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Novel Insights into the Direct Removal of Endotoxin by Polymyxin B Hemoperfusion

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Abstract
Aim: To demonstrate the capacity of polymyxin B-direct hemoperfusion (PMX-DHP) column Toraymyxin® 20R (PMX-20R) in removing endotoxin (LPS) from perfused blood, serum and plasma. ***Methods:*** Endotoxin-spiked bovine serum or plasma was perfused in PMX-20R as per the recommended performance testing protocol. Samples were taken at various time points to assess the amount of endotoxin removed during a 4-h session. In another set of experiments, FITC-labelled LPS (FITC-LPS) was spiked into a pool of human whole blood, followed by perfusion with the spiked blood for 2 h in order to allow FITC-LPS to bind PMX-20R. The amount of LPS was extracted from the columns and the amount of specifically bound LPS was determined by fluorometry. ***Results:*** PMX-20R columns perfused with bovine serum or plasma had an average binding rate of 88%, equivalent to approximately 12 μg of LPS. When PMX-20R was perfused with human whole blood, the columns bound an average of 20 μg of FITC-LPS. ***Conclusion:*** PMX-20R can bind LPS in all the biological fluids tested. The calculated binding capacity of 12–20 μg LPS suggests that in septic cases where endotoxin is present in the circulation, PMX-20R is able to adsorb clinically significant levels of endotoxin.

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Introduction

Endotoxin can enter the systemic circulation from different compartments and sources through a variety of routes [1]. This includes the transit from infected extravascular sites, release from blood born bacteria and movement across epithelial barriers. Notable reservoirs are the mouth, the upper respiratory tract, the gastrointestinal tract and the colon. Gram negative bacteria injected into the sterile peritoneal space will ultimately shed endotoxin and it can gain access to the circulation predominantly via lymphatics and the portal circulation. Munford [1] speculated that endotoxin in the venous circulation may be largely neutralized. It may exert its major effects in tissues and in the intracellular compartment. Due to its amphipathic character and ability to transport through the lymphatic system, it is likely that the extracellular compartment can act as a major reservoir of endotoxin. Hemoperfusion devices access only the endotoxin concentration in the vascular space, so that endotoxin removal

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from the interstitial compartment occurs only via thermodynamic and mechanical forces that tend to equalize the concentration between these 2 spaces. Since the extravascular interstitial compartment is larger than the vascular compartment, significant sequestration of endotoxin can occur in this compartment particularly in situations when bacterial source control is not achieved.

There have been several therapeutic strategies employed against endotoxin. These include mono and polyclonal antibodies, competitive inhibitors and drugs aimed at neutralizing endotoxin. All have failed to show clinical benefit [2]. Extracorporeal removal of endotoxin remains a viable alternative. This paper focuses on determining the removal capabilities of a specific PMX-DHP device, PMX-20R, which is widely used in Japan and parts of Europe.

PMX-20R was developed in Japan to leverage the specific binding ability of polymyxin B to endotoxin without its neurotoxic and nephrotoxic side effects that normally occur when this drug is administered systemically [3]. This device contains polymyxin B covalently bound to woven fibers of polypropylene/polystyrene packed into a cartridge. The manufacturer (Toray Industries, Tokyo, Japan) tests all production lots of PMX-20R prior to release in terms of their ability to adsorb endotoxin in vitro. To be released, each column must remove at least 60% of the initial 10 ng/mL endotoxin load perfused through the column within 90 min. To date, the number of in vivo clinical studies on the removal capability of PMX-20R is limited. In one Japanese trial, where 42 patients received 2 PMX-20R hemoperfusion episodes in a 24-h period, a mean reduction from 0.65 to 0.45 in endotoxin levels was demonstrated as measured by the endotoxin activity assay (EAA, Spectral Medical, Toronto, Canada) [4]. Novelli et al. [5], using EAA to follow endotoxin removal by PMX-DHP in post-abdominal surgery patients, have demonstrated that repeated hemoperfusion with PMX-DHP can lower endotoxin levels to normal (<0.4 EAA).

The experiments presented here analyze the capacity of the PMX-20R device in vitro for the removal of endotoxin in biologically relevant fluids.

