cascade reaction of coagulation factors. The third is fibrinolysis phase. In the second phase of hemostasis, fibrin forms through the activation of coagulation factors including extrinsic pathway, intrinsic pathway, and common pathway. Extrinsic pathway involves tissue damage and can be detected by PT. Intrinsic pathway involves

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surface contact and can be detected by aPTT. The extrinsic pathway starts with TF and triggers coagulation factor VII, which sequentially induces activation of factor X and prothrombin. The intrinsic pathway is triggered by autoactivated factor XII, which leads to cascade activation of factors XI, IX, X, and prothrombin. Deficiency in these coagulation factors results in bleeding diseases. The aim of this study was to investigate the effect of IA on coagulation system by assaying PT and aPTT in plasma of mice after intraperitoneal (IP) injection of LPS.

Matrix metalloproteinases (MMPs) (10) are a family of calcium-dependent zinc-containing endopeptidases and have the ability of cleaving collagen and extracellular matrix (ECM). In normal physiological condition, it involves in embryonic development, tissue remodeling, degradation of ECM, and angiogenesis, whereas in pathological condition, it involves in inflammation, tissue fibrosis, tumor cell invasion, and migration. Most MMPs are secreted as inactive form and are activated by the extracellular protease cleavage. Matrix metalloproteinase-2 (MMP-2, gelatinase A, proenzyme 72 kDa, active form 66 kDa) and matrix metalloproteinase 9 (MMP-9, gelatinase B, proenzyme 92 kDa, active form 86 kDa) (2) belong to type IV collagenase. MMP-9 protein expression would be stimulated by LPS (11) through MAPK (mitogen-activated protein kinases, including ERK1/2, p38, or JNK kinases) pathway. High mobility group box B1 (HMGB1) protein (12) was considered a nuclear protein before, but lately found it was released by cell necrosis or damage. According to the report (13) that, in early stimulation of inflammatory cytokines such as TNF, macrophages will release HMGB1 after LPS stimulated for 16 h. That means HMGB1 could be used as an inflammatory marker, a late mediator of lethal sepsis (14).

Hormesis (15) is an important concept in biological toxicology and medicine (16) that was discussed during the past decade, but was not the same as long-standing belief about the dose-dependent effect. The hormetic dose–response is characterized by being a stimulation in the low-dose zone, but an inhibitory response at higher dose. Many of dietary phytochemicals (17) induce hormetic biphasic dose effects in the low-dose limits. IA, containing in grape and related products, belongs to natural compounds and also possesses the similar feature of hormetic dose–response according to our study results. Impact of hormesis on biology (18) is a fundamental dose response, and will help the pharmaceutical industry and biomedical research to clarify the health implications where concentrations induce harmful responses below the threshold. Hormesis is a reproducible phenomenon with a frequency far greater than other dose–response models and this concept could be further applied in the development of new therapeutic medicine and will be applied to this study.

MATERIALS AND METHODS

Animals and materials

Male BALB/cByJNarl mice and C57BL/6JNarl (B6) mice, 5 to 6 weeks old, were purchased from the National Laboratory Animal Center, Taipei, Taiwan. Mice were used for experiment at least 1 week later.

Isopentylamine (Isoamylamine, IA) (M-820716) 250 mL was supplied by Merck, Germany.

LPS of *Escherichia coli* serotype O111:B4 (SI-L2630-100 mg) was obtained from Sigma-Aldrich.

PT and aPTT reagents were purchased from Dade Behring Company, Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany.

Treatments

IA was diluted with phosphate buffered saline (PBS) in the study to observe the survival rate of BALB/c mice and normal saline was used to study PT and aPTT in B6 mice at a concentration of 1,000 ppm via IP injection.

LPS 12.5 mg/kg was IP injected in the survival study of BALB/c mice and LPS 10 mg/kg in the PT and aPTT studies of B6 mice.

In the survival experiment, BALB/c mice were simultaneously IP injected with 1 mL IA 1,000 ppm and LPS 12.5 mg/kg of body weight. In the control group, PBS 1 mL and LPS were simultaneously injected. We determined the basis for survival by observing the mice 3 days after injection.

For the coagulation experiment, B6 mice were IP injected with a dose of 1,000 ppm IA in 1 mL and LPS 10 mg/kg of body weight. Then, plasma was collected at various time periods for PT and aPTT assays.

