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Original

Comparison of diagnostic ability of novel blood endotoxin measurement method using leukocyte-rich plasma sample and existing sepsis markers

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Abstract

Plasma samples are commonly used to measure endotoxin levels in blood. However, our previous research revealed the limited diagnostic ability of such plasma-based endotoxin assays. Therefore, we developed a unique method, the LRP37 method, for measuring endotoxin levels using leukocyte-rich plasma (LRP). This method capitalizes on the fact that the binding of endotoxins to leukocytes is the first step in endotoxin activity, along with complement activation. In this study, we examined two endotoxin measurement methods: a conventional turbidimetric kinetic assay (TKA) using LRP, and a semi-automated luminescent substrate method (SALS), which uses the leukocyte fraction obtained from the LRP. We compared the diagnostic performances of these methods with those of existing sepsis markers. A study consisting of 40 gram-negative and gram-positive patients revealed that the areas under the curve of TKA and SALS were 0.94 and 0.86, respectively. These diagnostic capabilities were the best among all the markers tested, underscoring the usefulness of the leukocyte-bound endotoxin assay.

Key words : leukocytes, endotoxins, sepsis, luminescent substrate method, turbidimetric kinetic assay

I. Introduction

Endotoxins, found in the cell walls of Gram-negative bacteria, exhibit various biological activities and play a significant role in the pathophysiology of sepsis. The limulus test, based on the gelation reaction in horseshoe crab amebocyte lysates, is commonly employed to measure endotoxin levels in plasma[1]. Methods utilizing this principle include the quantitative synthetic chromogenic substrate method[2] and turbidimetric kinetic assay (TKA)[3]. However, our investigation revealed that the sensitivity of TKA, which detects an increase in turbidity, was as low as about 50% despite its excellent detection limits[4,5]. We attribute the poor performance of TKA to the presence of endotoxins bound to or taken up by leukocytes, rendering them unmeasurable by this plasma-based assay[4]. Our previous studies indicated improved sensitivity of endotoxin measurements using

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leukocyte-rich plasma (LRP)[4]. In this study, we developed a unique and improved method for LRP preparation: the LRP37 method[5]. Addressing the time-consuming nature of TKA, which can take up to two hours, we employed a novel measurement system called the semi-automated luminescent substrate method (SALS)[6]. While a 10-fold dilution of LRP suffices for TKA, SALS requires at least a 75-fold dilution due to the high inhibitory factor activity, which is thought to inhibit the luciferase reaction in the plasma[7]. For SALS, we used the leukocyte fraction from the LRP to measure endotoxin levels in order to avoid the inhibitory factors in the plasma.

We compared endotoxin level measurements after TKA using LRP (LRP-TKA) and leukocyte fraction measurements by SALS (WBC-SALS). Additionally, we compared the diagnostic performances of these two methods with those of existing sepsis markers: C-reactive protein (CRP), white blood cells (WBC), procalcitonin (PCT), presepsin (PSEP), and interleukin-6 (IL-6).

II. Materials and Methods

1. Blood samples

Blood samples were collected from 57 patients admitted to the Iwate Advanced Critical Care Center between July 14, 2020, and December 31, 2021. Table 1 details the severity and names of the underlying diseases in the 57 patients. The cases include 17 non-infected patents, including trauma, 13 gram-positive infected patents, and 27 gram-negative infected patients. The severity and diseases responsible were identified using the results of blood, wound, sputum, urine, and cerebrospinal fluid cultures. Written informed consent was obtained from all patients or their family members prior to specimen collection. The International Consensus Definition of Sepsis and