Materials and Methods

Chemicals and Reagents

LPS (*Escherichia coli* O111:B4), FITC-labelled LPS (FITC-LPS; *E. coli* O111:B4), Proteinase K, Tris buffer, $CaCl_2$, SDS, guanidine HCl, and bovine serum and/or plasma were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sterile saline and sterile water were from Baxter Corporation (Mississauga, Canada). Sterile water for injection and physiological saline were from Otsuka Pharmaceutical Co. Ltd (Tokyo, Japan). Limulus Amoebocyte Lysate (LAL) reagents for the turbidimetric time assay were obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Equipment

PMX-20R (Toraymyxin® 20R) cartridges are commercially available from Toray Industries (Tokyo, Japan). Fifty-two production lots of PMX-20R, manufactured between November 2012 and November 2013, were tested in this study for adsorption capacity as per the procedure described below.

Toxinometer ET-6000 from Wako Pure Chemical Industries, Ltd (Osaka, Japan) was used as the instrumentation system for LAL-based turbidimetric time assay for endotoxin measurements in bovine serum/plasma preparations.

Fluoroskan Ascent Microplate fluorometer for assessing FTC-labelled LPS was from Thermo Fisher Scientific (Waltham MA, USA).

Preparation of Samples

Bovine serum/plasma: bovine serum was heat-inactivated by incubating at 54–56°C for 60 min. *E. coli* O111:B4 LPS stock was prepared at a 10,000 ng/mL concentration using sterile water and subsequently added to 1.5 L of serum at a final concentration of 10 ng/mL. The LPS-spiked blood was then incubated by stirring in a 37°C water bath for 60–120 min in order to prepare endotoxin-activated serum. Alternatively, bovine plasma was used; this was prepared by the centrifugation of bovine EDTA whole blood at 1,900 *g* for 60 min. The ensuing supernatant was collected and centrifuged again at 1,900 *g* for 15 min before recovering plasma for LPS spiking as mentioned above.

Human whole blood: 0.5 L of pooled whole blood was spiked with 100 μg of FITC-labeled *E. coli* O111:B4 LPS. Blood used in these runs was obtained from a discarded EDTA anti-coagulated blood pool where samples used were no more than 2 days old with storage at 4–8°C (St. Michael's Hospital REB approval 15-004).

Experimental Set-Up

Bovine Serum/Plasma experiments: a closed-loop system was set up that included a liquid water bath on a stir plate, a roller pump to drive the circulation at 100 mL/min and the PMX-20R device as per Figure 1. Prior to perfusion with LPS-spiked serum (or plasma), the PMX-20R cartridge was rinsed with 4 L of physiological saline. Thereafter, a vessel of 1.5 L LPS-spiked serum (or plasma) was placed in the 37°C water bath. A 0.3 L volume of the prepared sample was voided when changing over from the saline solution, leaving a total of 1.2 L of LPS-spiked serum (or plasma) to perfuse for 4 h at 100 mL/min. During this time, 1 mL of the circulating serum sample was collected at the beginning of the perfusion, at 30, 60 min, 2, 3, and 4 h into the perfusion.

Whole blood hemoperfusion experiments: 2 PMX-20R cartridges were tested from 2 different production lots (Lot 140202 and 140201). The columns were initially rinsed prior to whole blood exposure as mentioned above. To test LPS binding, each column was perfused with 0.5 L of LPS-spiked, EDTA anti-coagulated blood. Each column was perfused for 2 h at a flow rate of 100 mL/min. Each column was then perfused with 5 L of sterile depyrogenated saline and then 5 L of sterile depyrogenated water to remove blood. To elute bound LPS, each column was then incubated with 140 mL of 6M guanidine HCl for 1 h with agitation.

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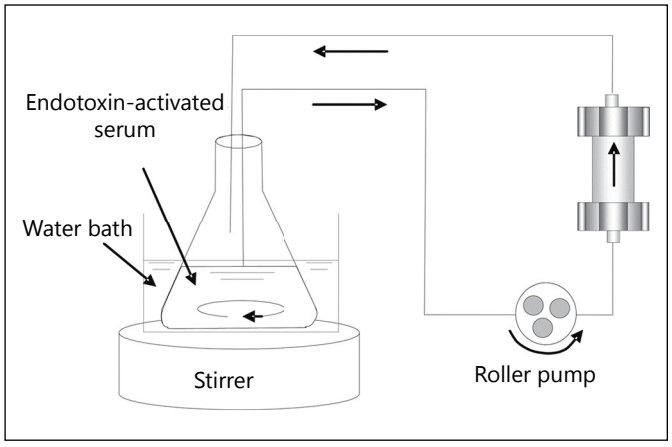

Fig. 1. Schematic drawing for the set-up of the hemoperfusion experiments. Endotoxin-activated serum or plasma was stirred at 37°C in a water bath and was recirculated through PMX-20R using a roller-pump at 100 mL/min for 4 h. The same set-up was used for the FITC-LPS-spiked whole blood experiments, except that the duration of perfusion was 2 h.

