Plasma Intermedin Level Indicates Severity and Treatment Efficacy of Septic Shock in Sprague-Dawley (SD) Rats

Su-Xian Yang
Yun-Xiu Chen
Jing Xu
Zhao-Hui Yang

Corresponding Author: Jing Xu, e-mail: xujing15953981086@163.com
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Background: The aim of this study was to investigate the value of plasma intermedin (IMD) in assessing severity and treatment efficacy of septic shock.

Material/Methods: Healthy male Sprague-Dawley (SD) rats were chosen and divided into a normal control group (n=15) and a shock model group (n=27) that received intravenous injection of lipopolysaccharide (LPS). Then, 3 specimens were taken from each group. The shock model group rats were divided into an LPS group and a treatment group with 12 rats each. The treatment group received intravenous injection of compound sodium lactate solution. Plasma IMD and IMD1-47 mRNA expressions were compared and analyzed.

Results: Mean arterial pressure (MAP) was lower while white blood cell count and TNF-α were higher in the shock model group than in the normal control group (P<0.05). After 10 h and 20 h, the treatment group had lower plasma IMD and IMD1-47 mRNA expressions compared with the LPS group (P<0.05). Plasma IMD and IMD1-47 mRNA expressions in the LPS group after 20 h were significantly higher than after 10 h (P<0.05). IMD was positively correlated with interleukins (IL-3, IL-6, and IL-8), white blood cell count, and body temperature (all P<0.05), but were negatively correlated with systolic pressure (r=–0.8474, P=0.0040).

Conclusions: Plasma IMD level can effectively reflect the severity of septic shock and can be used as an important indicator of septic shock treatment effectiveness.

MeSH Keywords: Aftercare • Receptors, Interleukin-3 • Severity of Illness Index • TNF-Related Apoptosis-Inducing Ligand

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**Background**

Septic shock is an acute and overwhelming inflammatory response that results from the dysregulation of the innate immune response after infection [1]. As a special type of sepsis, septic shock is also known as a systemic inflammatory response syndrome (SIRS) caused by infection, and the dysregulation of cytokines and inflammatory mediators play a crucial role in the occurrence and development of septic shock [2,3]. In general, the incidence of severe infection is much higher, reflecting the relative percentage of patients who develop organ dysfunction and thus are at greater risk of septic shock [4]. The mortality rate of patients with septic shock is much higher than that of patients with nonseptic critical illness and of the general population [5]. Furthermore, previous evidence demonstrated that septic shock has been associated with oxidative damage [6]. Traditional predictive markers for the diagnosis of septic shock include CRP, interleukin-6 (IL-6), and interleukin-8 (IL-8) [7]. Although somewhat effective in diagnosis, these factors have only limited value in early diagnosis and prognosis of septic shock, and more sensitive indicators are urgently needed.

Intermedin (IMD), also named as adrenomedullin-2, a member of the calcitonin/calcitonin gene-related peptide (CGRP) family, exerts a wide range of biological effects, especially in maintaining cardiovascular homeostasis [8]. The possible mechanism may be related to the upregulation in cAMP level in the circulatory system, and regulation of the proliferation of the smooth muscle and up-regulating NO system [9]. IMD also protects the mammalian vasculature, myocardium, and kidney from acute ischemia-reperfusion injury, chronic oxidative stress, and pressure-loading [10]. Severe infection may induce inflammatory response, as well as cell apoptosis, suggesting that IMD may serve as a sensitive indicator of septic shock [11]. IMD1-47 is deemed as a novel CGRP generated from prepro-IMD by proteolytic cleavage at the NH₂-terminal [12]. As one of the IMD members, it may have the same functions as IMD; therefore, we suspected that IMD level can serve as an important indicator for septic shock treatment effectiveness.

Septic shock rat models were established and plasma IMD levels were examined to verify the feasibility of plasma IMD level as an effective indicator for septic shock severity and treatment efficacy.

**Material and Methods**

**Ethics statements**

All animals were bought from the Laboratory Animal Center of Linyi People’s Hospital. All animal procedures were conducted after approval from the Medical Ethics Review Board of Linyi People’s Hospital. The experimental environment was set in accordance with China’s national standard for Experimental Animal Environment and Facilities (GB14925-2001). Animal feeding management and animal experimental operation were in line with Beijing Experimental Animals Management regulations.

**Animals grouping**

Healthy male SD rats (n=42) weighing 210–250 g were divided into a normal control group (n=15) and a shock model group (n=27). After the shock model was successfully constructed and samples were obtained, 3 rats in each group were randomly chosen and sacrificed. The remaining rats were divided into 3 groups: the normal control group (n=12), the lipo-polysaccharide (LPS) group (n=12), and the treatment group (n=12) (the shock model group was divided into an LPS group and a treatment group).

