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Evaluation of the use of the Endotoxin Activity Assay (EAA™) to Quantify Non-septic Exposure of Metabolic Endotoxemia

Natalie McPhee[1], **Kelton Tremellen**[1,2] **and Karma Pearce**[1*]

[1]*School of Pharmacy and Medical Sciences, Division of Health Sciences, University of South Australia, Adelaide, South Australia*

[2]*Repromed Adelaide Fertility Specialists, 180 Fullarton Road, Dulwich, South Australia*

[*]**Corresponding author**: Karma Pearce, PhD, Division of Health Sciences, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide 5001, South Australia, Tel: +61883021133; E-mail: karma.pearce@unisa.edu.au

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Abstract

Objective: Endotoxin, also known as lipopolysaccharide (LPS), is a potent immune stimulant. Low levels of endotoxin exposure (metabolic endotoxemia) play a pivotal role in metabolic disorders. However, there is no robust clinical assay to directly quantify metabolic endotoxemia. We aimed to validate the whole blood Endotoxin Activity Assay (EAA™) as a novel, rapid method to quantify low grade metabolic endotoxemia against the well-established lipopolysaccharide binging protein assay (LBP), a robust surrogate marker of endotoxaemia.

Methods: 67 women and 47 men aged 21 to 47 years (35.4 ± 5.5 years, 34.5 ±7.2 years respectively) were assessed for adiposity (BMI, waist circumference and % body fat using bio-impedance), endotoxin levels (LBP, EAA™) and inflammatory status (serum CRP, IL-6, IL-8).

Results: There was no direct relationship between EAA™ and LBP measures for quantitating metabolic endotoxemia for either women or men (R=0.146, p=0.284; R=0.283 p=0.09 respectively). In women, the traditional indirect marker of endotoxemia LBP correlated significantly with CRP and IL-6, measures of generalised immune activation and inflammation (R=0.664, p<0.001, R=0.296, p=0.028 respectively), but not with EAA™ assed endotoxemia. Supporting this relationship, LBP correlated with BMI and body fat percentage (R=0.306, p=0.022; R=0.301, p=0.024 respectively). However, the EAA™ only correlated with body fat percentage (R=0.382, p=0.014). In men, LBP was significantly related to CRP and IL-6 (R=0.345, p=0.046; R=0.421, p=0.009 respectively), but no relationship was observed between these inflammatory markers and EAA™ assed metabolic endotoxemia (R=0.206, p=0.243; R=0.280, p=0.093 respectively). There was no relationship between EAA™ or LBP and any of the three measures of adiposity.

Conclusion: In conclusion, the existing rapid whole blood EAA™ method of analysis was not suitable to detect low levels of endotoxemia known to be present in the obese state, while the results suggest LBP indirect analysis remains the superior tool for measuring low grade endotoxemia in this population.

Keywords: Endotoxins; Lipopolysaccharides; EAA™; Endotoxin activity assay; Lipopolysaccharide binding protein; lipopolysaccharide binding protein assay; Adiposity; Inflammation

Abbreviations CRP: C-Reactive Protein; EAA™: Endotoxin Activity Assay; IL-6: Interleukin 6; IL-8: Interleukin 8; LBP; Lipopolysaccharide Binding Protein Assay; LPS; Lipopolysaccharides; NSAID: Non-steroidal Anti-inflammatory Drugs; WHO: World Health Organisation

Introduction

Endotoxins, also known as lipopolysaccharides (LPS), are fragments derived from the outer membrane of gram negative bacteria [1]. Despite significant research interest on the effect of low levels of endotoxin exposure on health [2], there is no robust gold standard assay to directly test for metabolic endotoxemia, thereby limiting research advances in the field.

Currently, there are three very different analytical techniques to assess human endotoxin levels; The Limulus amebocyte assay (LAL) and Lipopolysaccharide binding protein assay (LBP) are both used for endotoxin detection over a wide range of endotoxin levels, while the Endotoxin Activity Assay (EAA™) is predominantly used to detect endotoxin in the septic high dose exposure range.

The LBP is reported to be an easy to conduct and robust indirect measurement of endotoxin exposure, with this acute-phase reactant protein being produced, predominantly by the liver [3] in response to endotoxin exposure. The biological role of LBP is to deliver endotoxin to the monocyte surface molecule CD14, a co-receptor allowing endotoxin to interact with toll- like receptor 4 (TLR4), triggering a signalling cascade that ultimately results in the up regulation in expression of pro-inflammatory cytokines [4].