Analysis

Bovine Serum/Plasma Samples

The 1 mL serum samples, collected during the 4-h perfusion, were diluted 10-fold with sterile water for injection and then heated at 70°C for 10 min before LAL testing. Endotoxin standards for the calibration curve were prepared by diluting the endotoxin stock solution to a final concentration of 0.5–10 ng/mL. Following this, the LAL lysate reagent was prepared in sterile water as per manufacturer's instructions, added to the samples and calibrators, and assessed for endotoxin levels using a Toxinometer. Once results were tabulated, the endotoxin elimination rate of the PMX-20R column at the various time points was calculated using the formula given below:

Endotoxin elimination rate (%) = {(baseline LPS concentration – sample LPS concentration)/baseline LPS concentration} × 100.

Human Whole Blood Samples

The FITC-LPS bound was determined by measuring the fluorescence of the eluent at 525 nm with excitation at 490 nm. For calibration of the LPS fluorescence, a standard curve was constructed using FITC-LPS concentrations in the same 6 M guanidine HCl matrix. Background fluorescence in the absence of FITC-LPS was determined in a separate series of perfusion experiments and subtracted from the results obtained.

Results

Bovine Serum/Plasma Experiments

In these in vitro column performance runs, in 1.5 L of endotoxin-activated serum (or plasma), the starting 10 ng/mL endotoxin concentration was routinely decreased from 10 to 2–3 ng/mL of endotoxin after column perfusion. Since the 10 ng/mL LPS in 1.5 L of serum (or plasma) equals 15 μg of total endotoxin, the decrease to 2–3 ng/mL LPS concentration during perfusion means a drop to 3–4.5 μg of total endotoxin. Therefore, the total amount of endotoxin removal was about 12 μg (range 10.4–12.7 μg). For the 52 lots of PMX-20R cartridges tested in this study, the mean endotoxin elimination rate according to the formula given above was 88% (range 76–93%).

Human Whole Blood Experiments

Based on the fluorescence measurements obtained from the eluted FITC-LPS, the 2 PMX-20R cartridges tested here bound a mean of 20 μg LPS.

Discussion

The adsorption capacity of PMX-20R cartridges was tested in these studies using matrices and conditions relevant to those used in patients for therapeutic purposes. LPS-spiked bovine plasma is used as a quality control measure to test and validate PMX-20R production lots prior to release. The minimum release specification is 60% removal of a total load of 15 μg of LPS based on the LAL assay. The mean endotoxin removal for the 52 column lots tested in this study was 88% or an average of about 12 μg of *E. coli* O111:B4 LPS. This is in line with information on the 17 column lots released for the EUPHRATES trial [6], where the average elimination rate was 89% (12.1 μg).

Conversion of the endotoxin mass obtained in this study to the nanogram/millilitre (ng/mL) mass concentration found in patients is important in order to relate the adsorption capacity of the column to the levels found in patients with septic shock. To this end, a supplementary experiment is also presented here in Figure 2 and Table 1. In septic patients with high measurable endotoxin levels in the range of 0.6–0.9 EA units (Fig. 2), a maximal load of endotoxin confined only to the vascular space would translate to approximately 20 μg of endotoxin at a concentration of 4 ng/mL in blood. A single PMX-DHP cartridge with an efficiency of 85% could remove a load of 17 μg of endotoxin, reducing the blood concentration from 4 to 0.65 ng/mL.

In an independent series of experiments, we evaluated the binding capacity of PMX-20R cartridges using pooled anticoagulated (EDTA) whole blood with a higher load of fluorescent labeled LPS (100 μg FITC LPS) as another method of determining the adsorption capacity in vitro.

