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Inhaltsverzeichnis

Zusammenfassung

Extrakorporale Blutreinigungssysteme werden verwendet, um Substanzen aus dem Blut von Patienten durch Filtrations- oder Adsorptionseinheiten zu entfernen. Bekanntestes Beispiel ist die chronische Dialyse bei chronischer Niereninsuffizienz. Diese langfristige bis lebenslange Behandlung ermöglicht Patienten mit schwacher oder keiner Nierenleistung ein Überleben bzw. eine beschwerdearme Wartezeit, bis ein geeignetes Transplantat zu Verfügung steht. In den letzten Jahrzehnten wurden Anstrengungen unternommen, auch bei Leber- und Sepsiserkrankungen extrakorporale Blutreinigungsverfahren als unterstützende Therapie zu verwenden. Zusätzlich zur Dialyse kommen Adsorber zum Einsatz, welche die Entfernung von Protein-gebundenen und hochmolekularen Substanzen aus dem Blut ermöglichen. Zielsubstanzen bei der extrakorporalen Leber- und Sepsistherapie sind unter anderem Bilirubin, Gallensäuren, Endotoxine, Zytokine und andere Entzündungsmediatoren.

Ziel dieser Arbeit war es, bestehende und neue, als Medizinprodukt noch nicht zugelassene Adsorber, hinsichtlich ihrer Effektivität und Blutverträglichkeit mit Hilfe von *in vitro*-Experimenten zu testen. Des Weiteren wurden Kombinationen von bestehenden Blutreinigungsverfahren für eine effizientere Behandlungsmöglichkeit bei Leber- und Sepsis-Erkrankungen untersucht.

Adsorptionsstudien mit kommerziell erhältlichen Endotoxinadsorbern wurden in Serum, Plasma und Blut mit Hilfe von Batchtests durchgeführt. Zusätzlich wurde untersucht, ob die Endotoxin-Inaktivierung durch die Verwendung von Polymyxin B (PMB), einem Antibiotikum mit hoher Affinität zu Endotoxinen, eine geeignete Alternative zu den Endotoxinadsorbern darstellt.

Polystyrol-Divinylbenzol basierte Adsorber mit unterschiedlichen Porengrößen wurden hinsichtlich Effizienz und Biokompatibilität mit kommerziell erhältlichen Zytokinadsorbern verglichen. Neben der Plasma- und Blutverträglichkeit wurde mit Hilfe von Batchtests in Plasma die adsorptive Entfernung ausgewählter Antibiotika untersucht. Zusätzlich wurde getestet, ob durch die Anwendung der regionalen Zitratantikoagulation anstatt von Heparin die Blutverträglichkeit in extrakorporalen Blutreinigungsverfahren gesteigert werden kann.

Die Poren von Adsorbern wirken als Molekularsieb und verhindern das Eindiffundieren von Molekülen, die größer als der Porendurchmesser sind. Die Ergebnisse dieser Arbeit zeigen, dass bei Polystyrol-Divinylbenzol-Copolymer basierenden Adsorbern 15 nm Poren für die Zytokin-Entfernung am geeignetsten sind. Hingegen sind für die Entfernung von Albumin-gebundenen Toxinen aus dem Blut Poren zwischen 30 und 40 nm am effektivsten. Poren mit noch größerem Durchmesser verringern die selektive Wirkung von Adsorbern und führen zu einer unkontrollierten Adsorption von allen Plasmaproteinen. Dadurch sind letztere nicht plasmakompatibel und erfüllen somit nicht die Anforderungen für die Verwendung in der extrakorporalen Blutreinigung. Die Blutverträglichkeit, Selektivität und Effizienz von Adsorbern, welche in der Blutreinigung eingesetzt werden, können somit durch die richtige Wahl der Porenverteilung verbessert werden. Bei der adsorptiven Entfernung von Zytokinen aus humanem Plasma zeigte der Adsorber CG161 die größte Effizienz. Zusätzlich konnte dieser Adsorber im Gegensatz zu den in der Klinik eingesetzten Adsorbern auch TNF-α großteils entfernen. In separaten *in vitro*-Experimenten konnte gezeigt werden, dass Adsorber, die in der Blutreinigung zu Anwendung kommen, den Serumspiegel von ausgewählten Antibiotika adsorptiv reduzieren. Für ein besseres und sicheres Antibiotika-Monitoring in der extrakorporalen