The LBP has been used extensively to detect low levels of metabolic endotoxemia associated with obesity [5-9]. LBP has been used to detect varying levels of endotoxemia in inflammatory bowel conditions, pancreatitis, cirrhosis and sepsis where levels in serum can increase 10 to 50 fold during acute inflammation [10,11].

However, the use of this indirect measurement is not ideal, as the accuracy of the test is dependent on normal hepatic function [12], and the response to endotoxin exposure is delayed, making LBP an

inappropriate test for quantifying rapidly changing levels of endotoxin exposure.

In contrast, the LAL assay offers a direct measurement of endotoxin through initiating a blood clotting cascade, as *Limulus polyphemus* clots with Gram negative bacteria during infection [13], presumably to prevent sepsis. The clotting is produced as a result of LPS initiating the coagulation of a cellular blood protein in amoebocytes of *L. polyphemus* (Horse Shoe Crab) by a signaling cascade [13]. Commercially, the LAL has been approved by the Food and Drug Administration FDA to evaluate the safety of medical devices screening for the absence of endotoxin Although the LAL assays are able to detect endotoxin in biological specimens, extensive sample preparation is required to neutralize the many components known to inhibit or activate the enzymatic cascade by heating, pre-treatment with harsh chemicals and dilution with pyrogen free water which increases the risk to the user, the complexity of the test and the time taken to determine the result [11]. Additionally, the assays are usually performed in batch mode with the subtraction of a baseline signal and determination of acceptability through the use of spiked positive controls increases the time taken to complete the analysis. Furthermore, the assay is highly sensitive to extrinsic endotoxin contamination [11].

The Endotoxin Activity Assay (EAA™) is a semi quantitative "bed side" test for endotoxin in whole blood, approved by the FDA for the detection of gram negative sepsis in intensive care patients, and quantifies the output on a relative scale from 0 to 1.0, based on an internal endotoxin standard that is added to each sample with results above 0.4 suggesting a significant risk of sepsis [14]. The assay comprises three separate reactions in samples of whole blood; a blank containing the whole blood and all reagents except the anti-lipid A antibody; a second tube containing whole blood and anti-lipid A antibody and the final maximal response tube containing whole blood and an excess of endotoxin.

The assay measures the endotoxin activity in whole blood by the priming of host neutrophil respiratory burst activity via complement opsonized LPS-IgM complexes and is not affected by extrinsic endotoxin contamination. The light energy produced as a result of a luminol reaction in the presence of immune complexes is detected as Relative Light Units (RLU) at 450 nm. The RLU is then converted to an Endotoxin Activity (EA) level (reported as a percentage of the total possible activity; (sample with antibody-sample without antibody)/(sample with maximal endotoxin-sample without antibody)) [14].

The EAA assay has been used to assess the risk of sepsis [14-20], as well as in healthy [21-23] and obese individuals [24], but is not marketed as a suitable test for the detection of low-grade endotoxemia. Unlike the LAL and LBP, the EAA has the advantage of neither require centrifugation nor sample treatment, can be performed on individual samples and therefore is capable of providing a real-time result within 30 minutes from blood collection, making it potentially an attractive assay format for quantifying metabolic endotoxaemia.

The aim of this research was to conduct a prospective observational study to validate the ability of the Endotoxin Activity Assay (EAA™) to measure low grade metabolic endotoxemia, against the well-established lipopolysaccharide binding protein assay (LBP) in a cohort of healthy (non-septic) men and women, with a range of body masses. We hypothesised that the LBP and EAA™ tools would correlate against each other and support positive relationships with both adiposity and inflammatory markers.

Methods

Participant recruitment

Men and women aged 18 to 50 years were recruited from a private infertility treatment medical unit (Repromed, Adelaide, South Australia) over a 6 month period in 2016. Exclusion criteria included inflammatory or infective disease and the consumption of immunosuppressive medication (NSAID, corticosteroids, fish oil) in the last month. The study was approved by the University of South Australia Human Research Ethics Committee in December 2015 (approval number; 0000030973), with informed written consent being obtained from all participants before enrolment.

Anthropometric measurements

Height was measured to within 1 cm using a stadiometer, and weight (kg) and percentage body fat measured to the nearest 0.1 kg and 0.1% respectively using bio-impedance digital scales (Tanita, UM051). Waist circumference was measured using an anthropometric tape measure and was defined as the midway point between the 12th rib and the iliac crest to an accuracy of 0.5 cm. Body mass index (BMI) was calculated as body weight (kg) divided by height (m) squared with the following ranges used for classification; underweight (<18.5 kg/m^2), normal weight (18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2) or obese (>30 kg/m^2) as per WHO guidelines (1996).