**Construction of septic shock model and sample collection**

The rats were fasted for 12 h, and intraperitoneal injection of 36 mg/kg of pentobarbital sodium (Guangzhou Land Biotech Corp., China) was performed for anesthesia with room temperature maintained at 26°C and body temperature at 37°C. The rats were fixed on the operating table and a 3-cm incision was made in the groin. Blunt dissection was performed to separate subcutaneous connective tissues and to expose the femoral artery and arteriovenous. Catheterization was performed in the femoral artery to connect the 4-channel polygraph (ABI, USA) to monitor mean arterial pressure (MAP). After blood pressure was stable for 30 min, rats in the shock model group were given intravenous injection of 10 mg/kg LPS (Sigma, USA), while rats in the normal control group were given equivalent intravenous injection of 10 mg/kg normal saline (Guangzhou Land Biotech Corp., China) for 5 min. The standard for successful septic shock model construction was: after LPS was injected for 1 h, MAP ≤60 mm Hg or the decrease of MAP ≥40 mm Hg; symptoms of agitation, skin flooding, cyanosis, and less urine appeared. After the model was constructed for 1 h, 1 ml of blood was drawn in the common carotid artery from rats in both the normal control group and the shock model group. An equivalent normal saline was filled to ensure enough blood volume. Half of the 1-ml blood sample was put into a low-temperature and high-speed centrifuge at a speed of 600 g for centrifugation for 15 min. The supernatant was extracted and stored in the refrigerator at −20°C for TNF-α detection with an ELISA kit (Sigma, USA) in strict accordance with the specifications. After blood anticoagulation, a 0.5-ml blood sample was preserved in the refrigerator at −20°C for examination of total number of white blood cells using an automatic whole blood chart instrument (ABI, USA). After going through
the above-mentioned procedures and specimen collection, 3 rats from each of the 2 groups were chosen and killed; their lungs, hearts, kidneys, livers, gastrointestinal tract, and other organs were dissected. An MBL2000-type optical microscope (Julabo Technology Co., Ltd., China) was used for pathological analysis. The remaining 12 rats in the normal control group were left without any treatment. Twenty-four rats in the shock model group were randomly divided into an LPS group (n=12) and a treatment group (n=12). Intravenous injection of 25 ml/kg compound sodium lactate solution was performed on every rat in the treatment group for treatment within 30 min; the same treatment was performed every other day within a cycle of 7 days. Rats in the LPS group were treated with equivalent normal saline. During the experiment, the 3 groups of rats were normally fed.

**Plasma IMD expressions by ELISA**

After the shock models in normal control group, LPS group, and treatment group were set for 10 h and 20 h, 2 ml of blood was drawn in the common carotid artery at 2 different stages. Then the blood was evenly mixed with 30 μl of 10% EDTA-Na2 (Guangzhou Land Biotech Corp., China) and 40 μl of aprotinin (Santa Cruz, USA) in the tube and centrifuged at 4°C at 600 g for 10 min to separate the plasma. The plasma was preserved at -80°C. An ELISA kit was used for testing contents of IMD in the plasma in strict accordance with the specifications.

**Detection of IMD mRNA Expression**

The primer design is shown in Table 1. After the models in the normal control group, LPS group, and treatment group were set for 10 h and 20 h, 1 ml of blood was drawn in the common carotid artery. Then the blood was centrifuged at 4°C at 600 g for 10 min to separate the plasma. TRIzol (Invitrogen, USA) was used in One Step Method to extract total RNA from the sample tissue; 2 μg of RNA was reversed into single-strand cDNA with MMLV reverse transcriptase and oligo (dT) 15 primer. A Primer 7000 Fluorescence Quantitative PCR Instrument (ABI, USA) was used to test interleukin-3 (IL-3), IL-6, and IL-8 in strict accordance with specifications. Then, 2.5 ml of blood was evenly mixed with 30 μl of 10% EDTA-Na2, and 40 μl of aprotinin in the test tube. After that, 0.5 ml was preserved at -20°C for testing total number of white blood cells with an automatic whole blood drawing instrument; 2 ml was centrifuged at 4°C at 600 g for 10 min to separate the plasma. The plasma was preserved at -20°C. An ELISA kit was used for testing contents of IMD in the plasma in strict accordance with the specifications.

