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Research Article

Endotoxin and cytokine removal with a new (CA) sorbent cartridge

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Short Title: Endotoxin & cytokine removal with CA cartridge

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Abstract
Introduction: Endotoxin, also referred to as lipopolysaccharide (LPS), is a major stimulus of the inflammatory response capable of leading to sepsis and septic shock. Extracorporeal blood purification therapies are increasingly employed in the treatment of sepsis. Timely and thorough removal of endotoxin and cytokines can help to mitigate the inflammatory cascade during septic shock. Hemoadsorption (HA) is a promising approach to achieve this goal. In this study, we analyzed different prototypes of a new CA cartridge: we performed *in vitro* circulations to define endotoxin and cytokine removal capacity.

Methods: *In vitro* HA was performed using GALILEO testing platform, customized circuits with mini-modules of CA cartridge were prepared. A batch of 500 mL of blood pre-conditioned with LPS was utilized for each circulation. We collected samples at different time points: endotoxin activity and removal ratio (RR) of LPS, Interleukin 6 (IL-6) and Interleukin-1 beta (IL-1β) were assessed.

Results: The prototypes showed different performances, highlighting promising adsorption affinity towards the target molecules.
A potentially clinically meaningful reduction of endotoxin activity by CA mini-module was observed. Concentration of the LPS, cytokine IL-6 and IL-1β showed a significant reduction over time, as confirmed by the RRs.

Conclusion: Our experiments allowed to characterize the prototypes in terms of adsorption capacity for both endotoxin and cytokines. After this preliminary approach, the final CA mini-module was tested under the same experimental conditions and displayed excellent adsorption capacity, indicating its potential application for endotoxin removal.

Introduction

Recent interest has grown concerning the use of extracorporeal blood purification (EBP) therapies in sepsis and other critical illness characterized by presence of PAMPS (Pathogen Associated Molecular Pattern) and DAMPS (Damage Associated Molecular Pattern) in blood [1]. Special interest has been placed in the application of new EBP based on adsorption [2–4]. The limited capacity of removal of membrane-based separation processes (hemodialysis/hemofiltration) has led to development of newer devices containing sorbent materials with various properties and porosity [5–9]. The pathophysiological complexity of sepsis and other cytokine release syndromes (CRS) lays in the cascade of events that initiates with bacterial or viral invasion of the host, followed by recognition of pathogen molecular patterns such as lipopolysaccharide (LPS) by the CD-14 receptors of the macrophage/monocyte system and subsequent activation of molecular effectors representing the chemical spectrum of the inflammation response [10,11]. These mechanisms have suggested a time-sensitive approach to blood purification strategy based on sequential application of specific and non-specific sorbent devices [12–15]. Specific adsorption has been advocated for removal of endotoxin [16] while non-specific adsorption has been proposed for removal of both pro and anti-inflammatory mediators according to the "peak concentration hypothesis" and the immunomodulatory effect of EBP [17].

While this sequential approach requires two different circuits and separate devices, we are proposing to merge specific and non-specific adsorption of endotoxin and mediators in one single unit containing a newly conceived sorbent designed to remove such target molecules. In order to achieve the above mentioned task, we studied different types of sorbents produced in the R&D department of one company and conducted experimental studies on different prototype cartridges. Once identified the most effective sorbent formulation, we used a miniaturized module *in vitro* to define effective endotoxin and cytokine removal capacity of the new sorbent.

Methods

In vitro hemoadsorption (HA) was performed using the GALILEO platform, a laboratory equipment developed specifically for *in vitro* experimental circulation, by the International Renal Research Institute of Vicenza (IRRIV, Vicenza, Italy). Five mini-module prototypes (XCA 1-2-3-4-5, shown in Fig. 1) containing the new CA sorbent material with slightly different chemical composition (Jafron Biomedical, Zhuhai, People's Republic of China) were tested *in vitro* to assess the best capacity of removal of endotoxin and cytokines from a batch of pre-conditioned blood. Once the most effective composition was identified, we assessed the final CA cartridge (Fig. 1). All the studied cartridges (XCA), including the final one (CA), were a downscaled version (25%) of the regular size available CA cartridge.

The sorbent material comes in beads made of styrene-divinylbenzene copolymer. The resin is one-step cross-linked. The beads size ranges between 355 µm and 600 µm, with an average pore size between 10-12 nm and the surface area (the sorbent surface in contact with the solution) is around 600 m^2/g. All the cartridges are filled with a total amount of 60 mL of beads. pH analysis highlighted differences in the internal storage liquids. The beads of the 5 prototypes have in common the carrier structure but they differ in the ligand, the substance immobilized onto the sorbent material. The exact chemical composition of the prototype sorbents (XCA 1–5) was not disclosed to the investigators at the time of testing. This blinded design was adopted intentionally to ensure unbiased evaluation of adsorption performance and to respect industrial confidentiality. Therefore, the interpretations are based solely on functional results rather than structural characteristics. No ligand is present onto the CA beads; the final CA cartridge (CA330) performs endotoxin removal via non-specific adsorption mechanisms based on the polymer matrix properties.

The customized circuits were assembled, connected to the cartridges after discharged the storage liquid and applied to the GALILEO testing platform. The circuits were primed according to manufacturer instructions for use of the cartridges: 3,000 mL of heparinized saline at a flow rate of 100 mL/min.