Following carbon dioxide anesthesia, blood was obtained by cardiac puncture using 3.2% sodium citrate mixed with blood at a ratio of 1:9. To get more plasma and blood unclotted, we drew blood from the heart. Mouse was sacrificed only one time after obtaining sufficient blood, so as to avoid the stress of mice. B6 mice plasma was separated and used immediately for PT and aPTT assay. BALB/c mice liver tissues were used for western blot analysis.

PT assay

PT was assayed in 4-fold dilution of PT reagent, composed of four parts including one part PT reagent, one part 25 mM CaCl₂ solution, and two parts sterile water.

On PT assay, 0.1 mL of plasma was placed into four independent incubation stations, each with one steel ball, and maintained at 37°C for 1 min in Stago ST4 coagulation analyzer (STart 4, Diagnostica Stago, France). Then, 0.2 mL of the diluted PT reagent was added for the PT detection by Stago ST4 coagulation analyzer.

aPTT assay

aPTT was assayed in 4-fold dilution of aPTT reagent using kit-provided buffer solution as diluent.

On aPTT assay, 0.1 mL of plasma was kept warm for 1 min at 37°C in four independent incubation stations in the Stago ST4 coagulation analyzer. Then, 0.1 mL of 4-fold diluted aPTT reagent and one steel ball were added. After 180 s, 0.1 mL calcium chloride (25 mM) was added for aPTT detection by the Stago ST4 coagulation analyzer.

Western blot analysis

BALB/c mice treated with 10 mg/kg LPS plus 1 mL of 1, 10, 100, or 1,000 ppm IA. Three hours later, liver was obtained by normal saline perfusion under mice anesthesia condition. Protein lysates of liver cell extract were prepared as PRO-PREP system (iNtRON Biotechnology Inc, Korea) and were fractionated on 10% SDS-PAGE. A Western blot analysis was performed with primary antibodies specific for MMP-9 (Catalog number: 10375-2-AP, proteintech), MMP-2 (#4022, Cell Signaling Technology, Danvers, Mass), HMGB1 (EPR3507, Abcam, Burlingame, Calif), p-ERK (#9102, Cell Signaling Technology), p-p38 (Material Number: 612281, BD Transduction Laboratories, Franklin Lakes, NJ), or Actin (Material Number: 612657, BD). The density of the specific bands was quantified using Image-Pro Plus software (Media Cybernetics, Silver Spring, Md).

Data analysis

Statistical analysis was carried out using SPSS 18.0 software. All data error bars are presented as mean (s) ± standard deviation. Comparisons between groups and estimations of *P* value were carried out by the Mann–Whitney *U* test.

RESULTS

Survival rates of BALB/c mice treated with LPS plus PBS or 1,000 ppm IA

BALB/cByJNarl mice were used to determine survival rates of LPS-induced endotoxemic shock. Among LPS plus PBS IP injected mice, five of 15 survived, whereas 14 of 15 LPS plus

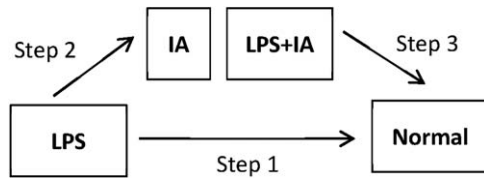


FIG. 1. According to Baron conceptual, strategic, and statistical considerations of social psychological research (19), we developed this flow chart. If there was statistical significance in step 1, we followed with comparisons in step 2. If steps 1 and 2 produced statistical significance, step 3 comparisons were carried out. If all three steps resulted in statistical significance (P value <0.05), the results were regarded as intervening effect. IA indicates isoamylamine; LPS, lipopolysaccharide.

1000 ppm IA IP injected mice survived. The survival rates (%) were 33.3% and 93.3%, respectively, as shown in Figure 1. The P value of the difference in survival rate between the two groups is 0.001, and this statistics shows significant differences. Thus, IA significantly improved the survival rate of mice with endotoxemic shock ($P < 0.05$).