**Table 1. Primer design.**

| Primer group | Sequence | Product length (bp) |
|--------------|----------|---------------------|
| IMD1-47      | 5’-CCTCACTTCGGCCTGTAGTCT-3’ | 297 |
|              | 5’-ACCCACCTCAGCCATAACTGT-3’ |     |
| β-actin      | 5’-ATCTGGACCACACCTTC-3’ | 291 |
|              | 5’-AGCCAGGTCCAGACGC-3’ |     |

**Statistical analysis**

SPSS 20 statistical software was used for statistical analysis. Quantity One Basic software was used to analyze gray value of electrophoresis bands of different groups. Measurement data are expressed as mean ± standard deviation (±SD); comparisons between 2 groups were examined by t test and comparisons among groups were conducted with variance analysis. The count data are expressed as percentages or rates; comparisons between 2 groups were examined by chi-square test and correlation analysis was performed with Pearson correlation analysis. P<0.05 means the difference was statistically significant.
Results

The establishment of rat septic shock model

Physiological characteristics after the model had been established for 1 h in the 2 groups are shown in Table 2. MAP in the shock model group was significantly lower than that in the normal control group (both P<0.05). Results of organ pathology analysis of the 2 groups are shown in Figure 1. Compared with those of the normal control group, diffuse bleeding lesions appeared in lungs, livers, kidneys, myocardium, and intestinal tissues of the shock model group (all P<0.05).

Plasma IMD expressions at different stages of shock

Plasma IMD in the 2 groups at shock stages of 10 h and 20 h are shown in Figure 2. After the model had been established for 10 h and 20 h, plasma IMD expressions in the LPS group and treatment group were significantly higher than those of the normal control group (both P<0.05), but plasma IMD expressions in the treatment group was significantly lower than those of the LPS group (P<0.05). After LPS group modeling for 20 h, plasma IMD expressions was significantly higher than that for 10 h (P<0.05). After treatment group modeling for 20 h, plasma IMD content was significantly lower than that for 10 h (P<0.05).

IMD mRNA Expression

Relative expressions of IMD1-47 mRNA in each group after the model had been established for 10 h and 20 h are shown in Figure 3. After modeling for 10 h and 20 h, plasma IMD1-47 mRNA expressions in the LPS group and treatment group were significantly higher than those in the normal control group, while plasma IMD1-47 mRNA expressions in the treatment group were significantly lower than those in the LPS group (all P<0.05). After LPS group modeling for 20 h, plasma IMD1-47 mRNA expressions were significantly higher than that for 10 h (P<0.05). After treatment group modeling for 20 h, plasma IMD1-47 mRNA expressions were significantly lower than that for 10 h (P=0.003).

Table 2. Comparisons of physiological characteristics between two groups after modeling for 1 h.

| Items                     | Normal control group | Shock model group | P    |
|----------------------------|----------------------|-------------------|------|
| Average MAP (mm Hg)       | 128.33±13.22         | 94.77±15.16       | <0.001|
| White blood cell count (×10⁹/L) | 7.64±3.24           | 9.56±2.18         | 0.027 |
| TNF-α (ng/ml)             | 2.60±1.24            | 3.43±1.15         | 0.035 |

MAP – mean arterial pressure; TNF-α – tumor necrosis factor-α.

Figure 1. Comparisons of pathological sections between 2 groups 1 h after modeling. (A1–E1) Lung, liver, kidney, myocardium, and intestinal tissues of the normal control group; (A2–E2) Lung, liver, kidney, myocardium, and intestinal tissues of the shock model group.
Comparison of the rat growth among different groups

The growth condition of rats is shown in Table 3 after 7 days of normal feeding. No rats died in the normal control group, with a survival rate of 100%. Two rats died in the treatment group, with a survival rate of 83.33%. The survival rates in the normal control group and treatment group showed no significant difference (P>0.05). All rats died in the LPS group, with a survival rate of 0%, which was significantly lower than those in the normal control group and treatment group (P<0.05).

Surviving rats in the normal control group and treatment group had normal activities and diets. Average weight of surviving rats in the normal control group was significantly higher than that in the treatment group (P<0.05).

Analysis of therapeutic evaluation of plasma IMD level

Correlation between plasma IMD level and endotoxin indices in the treatment group is shown in Table 4. From the 1st, 3rd, 5th, and 7th days, plasma IMD expressions decreased gradually and the corresponding contents of IL-3, IL-6, IL-8, white blood cell count, and body temperature decreased, while the systolic pressure increased gradually (all P<0.05). IMD expressions were positively correlated with contents of IL-3, IL-6, IL-8, white blood cell count, and body temperature (P=0.0031; P=0.0014; P=0.0114; P=0.0026; P=0.0177), while IMD expressions were negatively correlated with systolic pressure (P=0.0040) (Table 5).

Discussion

In this study, plasma IMD mRNA and cytokines levels had stronger expressions as the severity of septic shock increased; while the expressions decreased after treatment was given, which to some extent suggests plasma IMD level can indicate severity and treatment effect of septic shock.

Generally speaking, the pathogenesis of shock is complex, and is related to a variety of organs in the body, especially to the pathological process of cardiovascular system, cytokines, and inflammatory factors [13,14]. These factors include a variety of endogenous damage factors such as endothelin and...