The blood used in all experiments was obtained from the local blood center and derived from healthy volunteer donors. Specifically, we used whole blood units that were discarded due to insufficient volume for clinical use. All units used had the same blood group to minimize immunological variability.
Blood was stimulated by LPS (Escherichia coli-derived endotoxin, Sigma-Aldrich Co., St louis, USA). Adsorption capacity towards endotoxin and inflammation factors was assessed by Endotoxin Activity Assay (EAA) and the measured concentration of LPS, Interleukin 6 (IL-6) and Interleukin-1 beta (IL-1β).

Experiments with five CA prototypes
2,500 mL of blood (Hct 30%) were enriched with 50 mg of LPS and incubated at 37°C. After 24 h, blood was partitioned into five reservoirs: a batch of 500 mL for each circulation was used. Blood solution was heated to 37°C and continuously stirred during the circulation.
Circulation was maintained for 2 hours at a blood flow rate (Q_B) of 100 mL/min in a closed-loop configuration (Fig. 2).
Samples (3 mL each) were taken from:

- the experimental batch at 0, 10, 30, 60 and 120 minutes
- before and after the cartridge at 10 minutes (as described in Fig. 2).

The five experiments were performed in sequence and this explains different starting concentrations of the target molecules in the five batches.
We assessed the EAA for samples taken from the batch before and at the end of the experiments. The concentrations of LPS, IL-6 and IL-1 were assessed for each sample taken from the batch. LPS concentration likewise was measured in the samples taken before and after the cartridge.
The five prototypes (XCA 1–5) were tested in parallel in a blinded fashion, with no access to their specific formulations or to the identity of the one selected for further development. This academic evaluation represents a focused, comparative screening and was not designed to document the full industrial development process, which was independently carried out by the manufacturer.

Experiment with CA cartridge
800 mL of blood (Hct 30%) were used to test the final CA cartridge. 150 mL of them were considered as a negative control (CTR-), the remaining 650 mL were enriched with 15 mg of LPS. This solution was split in two reservoirs: a batch of 500 mL for the circulation, a batch of 150 mL maintained as positive control (CTR+). Blood solution was heated to 37°C and continuously stirred during the circulation.
Hemoadsorption simulation was performed with Q_B=100 mL/min and lasted two hours, similar to the previous modality.
Samples (3 mL each) were taken from:

- the experimental batch at 0, 10, 30, 60 and 120 minutes
- before and after the cartridge at 10 minutes and 120 minutes (as described in Fig. 2)
- control batches (CTR+ and CTR-) at 0, 10, 30, 60 and 120 minutes.

We evaluated EAA and concentration of LPS, IL-6 and IL-1β for each sample.

Calculation
We evaluated the EAA (Estor Spa, Milan, Italy): it is a quick and easy diagnostic test based on a monoclonal antibody that identifies endotoxin. With this method, LPS activity is measured based on the corresponding oxidative burst of primed neutrophils and is detected via the chemiluminescence method. With this approach, reliable quantification of the amount of endotoxin in a patient's whole blood can be easily obtained [18].
Quantification of LPS concentration was performed by the Human LPS ELISA kit (ELK Biotechnology, Denver, USA), an immunoassay method. IL-6 and IL-1β concentrations were measured with Human IL-6 ELISA Kit-ab178013 and Human IL-1β ELISA kit-ab214025 (Discovery Drive, Cambridge Biomedical Campus, Cambridge, CB2 0AX, UK) according to manufacturer's instructions. LPS, IL-6 and IL-1β plates

were read using VICTORX4 Multilabel Plate Reader (PerkinElmer Life Sciences, Waltham, MA, USA) at 450 nm for both molecules. The concentration values for these molecules were calculated by the extrapolation with standard curves. All tests were carried out in duplicate.
Adsorption was assessed considering the LPS and cytokine Removal Ratio (RR) and its kinetics. RR is the percentage reduction of the solute concentration after a period of circulation *C(t)* compared to the concentration at the beginning of the experiment *C(0)* and was calculated according to the formula:

$$RR\ (t) = 100 \cdot \frac{C(0) - C(t)}{C(0)}$$

Results

The experiments described below represent an academic contribution to a broader industrial R&D process. While the prototype materials were tested under the same experimental conditions, the analysis was intentionally descriptive, and no statistical inference was made. The aim was not to define mechanistic differences, but to observe functional trends that could inform further product refinement. The main focus was to characterize the CA cartridge as a potential tool for early extracorporeal endotoxin and cytokine removal.

Five CA prototypes

The five prototypes were identified with a serial number from 1 to 5 and randomly tested.
After 24 hours of incubation, blood was triggered by the LPS: baseline EAA values confirmed blood activation. In Table 1 EAA values are reported for each experiment: increased baselines are due to the delay in the execution, the five circulations were performed sequentially.
EAA values showed a significant decrease (on average -75±7%) over the course of the five experiments (Fig. 3). XCA 2 and XCA 4 displayed the major reduction of endotoxin activity, 83.3% and 81.1%, respectively.
Conversely, LPS concentrations didn't decrease so strongly over the five circulations. The highest LPS reductions were observed with XCA 1 and XCA 5, at the end of the experiments we found a RR of 13.2% and 13.3%, respectively (Table 2).
Promising results of XCA 1 have been confirmed by the instantaneous efficiency evaluated considering inlet and outlet LPS concentrations (Fig. 4): the sorbent removed 13% of the LPS entering the cartridge in a single pass. XCA 4 showed equal instantaneous efficiency, notwithstanding the limited reduction over time. XCA 5, in contrast, didn't highlight instantaneous removal capacity towards LPS after 10 minutes of circulation (Fig. 4), despite its efficiency in the long run (Table 2). XCA 2 and XCA 3 didn't exhibit a consistent LPS reduction.
Cytokine concentration over time in the five experiments showed different trends. IL-6 concentration before the circulation with XCA 3 cartridge resulted in 7,840 pg/mL and after 120 minutes of circulation, decreased to 1,500 pg/mL. We observed a RR of 80.9% with this cartridge, XCA 5, whereas, removed 28.2% of IL-6. XCA 1, XCA 2 and XCA 4 didn't exhibit a substantial IL-6 reduction (Table 3).
XCA 2 displayed a potentially clinically relevant reduction of IL-1β concentration, we observed a RR of 61.2% after 120 minutes of circulation. XCA 1 and XCA 3 removed about 20% of IL-1β, XCA 4 and XCA 5 didn't exhibit a consistent IL-1β reduction (Table 4).