Table 1. Severity and causative disease name of all 57 cases

	Non-infected cases (n = 17)	Gram-positive infected cases (n = 13)	Gram-negative infected cases (n = 27)	p-value
Female/Male	10/7	11/2	14/13	0.68
Age	69.8 ± 0.7	73.1 ± 3.9	69.1 ± 3.2	0.71
APACHE Ⅱ score	11.4 ± 6.1	15.2 ± 7.2※	17.7 ± 7.8※	0.02
SOFA score	2.4 ± 2.0	6.1 ± 5.1※	6.9 ± 5.5※	< 0.01
Gastroenterological diseases	0	8	12	
Respiratory diseases	0	2	1	
Traumas	6	0	0	
Circulatory diseases	1	0	0	
Cerebral diseases	3	0	0	
Renal diseases	0	1	5	
Burns	4	1	4	
Hepatobiliary diseases	0	1	0	
Gas gangrene	0	0	5	
Others	3	0	0	

A significant difference was observed between the non-infected cases and the Gram-negative infected cases in APACHE Ⅱ score and SOFA score.
※ $p < 0.05$ vs Non-infected cases.

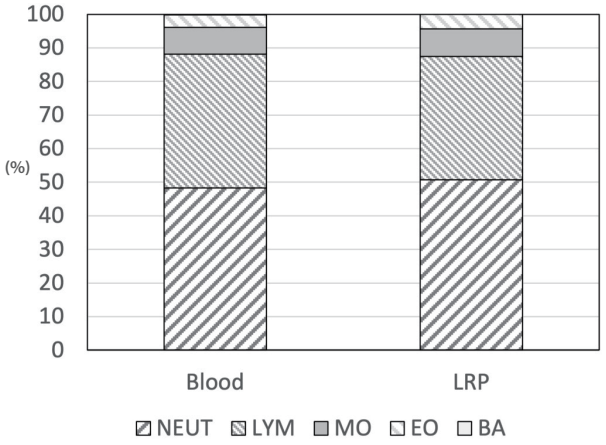

Fig. 1. Comparison of the differential leukocyte counts in whole blood and LRP
The differential leukocyte counts (neutrophils=NEUT, lymphocyte=LYM monocytes=MO, eosinophils=EO and basophils=BA) were performed using whole blood and LRP prepared by dextran sedimentation experiment obtained from 6 healthy donors. No statistical significances of the percentage of each cell between blood and LRP were observed ($p > 0.05$, t-test).

Septic Shock, 3rd Edition (Sepsis-3) was used to determine sepsis[8]. This study was approved by the Ethics Committee of Iwate Medical University School of Medicine (Ethics Committee No. MH2020-044) and all procedures were performed in compliance with the Declaration of Helsinki (World Medical Association 2013).

2. Reagents

1) Limulus amebocyte lysate (LAL)

Wako Lyophilized LAL Endotoxin Single Test (FUJIFILM Wako, Osaka, Japan) was used. This reagent is specific for endotoxins, owing to the presence of glucan[3].

2) Dextran T500

A 6%(W/V) solution of dextran T-500 (Pharmacosmo, Holbaek, Denmark) with an average molecular weight of 500,000 Da was prepared in physiological saline for injection (Otsuka Pharmaceutical Co., Ltd., Naruto, Japan). This solution was autoclaved at 121 ℃ for 90 min to ensure that it was free of endotoxin. Specifically, no gelation occurred even after the maximum measurement period (200 min) of incubation in TKA, suggesting an estimated endotoxin content of less than 0.05 pg/mL).