Assessment of metabolic endotoxemia: Endotoxin activity Assay (EAA™)

To assay levels of LPS, 10 ml of whole blood was placed in each of three tubes containing luminol buffer (300 µl/tube). The control tube contained buffer and blood only, whereas a positive control contained a maximum stimulatory concentration of endotoxin (2 ng/ml); the final tube contained the test sample. All three tubes, analysed in triplicate, were incubated at 37°C for 5 min. Chemiluminescence was instigated by the addition of 20 µl/tube human complement opsonized zymosan. Continuous measurements were made over 30 second intervals of light emissions over a total period of 20 minutes in a reciprocating tube luminometer (Autolumat, Germany).

Quantitation of endotoxin in whole blood was determined via a dose-response curve of endotoxin concentration verses averaged light emission [14]. A normalized response factor was calculated by subtracting the averaged 20 min light integral of the control from the assay tube and the maximally stimulated tube for each participant. The response factor was the difference light integral of the test sample divided by the difference integral of the maximally stimulated tube. The endotoxin concentration was extrapolated from the dose–response curve of response factor versus endotoxin concentration. LPS was quantified directly within 3 hours of blood collection using the EAA™ (protocol; EAA20-1, Spectral Medical Inc, Toronto, Canada).

The sensitivity was 0.042 EA units with a mean intra assay CV of 8.01% which was within the reported acceptability parameters. Upon completion, the remaining whole blood was centrifuged for plasma extraction (3800 rpm, 25°C, 8 minutes) and stored at -80°C. All assays were performed in duplicate and replicates with CV >20% were not included in the analysis.

Lipopolysaccharide Binding Protein (LBP) endotoxin measurement

Metabolic endotoxemia was quantified indirectly by LBP analysis using an ELISA system according to the manufacturer's guidelines (Hycult, Uden, Netherlands) with the minimum detectable concentration of LBP being 4.4 ng/ml.

Inflammation status

C-reactive protein (CRP) was measured in serum using an automated chemiluminesence machine (Integra 800, Roche Diagnostics, USA), with the limit of detection being 1 mg/l. Serum IL-6 (males and females) and IL-8 (females) were analysed in duplicate serum samples using a multiplex immunoassay (ProcartaPlex kit, eBioscience). The detectable range for each of these cytokines was 1.06 – 4340 pg/ml and 2.17 – 8900 pg/ml respectively.

Data interpretation

Statistical analyses were conducted using IBM Statistical Product and Service Solution software, version 22 (SPSS Inc., Chicago, IL, USA). All variables were reported as mean ± standard deviation where normally distributed, or as median ± inter quartile range when not normally distributed using the Shapiro-Wilk test. Correlations were assessed using the Pearson's method, with log transformation of non-normally distributed data prior to statistical analysis. Statistical significance was set at $p<0.05$.

Results

Participant demographics

Forty seven men and 67 women were recruited to this study. The mean (+SD) age, BMI and percentage body fat of the female participants was 35.4 ± 5.5 years, 29.0 ± 6.8 kg/m^2 and 37.6 ± 7.3% respectively, while the mean (+SD) age, BMI and percentage body fat of the male participants was 34.5 ± 7.2 years, 27.4 ± 3.6 kg/m^2 and 24.3 ± 5.9%. None of the participants were lean, with just over 60% of women overweight (26.8%) or obese (35.7%), compared to almost 80% of the men who were overweight 59.4% or obese (19%).

Metabolic endotoxemia

The mean (+SD) endotoxin level measured as EAA™ 0.32 ± 0.10 EA units for women and 0.26+0.1 EA units for men. Endotoxin as measured by LBP was 14.6+4.9 ng/ml for women and 11.4 (5.6-22.3) ng/ml for men. There was no direct relationship between EAA™ and LBP measures of endotoxin for either men or women, although a possible positive trend was noted for men (R=0.306, p=0.09).

Measures of inflammation

C-reactive protein, a well-established generalized measure of immune activation and the cytokines IL-6 and IL-8, measures of inflammation were also assessed. In the female cohort, CRP was strongly correlated with LBP (R=0.664, p<0.001), however no relationship was apparent with EAA™ (R=0.206, p=0.243). Similar results were observed when the two methods of endotoxin detection were correlated against IL-6 (EAA™: R=0.280, p=0.093), LBP: r=0.296, p=0.028). No relationship was observed with either EAA™ or LBP and serum IL-8 (p(R=-0.132, p=0.264); R=-0.001, p=0.996 respectively) (Table 1).

When the male cohort was examined, there was a positive association between LBP and both CRP and IL-6 (R=0.345, p=0.046; R=0.421, p=0.009 respectively), whilst no significant relationships were observed between EAA™ and either CRP or IL-6 (R=0.206, p=0.243; R=0.280, p=0.093) (Figure 1).