CA cartridge

A meaningful reduction of endotoxin activity by CA cartridge was observed: at the beginning of the experiment, the endotoxin activity was 2.1 and after circulation, it was reduced to 0.7 (reduction of 65.9%) (Fig. 5). Particularly, the major decay was noted within the first 10 minutes of circulation (Table 5).
The concentration of LPS during the circulation with the CA cartridge had a progressive and steady decrease (Table 6), as shown in Figure 6. a: the concentration in the reservoir before the experiment

was equal to 12,390 ng/mL, the cartridge removed 30.6% of LPS, reaching a concentration of 8,599 ng/mL at T120.
IL-6 concentration displayed an exponential decay over time: at T0 the concentration was 2,496 pg/mL and reached a final value of 403 pg/mL after 120 minutes (Fig. 6. b), with a corresponding RR of 83.9% (Table 6).
Concentration of the cytokine IL-1β showed a reduction (Fig. 6. c), particularly we observed the major reduction during the first 30 minutes of circulation (RR=81.8%). RR at the end of the experiment reached 92.6% (Table 6).
Samples taken before and after the cartridge allowed to estimate its instantaneous efficiency. Significant instantaneous LPS removal could be observed: the gap between inlet and outlet concentrations at 10 minutes and 120 minutes revealed a reduction of 10.9% and 11.3%, respectively (Fig. 7. a).
Regarding cytokines, significant results were obtained mainly at 10 minutes: IL-6 reduction was 25.7%, IL-1β decreased by 28.6% (Fig. 7. b, c).
Unchanged values over time of negative and positive controls concentrations confirmed the accuracy of the experiments and the consistency of our findings.

Discussion

Current sorbent devices utilized for sequential extracorporeal therapy in sepsis, are characterized by a specific function and are designed for a specific target molecule (endotoxin) or for a non-specific, broad spectrum of chemical mediators (cytokines). Polymer and surface modification of a new polystyrene-divinylbenzene based sorbent developed by Jafron company, is intended to create a new generation of sorbent cartridges indicated for combined removal of endotoxin and cytokines.
In our experiments, the company made available minimodules (XCA) with slightly different chemical composition of the beads, to identify the best sorbent candidate for the final CA cartridge. Our experiments allowed to characterize the five prototypes in terms of adsorption capacity for both endotoxin and cytokines and therefore to identify the best option for the final product.
After this preliminary approach, the final CA sorbent mini-module was tested under the same original experimental conditions and displayed excellent adsorption capacity for the target molecules. Of course, the efficiency over time demonstrated progressive reduction due to saturation of the sorbent, but this was clearly due to the fact that the mini-module only contains 25% of the commercially available cartridge (CA330).
It is important to note that EAA and LPS quantification by ELISA assess different aspects of endotoxemia. While LPS ELISA measures the total circulating endotoxin, EAA evaluates its biological activity and potential to stimulate neutrophils. Therefore, discrepancies between the two may arise, especially in the context of hemoadsorption, where the biological effect may be mitigated without complete removal of LPS. The stronger reduction in EAA compared to LPS concentration observed in our results suggests that the cartridge may effectively reduce the bioactive portion of endotoxin, thus attenuating its inflammatory potential, even if not all circulating LPS is physically removed.

Conclusion

These experiments suggest that the CA330 cartridge can be utilized in the early phases of the sepsis cascade with the possibility to target removal of endotoxin and the subsequent humoral effectors of the cascade. This enhances the possibility to perform the extracorporeal therapy at the right time obtaining both a pathophysiological effect on the primary cause of events, and an immunomodulatory effect due to reduction of peaks of mediators due to the activation of innate and adaptive immunity. Of course, this concept must be tested *in vivo* as suggested by the consensus group of ADQI [19,20], but the hypothesis follows the lines of the rationale based on the pathophysiology of the sepsis syndrome.
The collaboration between industry and the academic center resulted in identification of the best suitable sorbent material for the final product. This is a positive example of strict collaboration for the development of optimized, safe and effective medical devices.

Statements

Acknowledgement

The authors are grateful to the International Renal Research Institute of Vicenza and the R&D department of Jafron Medical Company.

Statement of Ethics

Blood from the Hospital blood bank was utilized for the experiments in agreement and compliance with the institutional review board recommendation for the good use of blood. Use of these samples for research purposes did not require ethical approval in accordance with local/ national guidelines. Informed consent is generally achieved for the donation in which it is stated that the hospital grants the good practice of utilization of the blood for any clinical and research purpose.