3. Preparations of samples

1) Preparation of LRP by the LRP37 method for LRP-TKA

Heparinized blood was collected and equilibrated at 37 ℃ for approximately 10 minutes. Then, 800 μL of the blood and 400 μL of the 37 ℃ equilibrated 6% dextran T-500 were mixed, and the resultant mixture was incubated at 37 ℃ for 15 to 20 min to produce a nearly clear upper layer[5], known as the LRP. The LRP was extracted and used for the endotoxin assay using the TKA method. The lower layer, that is, the dextran-aggregated erythrocyte layer, was discarded. The volume of the liquid portion of the LRP was diluted from the plasma volume by the value of the hematocrit and the addition of dextran solution. The dilution factor was ([A + amount of dextran]/A) (where A = blood volume - blood volume × hematocrit/100). For example, if the blood volume is 800 μL, the volume of dextran is 400 μL, and the hematocrit value is 30% (average hematocrit values of patient blood), in this illustration, A is 560 (800-800 × 0.3) and the dilution factor is calculated as (560 + 400)/560 = 1.71. Leukocyte counts remained unchanged between whole blood or LRP owing to the recovery rate of leukocytes, which was approximately 96% of the white blood cells. Comparisons were made with the mean of each differential leukocyte of whole blood and LRP of healthy subjects to confirm that there was no statistically significant difference. This confirmed that the number of fractions did not change during the process of LRP generation (Fig. 1).

2) Preparation of leukocytes from LRP for WBC-SALS

The LRP was centrifuged at 3000 rpm for 10 min using a benchtop centrifuge to obtain a precipitate, the leukocyte fraction. To lyse the leukocytes, the volume of water determined by the aforementioned formula was added to the precipitate. For example, when 500 μL of LRP was centrifuged, 300 μL of water was added to the precipitate according to the formula (500 / 1.71 ≒ 300 μL, with a hematocrit of 30 %). The mixture was vigorously agitated using a vortex mixer (MS-3; IKA Japan, Osaka, Japan) to ensure thorough lysis of the leukocytes. This sample was used for the SALS endotoxin assay.

4. Actual measurement methods

1) Pretreatment of LRP and leukocyte fraction

Since the blood comprises strong influencing factors for the Limulus reaction, LRP (also for the leukocyte fraction) was diluted 10-fold with a pretreatment solution (0.02 % Triton X-100, FUJIFILM Wako, Osaka, Japan) and the resultant solution was heat-treated at 70 ℃ for 10 minutes.

2) LRP-TKA

Lyophilized LAL was dissolved with 200 μL of the pretreated LRP and the test tube was placed in the Toxinometer ET-5500 (FUJIFILM Wako, Osaka Japan), and endotoxin levels were measured as previously described[3,4]. The endotoxin levels in the samples were calculated from a standard curve that had been plotted using *E. coli* O111:B4 LPS. The endotoxin value in pg/mL of LRP was obtained by multiplying the value obtained by 17.1. The detection limit of this assay was 0.86 pg/mL, which was calculated as 0.05 × 17.1 in LRP with a hematocrits value of 30 %. Because the SALS expresses endotoxin levels in EU/mL, TKA was also converted from pg to EU (1 pg = 7 mEU, information from the supplier).

3) WBC-SALS

SALS, developed by the DKK-TOA Corporation (Tokyo, Japan), was used for the detection of endotoxins in the dialysate during blood dialysis. The details of this method have been described previously[6, 8, 9]. Briefly, the system consists of a LAL tube, in which 200 μL of the pretreated leukocyte fraction was added, a bioluminescence (BL) tube, and a custom-made Luminutes®-ET luminometer connected to a Windows PC. The LAL tube contained LAL reagent (FUJIFILM WAKO, Osaka, Japan). The BL tube, which is smaller in diameter than the LAL tube, contains ATP, luciferase, and the synthetic luminescent substrate, benzoyl-Leu-Gly-Arg-amino-luciferin, which are automatically added to the reaction mixture in the LAL tube when the limulus reaction is completed. The amount of endotoxin (mEU/mL) in the leukocyte fraction of SALS cells was expressed by multiplying the obtained value by a dilution factor of 10. The detection limits in this instrument and the leukocyte fraction were 0.05 and 0.5 mEU/mL, respectively.

5. Measurement of infection markers

PCT (BioVendor R&D, Brno, Czech Republic) and IL-6 (R&D Systems, Minneapolis, MN, USA) levels were measured by enzyme-linked immunosorbent assay using plasma diluted 1:3 with phosphate-buffered saline and 1:10 with the diluent provided with the assay kit, respectively. PSEP values (LSI Medience, Tokyo, Japan) were measured by chemiluminescent enzyme immunoassay using undiluted blood.