In B6 mice, 1,000 ppm IA attenuates LPS-shortened PT, but not aPTT

C57BL/6JNarl (B6) mice were IP injected with LPS 10 mg/kg of body weight or LPS 10 mg/kg plus 1,000 ppm IA 1 mL. After administration for 1, 2, and 3 h, blood was drawn by heart puncture. The plasma was separated and immediately assayed on Stago ST4 coagulation analyzer. Normal group mice were not injected with LPS or IA reagent. When comparing the coagulation time of LPS group with normal control (normal coagulation time used as 100%), we can obtain that the value of LPS/normal at 1, 2, 3 h was 89.2%, 94.6%, 100.9% respectively in PT and 90.5%, 97.3%, 118.3% respectively in aPTT. It showed that PT and aPTT, as shown in step 1 of Tables 1 and 2, decreased and then increased, becoming prolonged, under LPS-treated mice.

The results of PT are shown in Table 1. By comparing 10 normal mice with 6 LPS-treated mice, there was statistical significance only at 1 h following treatment. For comparisons of 6 mice treated with LPS and 6 mice treated with 1,000 ppm IA, the differences in PT 1, 2, and 3 h after treatment were all statistically significant. For comparisons of six LPS plus IA-treated mice with normal group, differences were only statistically significant at 1 h following treatment. Therefore, only the group tested 1 h after treatment complied with the three steps and presented with statistical significance (P value <0.05).

The results of aPTT are shown in Table 2. The differences were statistically significant only at 3 h between 10 normal mice and six LPS-treated mice. For comparisons of six mice treated with LPS and six mice treated with 1,000 ppm IA, differences were only statistically significant at 1 and 2 h following treatment. For comparisons of six LPS plus IA-treated mice and normal group, there was statistically significant difference only at 3 h following treatment. None of the groups complied with all three steps of statistical significance (P value <0.05) (Fig. 2).

From Baron research methods (19), it shows that 1,000 ppm IA intervenes in the effect on PT by LPS-induced damage at 1 h (Table 1), but not on aPTT (Table 2). PT data are shown in steps 1, 2, and 3 of Table 1 and in Figures 3 to 5. aPTT data are shown in steps 1, 2, and 3 of Table 2 and in Figures 6 to 8.

LPS induced MMP-9 expression and was attenuated by IA via p-ERK/p-p38 signaling in BALB/c liver cells

Mouse hepatocyte extracts Western blotting analysis with mouse hepatocyte extract demonstrated that MMP-9 expression was slightly induced by lipopolysaccharide as shown in Figure 9A (upper diagram). Besides 100 ppm IA, LPS-induced MMP-9 protein expression was reversed by various concentrations of 1, 10, 1,000 ppm isoamylamine. However, the

TABLE 1. In B6 mice, treatment with 1,000 ppm IA attenuates LPS induced-shortening of PT

		Step 1. LPS versus Normal		
		Treatment time		
		1 h	2 h	3 h
LPS	n = 6	9.9 ± 0.8	10.5 ± 0.3	11.2 ± 0.6
Normal	n = 10	11.1 ± 0.7	11.1 ± 0.7	11.1 ± 0.7
P value [†]		0.007*	0.082	0.663
LPS/normal 89.2%, 94.6%, and 100.9%.				
		Step 2. LPS versus 1,000 ppm IA		
		Treatment time		
		1 h	2 h	3 h
LPS	n = 6	9.9 ± 0.8	10.5 ± 0.3	11.2 ± 0.6
1,000 ppm IA	n = 6	11.6 ± 1.0	12.2 ± 1.1	12.6 ± 1.2
P value [†]		0.005*	0.004*	0.043*
		Step 3. LPS + 1,000 ppm IA versus Normal		
		Treatment time		
		1 h	2 h	3 h
LPS + 1,000 ppm IA	n = 6	10.3 ± 0.4	10.4 ± 0.7	10.9 ± 0.7
Normal	n = 10	11.1 ± 0.7	11.1 ± 0.7	11.1 ± 0.7
P value [†]		0.030*	0.092	0.703

Data are presented as mean (s) ± standard deviation.

[†] Mann-Whitney U test, * $P < 0.05$.

IA indicates isoamylamine; LPS, lipopolysaccharide.