Conflict of Interest Statement

CR has been advisor or member of the speaker bureau or received honoraria for presentations in the last 3 years from the following companies: Asahi medical, Aferetica, Baxter, Biomerieux, B.Braun, Cytosorbents, Fresenius medical care, Medtronic, Jafron Medical, ESTOR, Nipro, Medica, GE. AL, MZ and CR were all a member of the journal's Editorial Board at the time of submission. All other authors have no conflicts of interest to declare.

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Author Contributions

CR, AL, NP, MdC, contributed in the study design and in the experimental settings. MdC contributed in the sample measurements. AL, NP, CR contributed in the data analysis. CR, AL, NP, MdC, MZ contributed in the interpretation and the writing process. All the authors revised critically the final manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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Figure Legends

Fig. 1. The five prototypes (on the left) and the mini-module of the CA cartridge (on the right) by Jafron Biomedical.

Fig. 2. Schematic representation of the *in vitro* circuit utilized for the experiments. Blood solution circulated in a closed-loop configuration through the tested cartridge. Samples were taken from the reservoir at different time point and from the inlet and outlet lines of the cartridge.

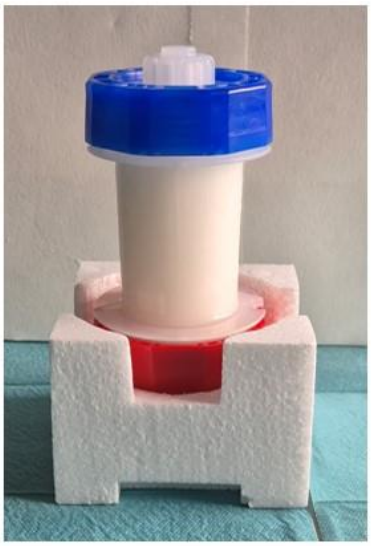
Fig. 3. Endotoxin Activity Assay (EAA) results obtained during the circulations with the five CA prototypes.

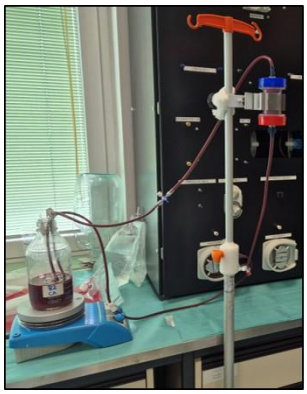
Fig. 4. LPS concentrations upstream and downstream of the five prototypes after 10 minutes of circulation.

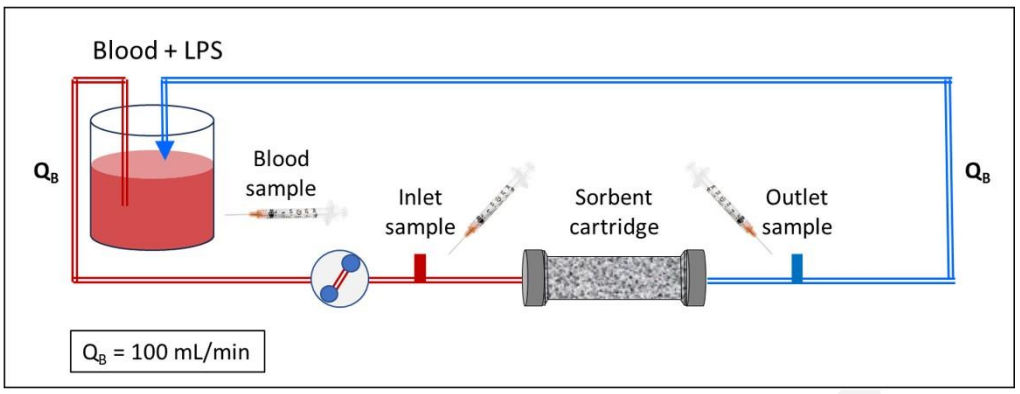
Fig. 5. Endotoxin Activity Assay (EAA) results obtained during the circulations with CA mini-module, results of negative (CTR-) and positive (CTR+) controls are also displayed.