Table 2. Values of parameters examined in 57 cases

	Non-infected cases (n = 17)	Infected cases		
		All cases (n = 40)	Gram-positive infected cases (n = 13)	Gram-negative infected cases (n = 27)
WBC (× 10^3/ μL)	10.68 ± 2.09	14.97 ± 1.36	10.37 ± 2.58	17.19 ± 1.69
CRP (mg/dL)	3.96 ± 2.68	13.88 ± 1.75	8.95 ± 3.42	16.26 ± 2.23
PCT (ng/mL)	1.06 ± 2.81	9.73 ± 1.82	7.93 ± 3.82	10.6 ± 2.05
IL-6 (pg/mL)	110.88 ± 1439.17	3465.9 ± 938.24	52375 ± 1892.32	4882.0 ± 1246.12
PSEP (pg/mL)	468.09 ± 1050.21	3152.7 ± 684.73	4596.04 ± 1414.9	2457.76 ± 599.36
LRP-TKA(mEU/mL)	13.57 ± 169.53	292.51 ± 110.57	16.74 ± 224.67	456.67 ± 148.28
WBC-SALS(mEU/mL)	59.69 ± 143.41	308.33 ± 93.49	14.54 ± 183.52	506.08 ± 122.03

WBC, white blood cells; CRP, C-reactive protein; PCT, procalcitonin; IL-6, interleukin-6; PSEP, presepsin; TKA, turbidimetric kinetic assay; LRP, leukocyte rich plasma; SALS, semi-automated luminescent substrate method. Reported cut-off values of PCT, CRP and PSEP in sepsis are 0.5 ng/mL, 8.7 mg/dL, and 671 pg/mL, respectively.

Table 3. Measured values and diagnostic abilities in 17 non-infected and 40 infected cases

	p-value	AUC (95% CI)	Cut-off value	Sensitivity	Specificity
WBC (× 10^3/ μL)	0.09	0.60 (0.45 − 0.75)	18.31	0.94 (0.85 − 0.99)	0.33 (0.21 − 0.45)
CRP (mg/dL)	0.03	0.81 (0.69 − 0.94)	4.04	0.71 (0.57 − 0.81)	0.80 (0.67 − 0.88)
PCT (ng/mL)	0.01	0.83 (0.72 − 0.95)	0.67	0.88 (0.77 − 0.94)	0.73 (0.61 − 0.83)
IL-6 (pg/mL)	0.06	0.77 (0.63 - 0.90)	8.0	0.47 (0.39 − 0.59)	0.95 (0.85 − 0.99)
PSEP (pg/mL)	0.03	0.81 (0.69 - 0.93)	1136	1.00 (0.92 − 1.00)	0.50 (0.36 − 0.62)
LRP-TKA (mEU/mL)	0.15	0.80 (0.71 - 0.93)	33.6	0.94 (0.85 − 0.99)	0.60 (0.47 − 0.72)
WBC-SALS(mEU/mL)	0.17	0.67 (0.56 - 0.84)	22.7	0.70 (0.57 − 0.80)	0.65 (0.52 − 0.76)

WBC, white blood cells; CRP, C-reactive protein; PCT, procalcitonin; IL-6, interleukin-6; PSEP, presepsin; TKA, turbidimetric kinetic assay; LRP, leukocyte rich plasma; SALS, semi-automated luminescent substrate method; AUC, area under curve. Parameters with higher values are underlined (e.g., significant p-value ($p < 0.05$), area under the curve greater than 0.8, and specificity or sensitivity greater than 0.9).

6. Statistical analysis

Comparisons between the two groups were made using the student's t-test and Mann-Whitney U test. The Spearman rank-order correlation coefficient was used to examine the correlations. We used receiver operating characteristic (ROC) analysis cutoff values. P-values < 0.05 were considered statistically significant. Two-group comparisons, ROC analysis, correlations, and multiple linear regression analyses were performed using the JMP software (SAS Institute, Cary, NC, USA).