TABLE 2. In B6 mice, treatment with 1,000 ppm IA does not attenuate LPS-shortened aPTT

		Step 1. LPS versus Normal		
		Treatment time		
		1 h	2 h	3 h
LPS	n = 6	23.8 ± 4.8	25.6 ± 4.4	31.1 ± 3.8
Normal	n = 10	26.3 ± 3.6	26.3 ± 3.6	26.3 ± 3.6
<i>P</i> value [†]		0.158	0.828	0.030*
LPS/normal 90.5%, 97.3%, and 118.3%.				
		Step 2. LPS versus 1000 ppm IA		
		Treatment time		
		1 h	2 h	3 h
LPS	n = 6	23.8 ± 4.8	25.6 ± 4.4	31.1 ± 3.8
1,000 ppm IA	n = 6	30.8 ± 1.5	32.0 ± 3.5	33.7 ± 2.1
<i>P</i> value [†]		0.037*	0.025*	0.150
		Step 3. LPS + 1,000 ppm IA versus Normal		
		Treatment time		
		1 h	2 h	3 h
LPS + 1,000 ppm IA	n = 6	22.6 ± 3.7	25.6 ± 2.6	31.8 ± 3.6
Normal	n = 10	26.3 ± 3.6	26.3 ± 3.6	26.3 ± 3.6
<i>P</i> value [†]		0.193	0.745	0.020*

Data are presented as mean (s) ± standard deviation.

[†] Mann–Whitney *U* test, **P* < 0.05.

IA indicates isoamylamine; LPS, lipopolysaccharide.

changes of MMP-2 and HMGB1 contents were less obvious and almost constitutively expressed under a variety of different treated conditions. Actin was used as loading control.

Furthermore, we elucidated IA in the character of MAPK pathway when mice were treated with LPS via IP injection. Our study found IA could prevent phosphorylation of ERK and p38 kinases in Figure 9A (lower diagram), rather than JNK (data not show). These results may deduce that IA attenuating MMP-9 expression through p-ERK/p-p38 signaling at early stage.

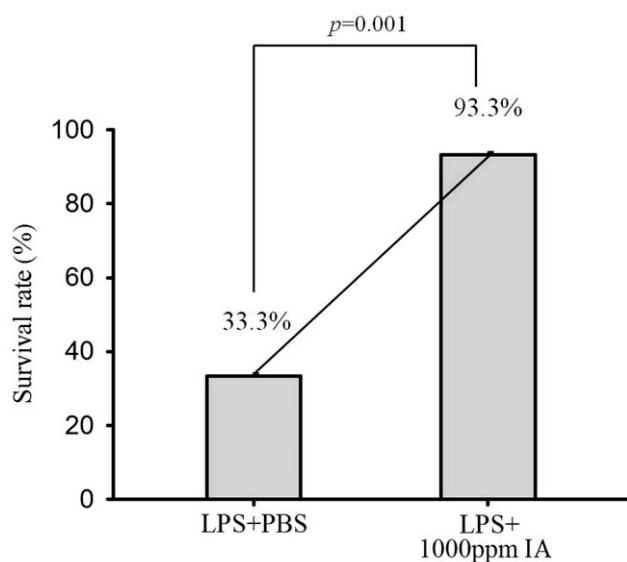


FIG. 2. Survival rates of BALB/c mice treated with LPS plus PBS or 1,000 ppm IA and their survival rates (%) were 33.3% and 93.3%, respectively. The *P* value was used to clarify the difference of survival rates having statistical significance between control group and experimental group. IA indicates isoamylamine; LPS, lipopolysaccharide.

DISCUSSION

At present, there is no specific drug to effectively rescue patients with SS. SS can result in numerous symptoms, including systemic inflammatory response syndrome (20–23), oxidative damage (23, 24), hypoxia (23, 25), multiple organ dysfunction syndrome (1, 2, 21–23, 26), and DIC (26). DIC is a serious phenomenon, mainly caused by endotoxin LPS releasing from cell wall of gram-negative bacteria. DIC presents under conditions of normal or shortened coagulation time due to activated circulating clotting factors, and followed by bleeding (4). Clinical studies have demonstrated that raised levels of circulating LPS are associated with severe illness, inflammation, multiple organ dysfunction, and mortality. LPS is the most powerful inducer of DIC through TF (1). We made