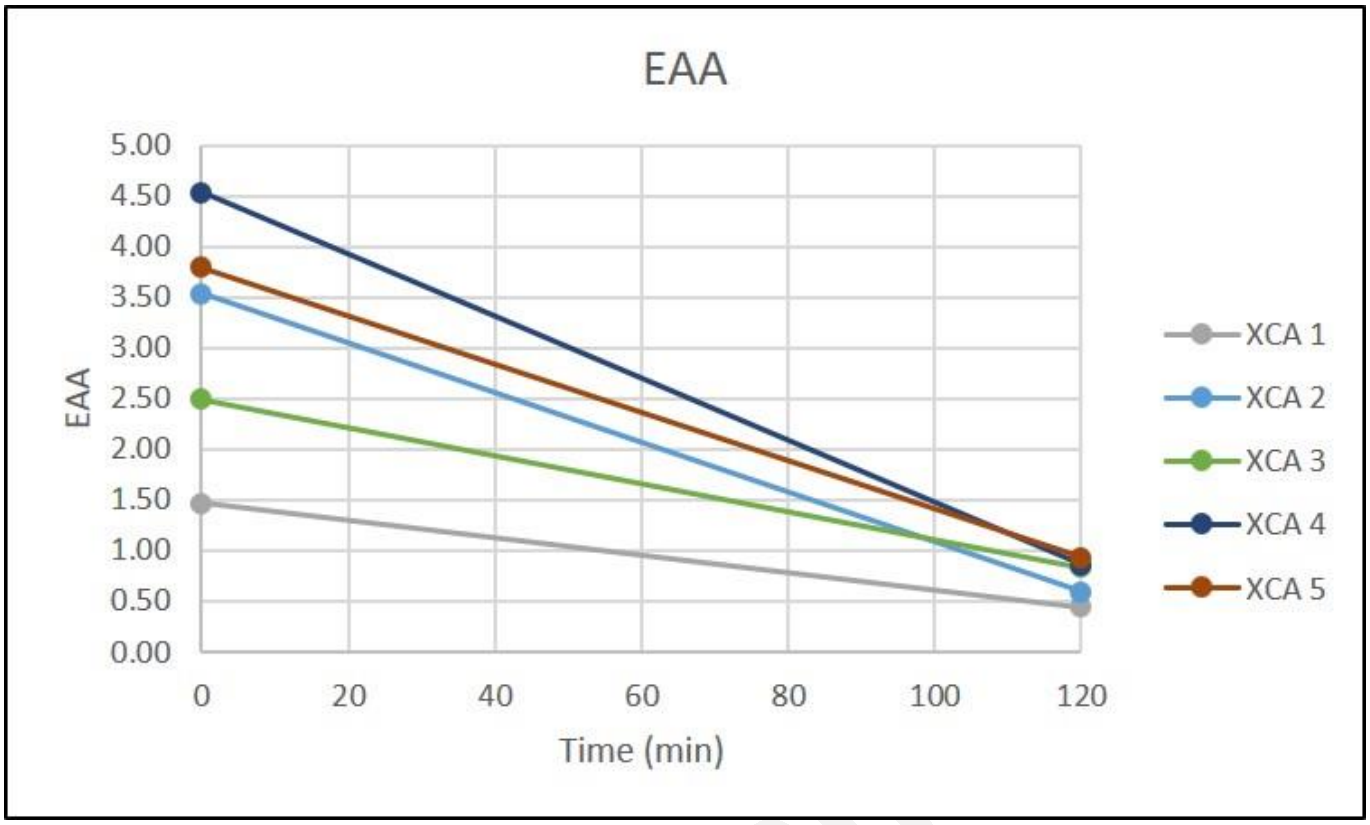
Fig. 6. Curves of concentration values for the different molecules analysed: a. LPS, b. IL-6, c. IL-1β. Concentration of negative (CTR-) and positive (CTR+) controls are also reported.

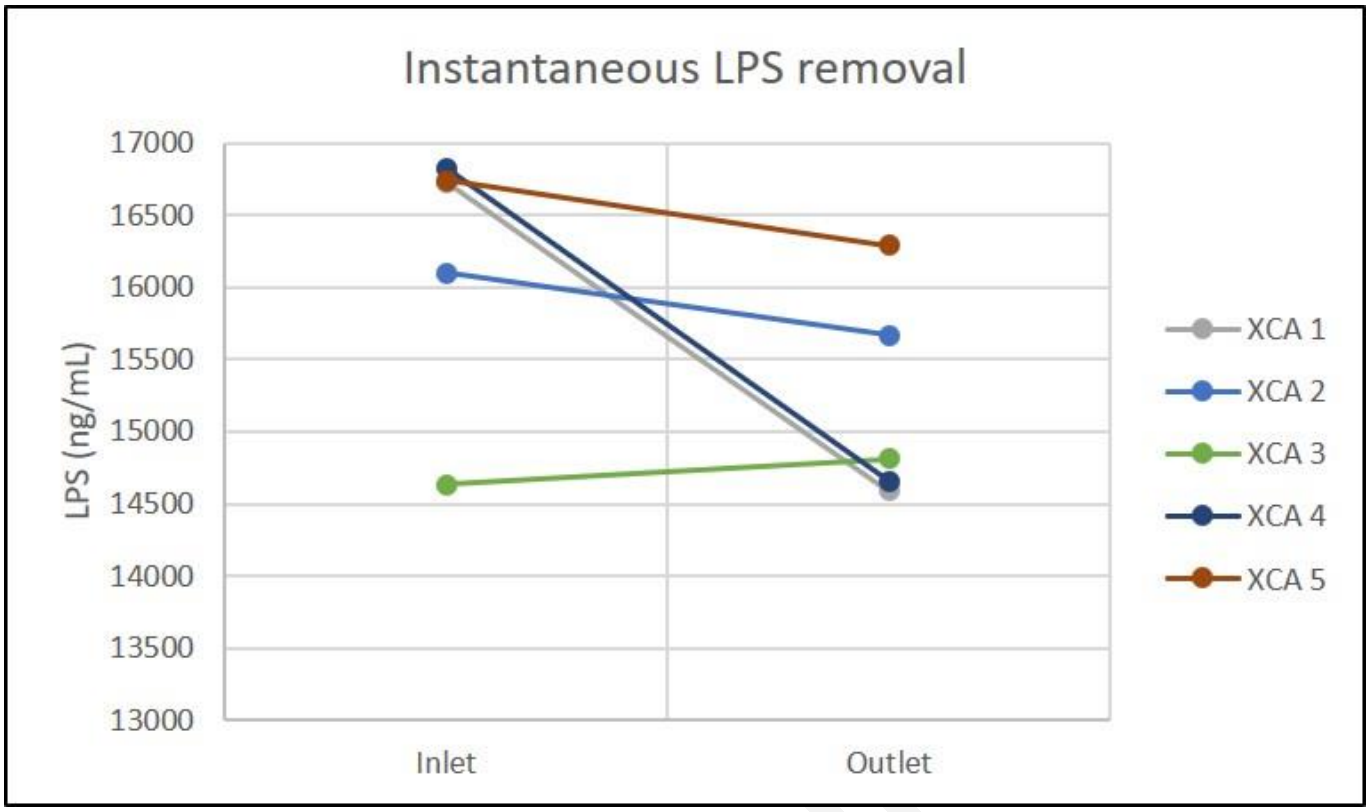
Fig. 7. Concentrations of different molecules upstream and downstream of the CA mini-module after 10 and 120 minutes of circulation: a. LPS, b. IL-6, c. IL-1β.