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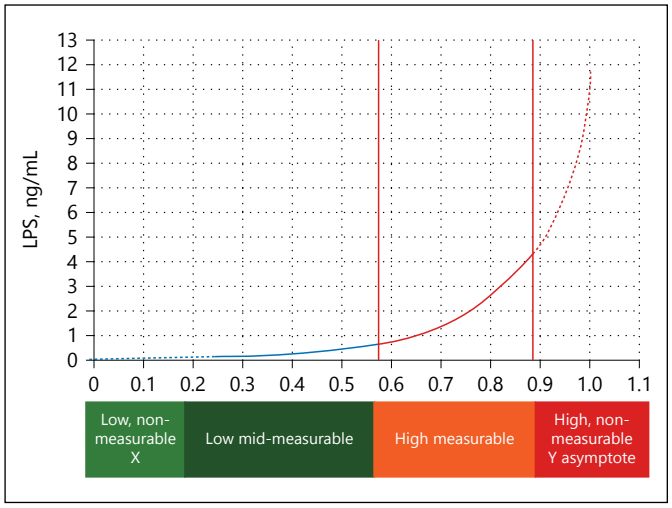

Fig. 2. Relationship of endotoxin burden (LPS in ng/mL, Y axis) and endotoxin activity (EA) values (X axis, no units) as measured by endotoxin activity assay (EAA). The curve is divided up into 4 segments: a low, non-measurable, a low mid-measurable segment from 0.2 to 0.59 EA, which corresponds approximately to 0.65 ng/mL or 3 μg of endotoxin; a high measurable region with EA values 0.6–0.9 corresponding to 1–4 ng/mL or 5–20 μg of endotoxin, and a high, non-measurable section above EA values of 0.9.

Table 1. Samples of EDTA-coagulated whole blood samples were obtained from 20 healthy donors. For each donor, the sample was divided in 4 aliquots of 2 mL spiked with different known concentration of *E. coli* O55:B5 LPS, randomly chosen as per the following diagram

LPS ng/mL	Donors				
	1	2	3	…	*n*
11.500	×		×		×
4.600	×	×		×	
3.060		×	×		
2.300		×		×	×
1.150	×		×	×	×
Baseline 0.100	×	×	×	×	×

The spiked samples were analyzed using standard protocols of the EAA. Values were then plotted as LPS concentration versus EA values. For this curve, a mean square best fitting trend line was found, by evaluating linear, polynomial and exponential best-fit lines. Results are summarized in dose response curve of Figure 2, where it was shown that above an EA value of 0.9 (corresponding to >4 ng/mL of LPS) the curve exhibits an asymptotic behavior and thus cannot be used to quantitate LPS levels in this range.

In these experiments, the amount of LPS bound to the PMX-20R matrix was about 20 μg. These results suggest that the maximal column removal capacity is in the range of 0.3–3 nmoles of LPS assuming mean molecular weights between 100 and 10 kilodaltons for LPS molecules. Based on past clinical information, high concentrations of endotoxin in septic human blood could range from approximately 1 to 10 ng/mL, translating to a total plasma load of 3–30 μg.

There is no definitive information concerning the quantity of endotoxin that needs to be removed from sepsis patients per treatment in order to realize a clinical benefit. However, when an extracorporeal device is considered for endotoxin removal in the clinical setting, the calculated column capacity from this study indicate that the complete removal of endotoxin in the blood stream using a single column is unlikely, as the burden may exceed the adsorption capacity. Since the extravascular interstitial compartment is larger than the vascular compartment, significant sequestration of endotoxin can occur outside of the circulation, particularly in situations when bacterial source control is not achieved. A simplified schematic of the complex kinetics of endotoxin is depicted in Figure 3. Thus, blood levels of endotoxin post hemoperfusion may remain elevated due to the equilibration of endotoxin between these compartments. In certain patients, this discrepancy between the endotoxin burden and the adsorption capacity may be large. Figure 2 demonstrates that when EAA levels are greater than 0.9, endotoxin mass concentrations can be much greater than 4 ng/mL; this means that in a total whole blood volume of 5 L with endotoxin equally distributed between cells and plasma, a total blood load greater than 20 μg could be achieved. If endotoxin is distributed additionally into the extracellular interstitial space (10 L), then the total extracellular endotoxin load could be significantly higher than the adsorption capacity of a single PMX-20R cartridge.

Conclusions

The in vitro studies evaluating the adsorptive capacity of PMX-20R have suggested that the total binding capacity of the columns is in the range of 10–20 μg of endotoxin. If one assumes an average molecular weight of 10–100 kilodaltons for LPS molecules, then this translates to a maximal binding capacity of approximately 0.3–3.0 nmoles of LPS under physiological conditions. This capacity should be taken into account when determining the treatment of patients with this hemoperfusion device.