III. Results

Each marker tended to have higher measured values in the infected group than in the non-infected group. CRP, PCT, and PSEP values were significantly higher in the infected group; however, there was no significant difference in WBC, IL-6, LRP-TKA, and WBC-SALS (Table 2). With an area under the curve (AUC) of 0.83, PCT had the best ability to distinguish 17 non-infected cases from 40 infected cases (Table 3). Receiver Operating Characteristic curve for each marker in 17 non-infected cases and 40 infected cases

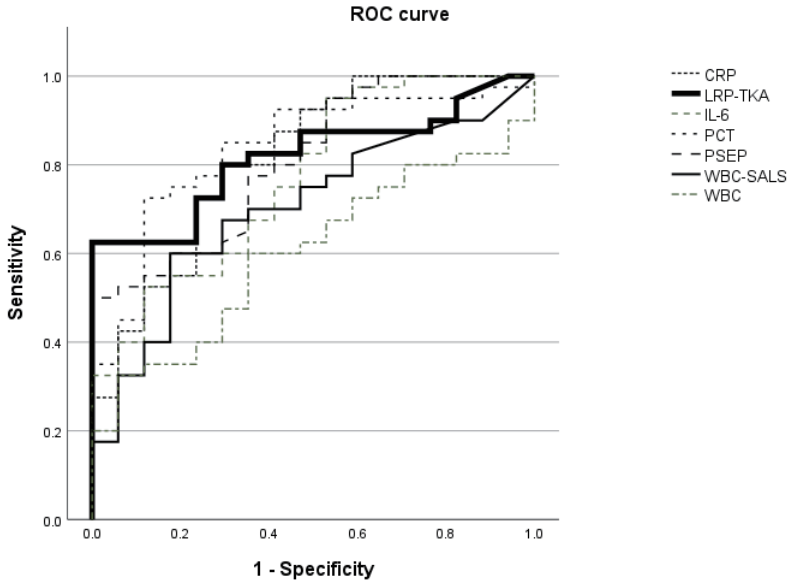

Fig 2. Receiver Operating Characteristic curve for each marker in 17 non-infected cases and 40 infected cases
No significant difference was observed in each area under the curve. WBC, white blood cells; CRP, C-reactive protein; PCT, procalcitonin; IL-6, interleukin-6; PSEP, presepsin; TKA, turbidimetric kinetic assay; LRP, leukocyte rich plasma; SALS, semi-automated luminescent substrate method; AUC : area under curve.