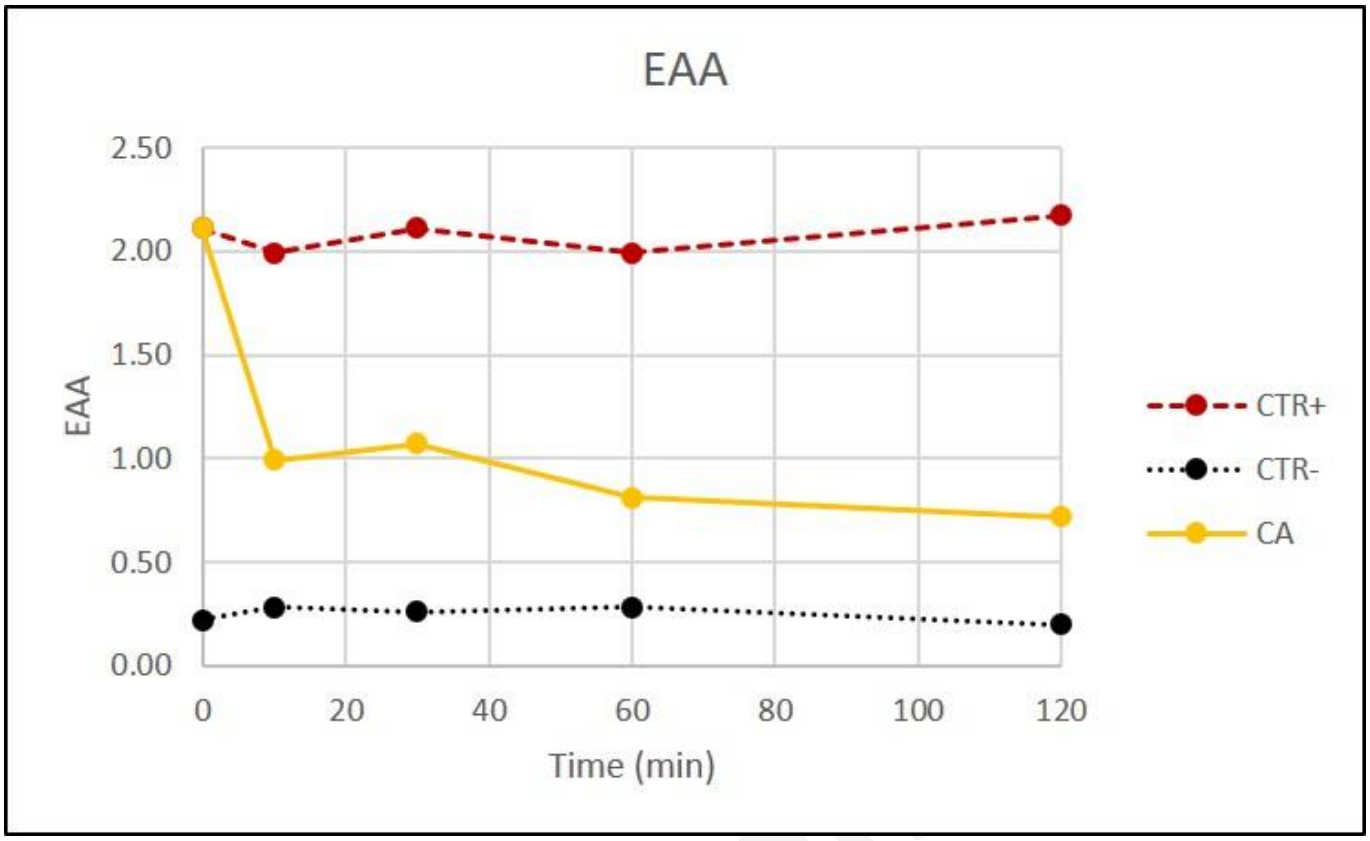


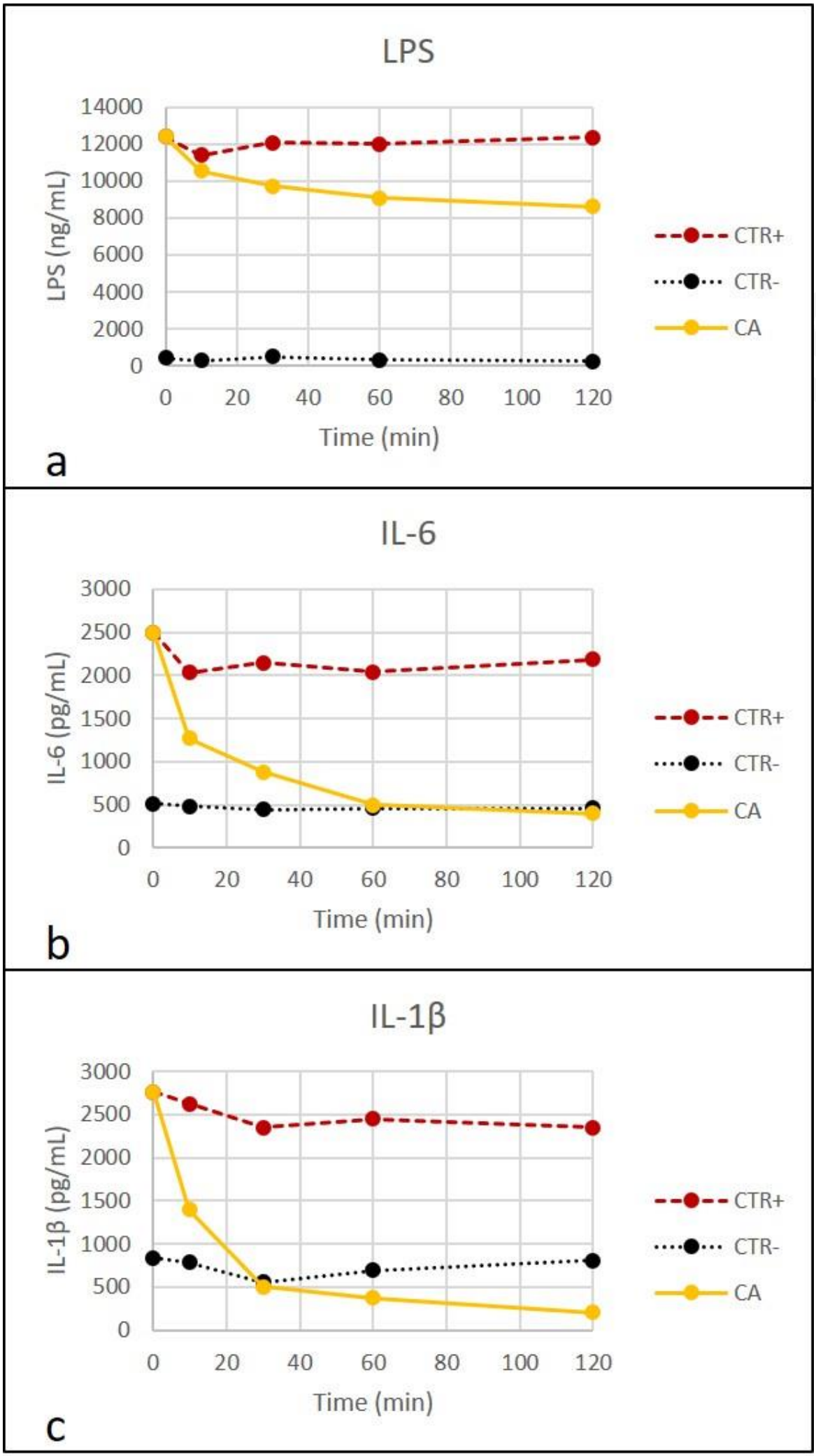












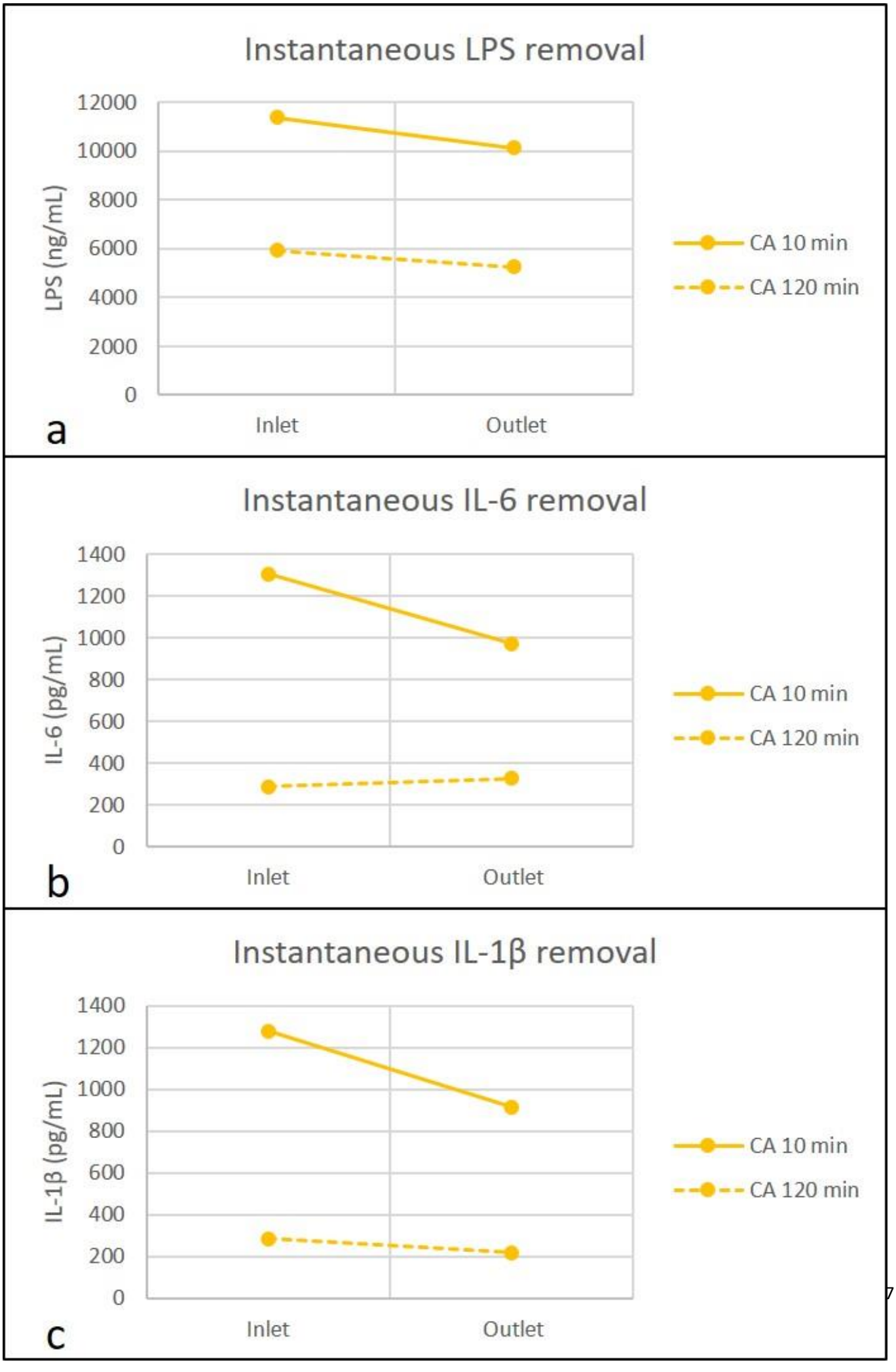


Table 1. Values of Endotoxin Activity Assay (EAA) obtained before and after the circulations with the five CA prototypes.

EAA	T0	T120
XCA 1	1.47	0.44
XCA 2	3.54	0.59
XCA 3	2.49	0.83
XCA 4	4.54	0.86
XCA 5	3.80	0.93

Table 2. LPS concentrations and Removal Ratios (RR) of the five prototypes at the different time points.

LPS	T0	T10		T30		T60		T120	
	C (ng/mL)	C (ng/mL)	RR (%)	C (ng/mL)	RR (%)	C (ng/mL)	RR (%)	C (ng/mL)	RR (%)
XCA 1	17,363	15,610	10.1	13,945	19.7	15,752	9.3	15,077	13.2
XCA 2	14,780	14,224	3.8	14,164	4.2	15,503	0.0	14,526	1.7
XCA 3	15,112	13,702	9.3	14,247	5.7	12,991	14.0	13,904	8.0
XCA 4	13,702	13,501	1.5	13,678	0.2	15,681	0.0	14,366	0.0
XCA 5	14,852	15,610	0.0	15,160	0.0	14,366	3.3	12,873	13.3

Table 3. Interleukin-6 (IL-6) concentrations and Removal Ratios (RR) of the five prototypes at the different time points.