Parameters	Variable Mean + SD or Median (IQR)	
	Women	Men
CRP mg/l	2.5 (4.5)	1.6 (2.0)
serum IL-6 pg/ml	2.1 (2.0)	5.6+2.4
serum IL-8 pg/ml	1.8 (3.3)	

Table 1: Measures of inflammation. CRP: C-reactive protein; IL-6: Interleukin 6; IL-8: Interleukin 8. Normally distributed data expressed as mean + SD and non-normally distributed as median+Inter-quartile range.

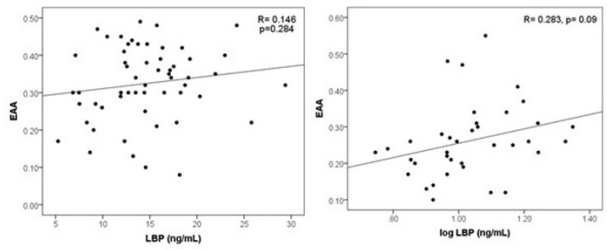

Figure 1: The relationship between the measures of endotoxin, EAA™ and LBP. 1a: Women, 1b: Men.

In women, LBP positively correlated with BMI and body fat percentage, whilst the relationship with waist circumference also approached significance (R=0.306, p=0.022; R=0.301, p= 0.024; R=0.259, p=0.054 respectively). However, when the EAA™ assay was used to quantify endotoxin levels in women, there was only a relationship with body fat percentage (R=0.382, p=0.014), a known trigger for metabolic endotoxaemia. In the male cohort, there was no relationship between LBP or EAA™ and any of the three measures of adiposity (LBP; body fat; R=0.223, p=0.185; BMI: R=0.194, p=0.249; waist: R=0.141, p=0.406, EAA™; body fat: R=0.026, p=0.877; BMI: R=0.049, p=0.773; waist: R=-0.026, p=0.878) (Figure 2).

Discussion

The key finding of this study was that the EAA™ was not a suitable tool to measure low grade endotoxemia typically associated with obesity. Firstly, the EAA assay results bore no correlation with the well-established LBP analysis (women; p=0.289, men; p=0.09). Secondly, the EAA assay showed no relationship between any of the measures of adiposity, with the exception of % body fat in women alone. This is important as endotoxin levels are known to increase proportionally with increasing levels of obesity [7,11,25], as increasing adiposity is associated with a breakdown in the intestinal mucosal barrier, allowing the translocation of gut bacteria into the systemic circulation. Thirdly,

the EAA™ assay did not display any relationships between well-established markers of immune activation and inflammation (CRP and serum cytokines), providing further evidence of the lack of suitability of the EAA™ for use in this context.

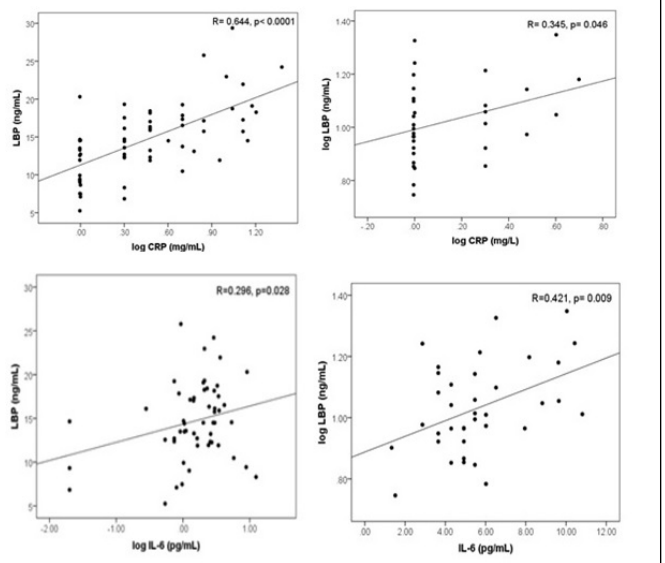

Figure 2: The relationship between endotoxin and measures of inflammation. 1a: Women, 1b: Men, 1c: Women, 1d: Men.