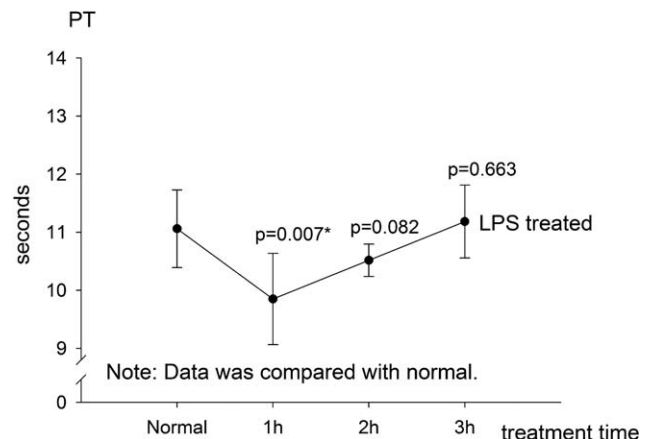


FIG. 3. PT changes in B6 mice plasma after LPS treatment (LPS vs. Normal). The *P* value was used to demonstrate whether PT has statistical significance among data of LPS treated 1, 2, and 3h compared with normal. LPS indicates lipopolysaccharide; PT, prothrombin time.

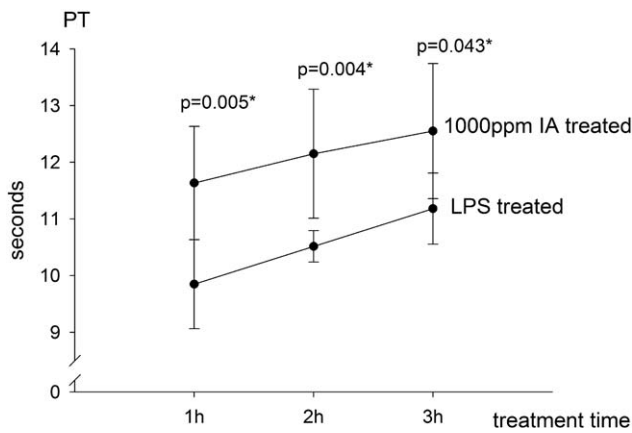


FIG. 4. PT changes in B6 mice plasma after LPS or 1,000 ppm IA treatment (LPS vs. 1,000 ppm IA). The *P* value was used to demonstrate whether PT has statistical significance among data of 1,000 ppm IA compared with LPS treated 1, 2, and 3 h, individually. IA indicates isoamylamine; LPS, lipopolysaccharide; PT, prothrombin time.

use of endotoxin model to study on the coagulation and inflammation of sepsis.

Schochl et al. (5) studied a pig model of endotoxemia and observed significant shortening of clotting time (s) 2 h after LPS infusion, followed by gradual increase. If compared the LPS group data with normal group data (normal coagulation time used as 100%), we can obtain that the LPS/normal percentage of treatment time 1, 2, and 3 was 89.2%, 94.6%, 100.9% in PT, and 90.5%, 97.3%, 118.3% in aPTT. This showed in LPS-induced mouse DIC (see step 1 in Tables 1 and 2), PT and aPTT were initially shorten and then prolong later on. In our study, the abnormal variation in PT was significantly attenuated by IA, indicating a protective effect of IA on LPS-induced mouse DIC coagulopathy. This trend in coagulation time (s) in the early stage of mouse endotoxemia in our study is in accordance with that of the previous pig model (5).

That novel function of isoamylamine improves survival in endotoxemic mice was first discovered in BALB/cByJNarl

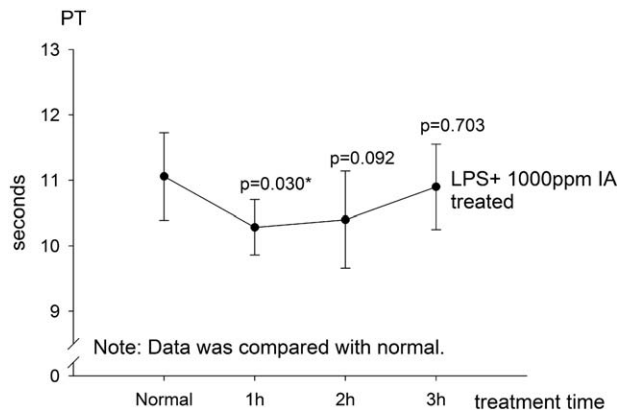


FIG. 5. PT changes in B6 mice plasma after LPS plus 1,000 ppm IA treatment (LPS + 1000 ppm IA vs. Normal). The *P* value was used to demonstrate whether PT has statistical significance among data of LPS plus 1,000 ppm IA treated 1, 2, and 3 h compared with normal. IA indicates isoamylamine; LPS, lipopolysaccharide; PT, prothrombin time.