IL-6	T0	T10		T30		T60		T120	
	C (pg/mL)	C (pg/mL)	RR (%)	C (pg/mL)	RR (%)	C (pg/mL)	RR (%)	C (pg/mL)	RR (%)
XCA 1	2,998	3,183	0.0	2,944	1.8	2,247	25	2,749	8.3
XCA 2	3,008	2,911	3.3	1,470	51.1	4,860	0.0	9,041	0.0
XCA 3	7,840	2,210	71.8	3,534	54.9	2,995	61.8	1,500	80.9
XCA 4	1,621	5,638	0.0	9,075	0.0	8,469	0.0	7,685	0.0
XCA 5	2,944	1,968	33.2	665	77.4	2,225	24.4	2,115	28.2

Table 4. Interleukin-1β (IL-1β) concentrations and Removal Ratios (RR) of the five prototypes at the different time points.

IL-1β	T0	T10		T30		T60		T120	
	C (pg/mL)	C (pg/mL)	RR (%)	C (pg/mL)	RR (%)	C (pg/mL)	RR (%)	C (pg/mL)	RR (%)
XCA 1	711	1,297	0.0	2,611	0.0	2,483	0.0	540	24.1
XCA 2	4,883	897	81.6	954	80.5	1,211	75.2	1,897	61.2
XCA 3	1,683	1,183	29.7	897	46.7	2,440	0.0	1,340	20.4
XCA 4	111	197	0.0	1,183	0.0	2,683	0.0	1,297	0.0
XCA 5	1,140	1,851	0.0	1,326	0.0	898	21.2	1,339	0.0