In contrast, our study confirmed positive relationships between increasing adiposity and the indirect endotoxin measure LBP, in agreement with multiple previous studies [11] confirming LBPs capacity to quantify metabolic endotoxaemia. Furthermore, in women the LBP measure showed significant positive relationships with CRP and IL-6, and the three measures of adiposity as well as positive relationships in the male cohort between LBP and both CRP and IL-6, but not adiposity. We believe the absence of a significant association between LBP and adiposity in the male cohort may be due to the study not reaching a sufficiently powered sample size of obese men. This is evidenced by the fact a relationship was observed in the female cohort, which included a larger sample size (57 women compared to 37 men), as well as a greater proportion of obese participants in the female cohort (35.7% versus 19.0%). Furthermore, we have recently reported in a larger separate study, a significant positive relationship between LBP and adiposity in 50 overweight and obese males [26]. Others have also reported highly significant differences in LBP values in overweight and obese individuals compared to healthy controls, with differences stratified by BMI [5]. All these findings suggest LBP is a robust assay for obesity related low grade metabolic endotoxemia.

Collectively, the findings suggest the EAA™ is not a useful test for metabolic endotoxemia in its current form. Previous studies have also questioned the usefulness of this test under conditions of low level endotoxemia. Two research groups have failed to report a significant relationship between EAA™ and LAL values [27,28]. Additionally, Matsumoto et al. reported a dose dependant elevation in EAA results in response to IL-8 administration to blood samples, and suggested that EAA™ values represented the primed state of neutrophils [29]. However, the current study failed to detect a relationship between endotoxin (EAA™) and serum IL-8 (R=-0.132, p=0.264), which counters this argument. Pendyala et al. have demonstrated in a cross sectional study that EAA™ values increase in response to the adoption of a high fat diet, a known stimulus for metabolic endotoxaemia [23]. This small scale study included a cohort of only 3 men and 5 women, and reported EAA™ values ranging between approximately 0.05 and 0.4, similar in range to the present study. While the differences suggest that the EAA™ may be suitable for assessing the effect of different dietary components on endotoxin activity within the blood, neither LAL or LBP levels were reported. Future research in this area should validate the EAA™ against other well established analytical assays such as LPB.

A potential explanation for why this study failed to observe a significant relationship between the EAA™ and the LBP assay in the context of metabolic endotoxemia could relate to the fact the assay was designed specifically for use in individuals at risk of sepsis. The EAA™ normalises endotoxin induced neutrophil chemilumiescence against a maximal control tube spiked with exogenous endotoxin levels to simulate sepsis. Romaschin et al. in their review of the EAA™ assay report the most sensitive part of the dose response curve (change in EU per LPS dose) in the revised calibration using WHO standardised LPS (200 pg/EU) was greatest in the range of 0.1 to 0.3EU, yet these results remain unpublished [30]. It is therefore plausible that manipulation of the EAA™ protocol through decreasing the endotoxin concentration within the maximum response tube may yield an assay that can accurately measure endotoxin activity in a "low range" metabolic endotoxemia context [31]. We call on the manufacturers of the EAA assay (Spectral Diagnostics) to investigate this possibility. Furthermore, the EAA™ is an indirect measure of endotoxin, measuring the production of reactive oxygen species (ROS) in response to endotoxin. Chronic exposure to endotoxin has previously been shown to decrease the production of ROS in in-vitro studies, a concept referred to as endotoxin tolerance [32]. The presence of endotoxin tolerance could not be identified using the methods employed in this study, as this would require an interventional design, however, its occurrence could in part explain why the EAA™ did not detect metabolic endotoxemia as originally hypothesised. As the presence of chronic low levels of endotoxin within the blood is a central concept to the metabolic endotoxemia hypothesis, the measurement of ROS may therefore make the assay inherently unsuitable for this context.

The strengths of this study include the quality control procedures employed. All duplicates performed using the EAA™ protocol conformed to the manufacturer's instructions and all coefficients of variation were below the recommended upper limit of 20%. Similarly, the standard curves obtained for the LBP assay exceeded the required R value (R>0.99). Accordingly, the data generated from these two assays was likely to reflect true EAA™ and LBP values. However, several limitations should be noted; the participants in this study were all aged 18 to 50 years and did not represent a diverse range of ethnicities. It is therefore possible these results may vary in different populations and this warrants further investigation.

Conclusion

In conclusion, the rapid whole blood EAA™ method of analysis was not suitable to detect low levels of endotoxemia, while the results suggest LBP analysis remains the superior tool for measuring low grade endotoxin concentrations in this population. We suggest all future studies employing the EAA™ in the context of low level endotoxemia also validate the tool against either the more established LAL or LBP methods of analysis. We also call on the manufacturers of

the EAA assay (Spectral Diagnostics) to investigate this possibility that manipulation of the EAA™ protocol through decreasing the endotoxin concentration within the maximum response tube may yield an assay that can accurately measure endotoxin activity in a "low range" metabolic endotoxemia context.

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