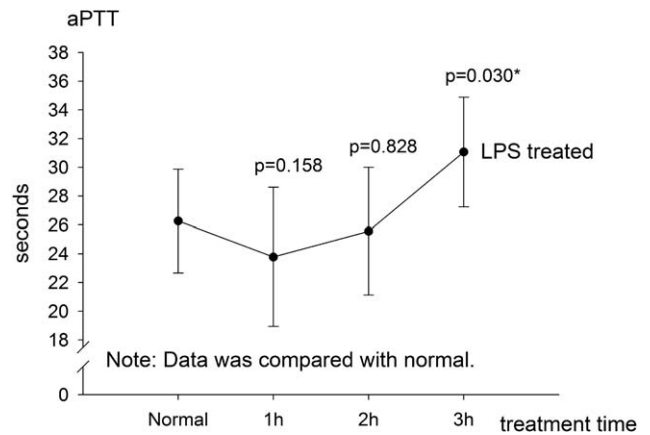


FIG. 6. aPTT changes in B6 mice plasma after LPS treatment (LPS vs. Normal). The *P* value was used to demonstrate whether aPTT has statistical significance among data of LPS treated 1, 2, and 3 h compared with normal. aPTT indicates activated partial thromboplastin time; LPS, lipopolysaccharide.

mice. After the literature search (27, 28), we used the B6 mice to carry out coagulation assay. In this study, we found that 1,000 ppm IA increased survival rate up to 93.3% in comparison with control group (33.3%) ($P = 0.001$). The lethal dose of IA was as high as 30,000 ppm of 1 mL in mouse by IP injected (unpublished). Then, we made use of mouse plasma to test the effect of IA on coagulopathy. Because the reference values of PT and aPTT are very important for comparison with others, we made use of more mice in the reference group than the experimental group (10 for the control normal group and 6 for LPS treated). We found that 1,000 ppm IA affects PT at 1 h, but not aPTT. In other words, IA may alleviate endotoxemic shock by shortening PT at the onset, but with no effect on aPTT. In this study, the abnormal variation in PT was significantly attenuated by IA, indicating a protective effect of IA on LPS-induced mouse DIC coagulopathy.

The data of biologic relevance are often very slight, but we can use biometrics to see whether the change is statistical significance or not.

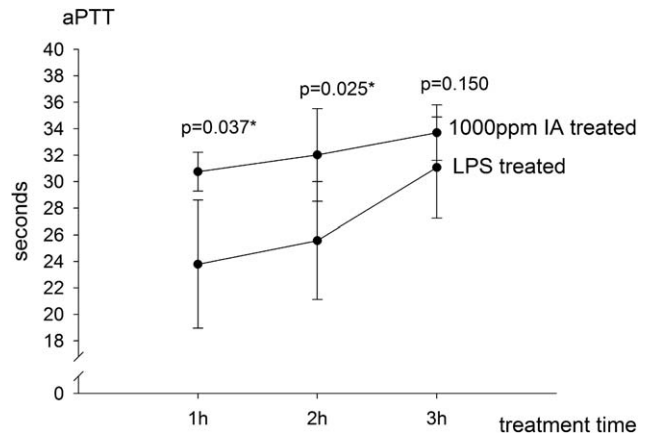


FIG. 7. aPTT changes in B6 mice plasma after LPS or 1,000 ppm IA treatment (LPS vs. 1,000 ppm IA). The *P* value was used to demonstrate whether aPTT has statistical significance among data of 1,000 ppm IA compared with LPS treated 1, 2, and 3 h, individually. aPTT indicates activated partial thromboplastin time; IA, isoamylamine; LPS, lipopolysaccharide.