Table 3. Growth condition of rats in different groups.

| Items         | Normal control group (n=12) | LPS group (n=12) | Treatment group (n=12) |
|---------------|-----------------------------|------------------|------------------------|
| Survival rate (%) | 100                          | 0*               | 83.33                  |
| Weight (g)     | 218.24±25.56                | 188.57±25.61*    | 193.22±22.55*          |

* Compared with the normal control group, P<0.05.
Our experimental results show that with aggravation of septic shock, high expressions of IMD mRNA and cytokines levels occur, together with a low level of MAP and high white blood cell copy count and TNF-α, suggesting that an obvious inflammatory response appears in the process of septic shock. Severe infection may cause cell damage and the damaged endothelial cells can promote the secretion of inflammatory cytokines TNF-α and IL-6 due to increased expression of intercellular adhesion factor and mediated inflammatory cell dissociation [22,23]. The factors that mediate the apoptosis include the lipid peroxidation of oxygen free radicals, energy metabolism disorder, the activation of neutrophils [23], which may influence the growth of the body and even damages tissues or organs. Our experimental results confirmed that in the course of shock, plasma IMD level is positively correlated with the severity of shock [24,25].

Actually, the increase of IMD level in the course of septic shock is beneficial for body protection. According to the results, at the beginning of treatment, IMD level had a high expression. A study conducted by Wang et al. also concluded that the secretion of CGRP was increased in the animal models of septic shock [26], suggesting that animal models of septic shock had higher IMD levels. The level of IMD decreased as the body recovered. From the perspective of regulation of IMD on the body, as the expression of IMD is regulated by the NOS signal, the upregulation of IMD suggests that the efficacy of the NOS regulation pathway is improved, contributing to the mediation of ADM factor, which is one of the protective mechanisms of IMD [27]. The increase of IMD level also increases the expression of NO. Through regulating the pathway activity of NO-CAMP, the IMD can down-regulate the metabolic level in the course of shock, thus protecting the body [28]. IMD is also involved in the regulation pathway of CL/RAMPs receptor binding, which plays an important role in maintaining the stability of the internal environment and enhancing effective perfusion of cells and damaging lipid peroxidation [29]. Generally speaking, IMD exerts a similar biological effect as the CGRP family and does to activate the CAMP signaling pathway to prevent cell damage in the course of shock. A study conducted by Sexton et al. also reported that CGRP treatment appears to be beneficial in animal models of sepsis [30].

| Group | IMD content (ng/L) | IL-3 content (ng/ml) | IL-6 content (ng/ml) | IL-8 content (ng/L) | White blood cell count (×10⁹/L) | Temperature (°C) | Systolic pressure (mmHg) |
|-------|------------------|---------------------|---------------------|-------------------|-------------------------------|-----------------|------------------------|
| Day 1 | 457.6±51.55      | 0.17±0.09           | 0.29±0.13           | 2.58±1.01         | 10.27±1.51                    | 40.8±0.8        | 62.36±12.55            |
| Day 3 | 402.9±55.43      | 0.14±0.08           | 0.23±0.07           | 2.49±1.34         | 9.35±1.64                     | 40.2±0.7        | 75.69±14.78*           |
| Day 5 | 338.3±56.72      | 0.08±0.01*          | 0.21±0.08           | 1.33±0.61*        | 8.63±1.45*                    | 39.7±0.5*       | 93.08±17.65*           |
| Day 7 | 284.5±52.12      | 0.05±0.02*          | 0.18±0.05*          | 0.86±0.14*        | 8.04±1.26*                    | 39.4±0.5*       | 99.62±16.88*           |
| F     | 26.500            | 5.643               | 3.375               | 4.913             | 5.123                         | 8.437           | 14.100                 |
| P     | <0.001            | 0.002               | 0.027               | 0.005             | 0.004                         | <0.001          | <0.001                 |

* Compared with one-day treatment, P<0.05.

Table 4. Plasma IMD level and endotoxin indices changes in treatment group at different times.

Table 5. Correlation between plasma IMD level and endotoxin indices in treatment group.
Conclusions

Above all, with the application of rat shock model, our results supported that the expression level of IMD can act as an indicator for the severity and prognostic evaluation of septic shock. However, our results were not evidenced by clinical experimental samples. Compared with those of rats, actual situations of human patients are much more complex; therefore, the diagnostic role of IMD level in the severity and prognostic evaluation of septic shock remains to be proved by further evidence. However, the correlation between IMD and septic shock was largely demonstrated, and its potential clinical utility as an indicator of severity and treatment efficacy of septic shock, as well as showing its role in shock, are promising.

Competing interests

None.

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