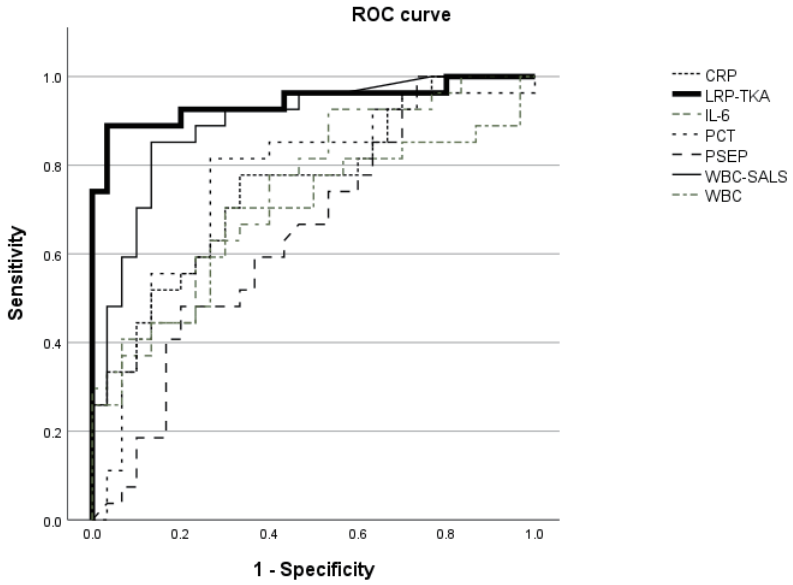

Fig 3. Receiver Operating Characteristic curve for each markers in 17 non-infected and 27 Gram-negative infected cases
There was a significant difference between the AUC of LRP-TKA and the AUC of WBC (P = 0.03). WBC, white blood cells; CRP, c-reactive protein; PCT, procalcitonin; IL-6, interleukin-6; PSEP, presepsin; TKA, turbidimetric kinetic assay; LRP, leukocyte rich plasma; SALS, semi-automated luminescent substrate method; AUC, area under curve.

showed no significant differences in each area under the curve (Fig. 2).

Excluding the 17 non-infection cases, comparison of the Gram-positive and Gram-negative groups revealed that Gram-negative bacteria tended to have higher WBC-SALS

Table 4. Measured values and diagnostic abilities in 17 non-infected and 27 Gram-negative infected cases

	p-value	AUC (95% CI)	Cut-off value	Sensitivity	Specificity
WBC ($\times 10^3/\mu L$)	0.02	0.67 (0.51 - 0.83)	10.74	0.64 (0.49 − 0.76)	0.71 (0.56 − 0.82)
CRP (mg/dL)	< 0.01	0.83 (0.71 - 0.95)	4.95	0.76 (0.61 − 0.86)	0.78 (0.63 − 0.88)
PCT (ng/mL)	< 0.01	0.86 (0.75 - 0.98)	0.69	0.88 (0.75 − 0.95)	0.82 (0.57 − 0.81)
IL-6 (pg/mL)	0.02	0.80 (0.67 - 0.93)	136.18	0.88 (0.75 − 0.95)	0.60 (0.57 − 0.81)
PSEP (pg/mL)	0.04	0.80 (0.67 - 0.93)	1136	0.94 (0.84 − 0.99)	0.49 (0.57 − 0.81)
LRP-TKA (mEU/mL)	< 0.01	0.94 (0.88 - 1.0)	33.6	0.89 (0.76 − 0.96)	0.89 (0.76 − 0.96)
WBC-SALS (mEU/mL)	0.02	0.86 (0.74 - 0.98)	43.8	0.82 (0.68 − 0.90)	0.85 (0.71 − 0.93)

WBC, white blood cells; CRP, C-reactive protein; PCT, procalcitonin; IL-6, interleukin-6; PSEP, presepsin; TKA, turbidimetric kinetic assay; LRP, leukocyte rich plasma; SALS, semi-automated luminescent substrate method; AUC, area under curve. Parameters with higher values are underlined (e.g., significant p-value ($p < 0.05$), area under the curve greater than 0.8, and specificity or sensitivity greater than 0.9).

and LRP-TKA values, with a particularly significant difference in the latter (Table 2,3).

When it came to differentiating the 17 non-infected cases from the 27 Gram-negative bacteria cases, excluding the Gram-positive bacteria group, all markers were significantly higher in the Gram-negative group (Table 2). With an AUC of 0.94, the TKA with LRP method had the best AUC. SALS had an AUC of 0.86, which was better than that of the other parameters, but comparable to that of PCT (Table 4). Receiver Operating Characteristic curve for each markers in 17 non-infected and 27 Gram-negative infected cases showed a significant difference between the AUC of LRP-TKA and the AUC of WBC ($P = 0.03$) (Fig. 3).

The WBC-SALS cutoff value was calculated to be 22.7 mEU/ml (Table 3), while the mean endotoxin value for uninfected patients was 59.69 mEU/ml, indicating that endotoxins are present even in uninfected patients in some cases (Table 2). In such cases, the bacterial culture may have shown false-negative results, or the blood samples might have contained residual endotoxin from non-colony-forming bacteria.