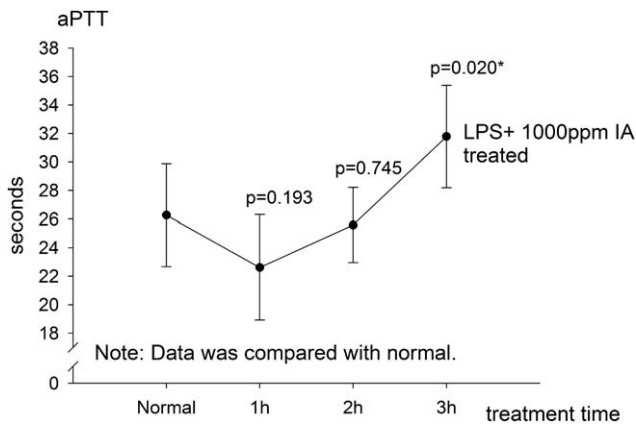


FIG. 8. aPTT changes in B6 mice plasma after LPS plus 1,000 ppm IA treatment (LPS + 1,000 ppm IA vs. Normal). The P value was used to demonstrate whether aPTT has statistical significance among data of LPS plus 1,000 ppm IA treated 1, 2, and 3h compared with normal. aPTT indicates activated partial thromboplastin time; IA, isoamylamine; LPS, lipopolysaccharide.

By statistical analysis, we would clarify the relevance between normal and experimental groups. If researchers would know the effect of drug or reagent intervene coagulation time between control and experimental groups, they could have used Baron research methods (19) to find meaningful change slightly.

Although also resulting in SS, results differ from those of dengue virus infection. Dengue virus (29) belongs to the family Flaviviridae. Its genetic material consists of a linear single-strand positive sense RNA (linear (+) SS-RNA), with length of about 11Kb RNA (30), and outer membrane. The virus is mainly transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes. Dengue fever can be divided into four types. In none of these types do antibodies provide protective effect. The severe form of dengue fever is characterized by coagulopathy. This phenomenon of dengue hemorrhagic fever (DHF) results in normal PT and prolonged aPTT (31–33). There have been reports that the hemostatic change in DHF involves low level intrinsic pathway coagulation factors VIII and XII (34).

LPS-stimulated monocytes secrete TF, named coagulation factor III, which primarily activates the extrinsic pathway and