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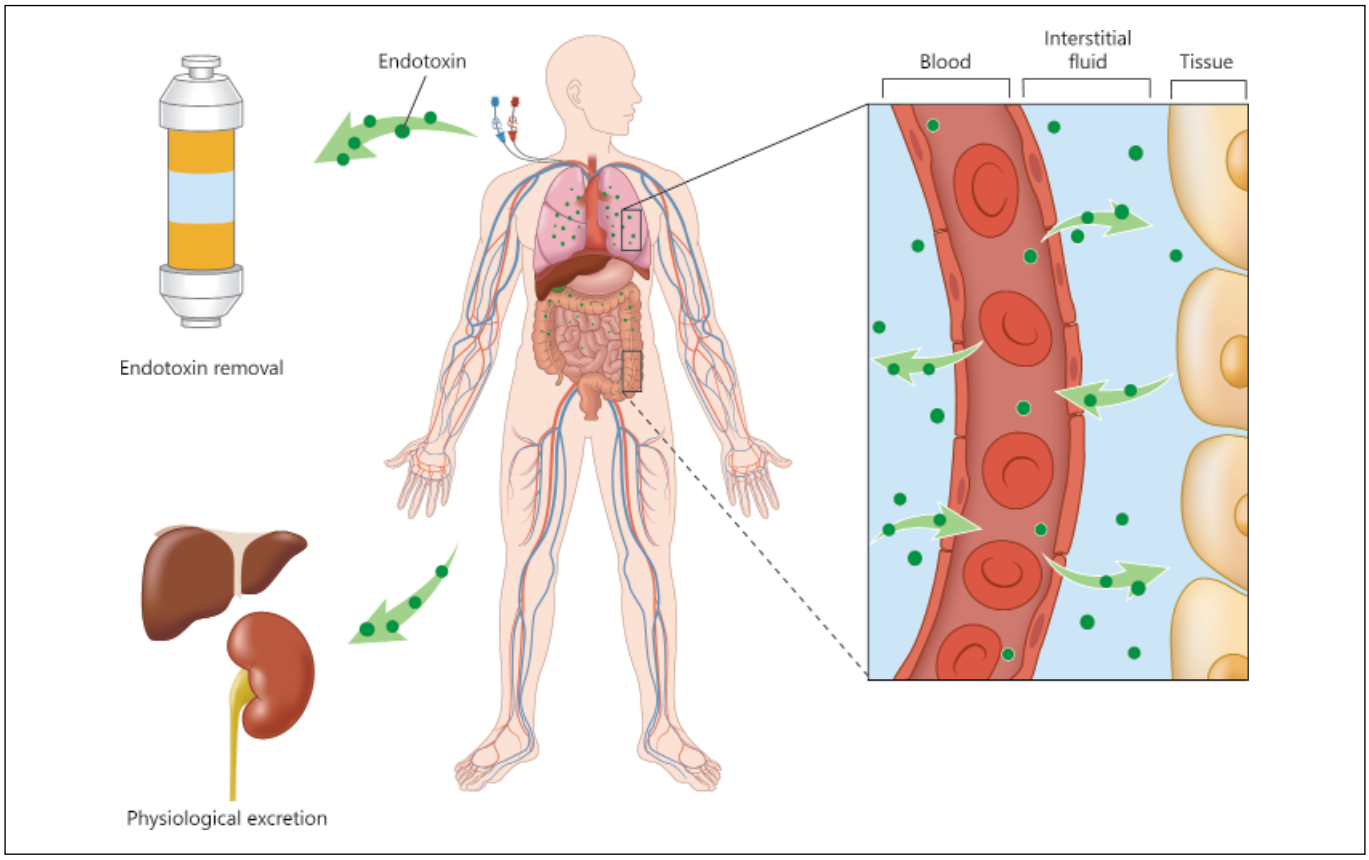

Fig. 3. Diagram of endotoxin kinetics. Endotoxin (depicted as green spheres) can reside in the circulation where it is excreted via the kidney and the liver. At the same time, it traffics in and out of the circulation (enlarged inset) to distribute to the interstitial fluid. PMX-20R can thus remove endotoxin indirectly from interstitial fluid when connected to the patient's circulation.

The results presented in this study suggest that the adsorption capacity of PMX-20R is sufficient to remove a clinically significant amount of endotoxin in a majority of endotoxemic septic shock patients; however this may not be the case in patients with a high EAA burden >0.9.

Disclosure Statement

A.D.R. and D.J.K. are consultants for Spectral Medical Inc. C.V.O.-F. is an employee of Spectral Medical Inc. H.S. is an employee of Toray Medical Co.

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