IV. Discussion

To date, reports have compared the diagnostic performance of existing sepsis markers, including CRP, PCT, IL-6, and PSEP levels; however, no discernible differences have been found. In an evaluation of the diagnoses of 33 Intensive Care Unit patients, Bacil et al.[11] found that PCT performed better than IL-6. Meynaar et al.[12] also reported that PCT is superior to IL-6 and CRP. In contrast, Du et al.[13] found no difference in the diagnostic performance of IL-6 and PCT, and Takahashi et al.[14] reported that IL-6 was the most effective in diagnosing sepsis. There also have been reports in the past few years that PSEP[15-17] is the best, and a comprehensive investigation of this subject is anticipated. At this point, an analysis of multiple sepsis markers plus clinical indications, such as vital signs, is required, as there is no single sepsis marker known to be effective in diagnosing sepsis. The results of this study can contribute to improving the clinical treatment of sepsis.

One implication of this study is the

excellent diagnostic ability of PCT and LRP TKA. Our investigation found that the AUC of PCT was the highest among other markers when distinguishing the noninfected group from the infected groups, even though the difference in effectiveness between CRP and PSEP was minor, as previously reported. Second, LRP-TKA demonstrated an outstanding ability to discriminate between the uninfected group from the gram-negative bacterial infection groups.

This study is the first to demonstrate the validity of using only the leukocyte fraction as a target for endotoxin measurement in the diagnosis of sepsis. We conducted this study based on the premise that conventional endotoxin measurement methods using plasma may not be able to accurately evaluate endotoxins. We confirmed that no leukocytes were found in the plasma and most leukocytes of any type were recovered from the LRP. We also examined samples containing only leukocytes for the clinical application of SALS (i.e., WBC-SALS). However, the AUC of WBC-SALS was 0.86, which was slightly inferior to that of LRP-TKA. Nevertheless, SALS has the advantage of being easy to use and quick to implement, as the majority of its procedures are automated and can take as few as 20 minutes. We intend to continue our research into the clinical effectiveness of SALS.

V. Conclusions

In this study, 1) PCT was superior in diagnosing infected groups compared to uninfected groups; 2) LRP-TKA was superior in diagnosing gram-negative infected groups compared to uninfected groups, but WBC-SALS was also considered a comparable method; and 3) significant correlations were found between the SOFA score and CRP, PSEP and APACHE II and CRP, WBC-SALS. Considering the ease of measurement and short time required, WBC-SALS is considered a better endotoxin assay than LRP-TKA. This will be confirmed in future studies with a larger number of cases.

Conflict of interest: The authors have no conflict of interest to declare.

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多白血球血漿検体を用いた新規エンドトキシン測定法の敗血症検査としての有用性の検証

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要旨

血中エンドトキシン（endotoxin: ET）濃度を測定するためには，一般的に多血小板血漿検体が用いられる．しかし，従来の多血小板血漿検体を用いた測定法は, 診断能が低いことがよく知られている．そこで我々は，ET 活性化の第一段階が ET と白血球の結合であるという事実を利用し，測定検体として多白血球血漿（leucocyte - rich plasma：LRP）を用いた新たな ET 濃度測定法を開発した．本研究では，測定検体と測定方法の組み合わせとして，LRP の ET を比濁時間測定法（turbidimetric kinetic assay: TKA) で測定する方法と，LRP から得られた白血球分画の ET を半自動発光基質法（semi-automated luminescent substrate method: SALS）で測定する方法を検討し，これらの方法の診断能を既存の敗血症マーカーと比較した．40 例のグラム陰性および陽性患者からなる試験の結果から，TKA および SALS の曲線下面積（AUC）はそれぞれ 0.94 および 0.86 であり，これらの診断能は比較したすべてのマーカーの中で最も優れており，白血球結合 ET を利用した ET 濃度測定法の有用性が確認された．