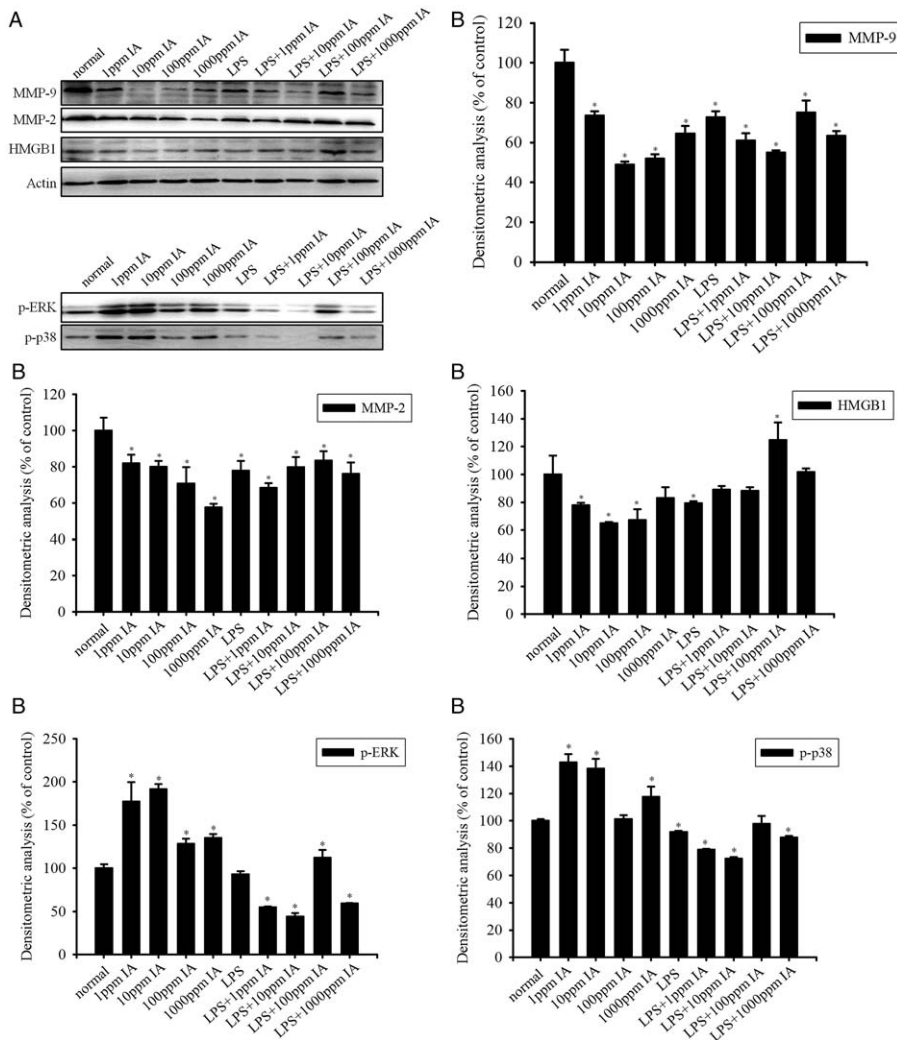


FIG. 9. Western blot analysis was performed that Isoamylamine could inhibit MMP-9 expression through p-ERK/p-p38 signaling. A, Effect of IA was in expression of MMP-9, MMP-2, HMGB1, p-ERK, p-p38. Actin used as loading control. B, The density of MMP-9, MMP-2, HMGB1, p-ERK, p-p38 bands was quantified using Image-Pro Plus software (Media Cybernetics, Silver Spring, Md). *P<0.05, compared with the control group. IA indicates isoamylamine.

inhibits fibrinolysis (1, 35). IA also affects extrinsic pathway PT. The extrinsic pathway is triggered mainly by coagulation factors III and VII (9). In future studies, we may investigate the role of IA in the relationship between TF and coagulation factor VII.

To clarify the IA in an anti-inflammatory role in mice, we did assay MMP9, MMP2, HMGB1, and so on. Our experiments analyzing extracts from hepatocyte showed MMP-9 expression slightly induced by lipopolysaccharide administrating for 3 h shown in Figure 9A (upper diagram); however, normal MMP-9 band was heavier than LPS-treated MMP content, we speculated that MMP had all its function at physiology and pathology. Under IA administration, MMP-9 protein was apparently lighter than LPS treatment or normal. The change of MMP-2 content was less obvious under a variety of different treated conditions. These results were similar to that of Woo et al. (11) previously published. Their results demonstrated MMP-9 amounts increased distinctly after LPS treatment for 12 h and MMP-2 was shown as a control. Inflammation marker HMGB1 commonly used as a late cytokine mediator of lethal sepsis (14). In our study treating at early phase, liver extracts HMGB1 contents were little apparent changes in mice after IP injection of LPS or IA. It was speculating that variation of HMGB1 protein to be observed until in the late phase.

Biphasic dose responses of multiple-signaling pathways mediating hormetic dose responses have been reported (18), and one of these is MAPK pathways, including the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway, the c-Jun Nterminal kinase (JNK), and the p38 MAPK pathway. According to our study, IA attenuated MMP-9 expression via phosphorylation of ERK and p38 in Figure 9A (lower diagram). Because the inhibitory effects of various IA concentrations were not linear relationship, we suggested that was an impact of hormesis.

In conclusion, in view of currently no effective treatment for SS, IA can increase the survival rate. This study focused on the effects of IA on blood coagulation function and possession of anti-inflammatory ability. Further studies are needed to elucidate its role in other inflammation conditions as well as the other functions of IA, especially at late phase and pathways.

We summarize our study in flowchart and outline figure of TOC (Table of Contents)/Abstract Graphics in the end (see Supplemental Digital Content, <http://links.lww.com/SHK/A504>). We had recorded the video demonstrating novel function of IA and attached as Supplemental Digital Content (<http://links.lww.com/SHK/A505>). This video was the activity of B6 mice injected with LPS or IA reagent. We could see the activity difference between mice injected LPS and LPS plus IA. The mouse group injected LPS reagent was weaker than the ones injected LPS plus IA or normal.

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