Table 5. Values of Endotoxin Activity Assay (EAA) obtained at different time points of the circulations with CA mini-module.

EAA	T0	T10	T30	T60	T120
CA	2.11	0.99	1.07	0.81	0.72
CTR+	2.11	1.99	2.11	1.99	2.17
CRT-	0.22	0.28	0.26	0.28	0.20

Table 6. Concentrations and Removal Ratios (RR) obtained at different time points of the circulations with CA mini-module: Lipopolysaccharides (LPS), Interleukin-6 (IL-6), Interleukin-1β (IL-1β).

		T0	T10		T30		T60		T120	
		C	C	RR (%)	C	RR (%)	C	RR (%)	C	RR (%)
LPS (ng/mL)	**CA**	12,390	10,532	15.0	9,726	21.5	9,061	26.9	8,599	30.6
	CTR+	12,390	11,405	-	12,086	-	11,990	-	12,357	-
	CRT-	428	278	-	474	-	301	-	240	-
IL-6 (pg/mL)	**CA**	2,497	1,269	49.2	881	64.7	501	79.9	403	83.9
	CTR+	2,497	2,034	-	2,147	-	2,038	-	2,184	-
	CRT-	517	487	-	445	-	462	-	459	-
IL-1β (pg/mL)	**CA**	2,759	1,396	49.4	503	81.8	374	86.5	204	92.6
	CTR+	2,759	2,621	-	2,349	-	2,446	-	2,346	-
	CRT-	837	786	-	556	-	697	-	810	-