Leber- und Sepsis-Behandlung müssen weitere Untersuchungen zur Bestimmung der Clearance von Antibiotika während der extrakorporalen Therapie durchgeführt werden.
In Adsorptions- und Filtrationsexperimenten mit Blut konnte gezeigt werden, dass die Zitratantikoagulation im Vergleich zu Heparin die Bioverträglichkeit von Materialien, welche in der Blutreinigung zur Anwendung kommen, deutlich verbessert.
Von allen in dieser Arbeit untersuchten Endotoxinadsorbern zeigte die DEAE-Sepharose die effektivste Endotoxin-Elimination. DEAE-Sepharose kann jedoch durch seine schlechte Bioverträglichkeit in Blut und Plasma nicht eingesetzt werden. Von den übrigen Adsorbern konnten nur die PMB basierenden Adsorber eine Endotoxin-Reduktion bewirken. Jedoch konnte auch gezeigt werden, dass die Abnahme der LPS-Aktivität durch desorbiertes PMB verursacht wurde, welches Endotoxine als LPS-PMB-Komplex bindet. Liegen Endotoxine in Form des LPS-PMB-Komplexes besitzen eine geringere Immunstimulierende Wirkung im Blut und führen dort zu einer stark reduzierten Zytokinsekretion durch Leukozyten. Auf Grund der *in vitro* Ergebnisse konnten wir zeigen, dass der ideale PMB-Serumspiegel für die LPS-Inaktivierung zwischen 100 und 200 ng/ml liegt. Durch die Beschichtung des Zytokinadsorbers CG161 mit PMB konnte die adsorptive Zytokinentfernung und eine kontinuierliche PMB-Freisetzung durch ein und denselben Adsorber erreicht werden.
Eine neue extrakorporale Therapiemöglichkeit für die Gram-negative Sepsis könnte durch eine Kombination von bestehenden und neu entwickelten Verfahren sein. Dieses könnte sich zusammensetzen aus:

- Einem Zytokinadsorber, der auch in der Lage ist Protein-gebundene Toxine aus dem Blut zu entfernen.
- Einem Verfahren, welches die immunstimulierende Eigenschaft von Endotoxinen inaktiviert. Dies kann durch eine kontinuierliche Polymyxin Infusion oder durch einen Polymyxin-beschichteten Adsorber erreicht werden.
- Einem High-Cut off Filter, welcher effizient urämische Toxine entfernt und zusätzlich kleine bis mittelmolekulare Entzündungsmediatoren entfernt.
- Einer regionalen Zitratantikoagulation, welche die Plasma und Blutverträglichkeit der Materialien verbessert.

Weitere *in vitro*-Versuche müssen noch folgen, um einen sicheren Einsatz in der Klinik zu ermöglichen.

Abstract

Extracorporeal blood purification systems are used to remove substances from the blood of patients through filtration or adsorption units. The best-known extracorporeal blood purification system is the chronic dialysis which is used in chronic renal failure. This long-term to lifelong treatment enables patients with weak or no kidney function to survive or prolong life until a suitable transplant is available. In recent decades, efforts have also been made to use extracorporeal blood purification methods as a supportive therapy for liver failure and sepsis. In addition to dialysis, adsorbents are used to remove protein-bound and large molecular substances from the blood. Target substances in extracorporeal liver support and sepsis therapy are, among others, bilirubin, bile acids, endotoxins, cytokines and other inflammatory mediators.

This work aimed to test by *in vitro* experiments existing and new adsorbents, which are not approved as a medical device, for their effectiveness and blood compatibility. Furthermore, combinations of existing blood purification methods were tested to develop an efficient extracorporeal blood purification system for liver and sepsis therapy. Adsorption studies with commercially available endotoxin adsorbents were conducted in serum, plasma, and blood. Also, it has been investigated whether endotoxin inactivation by the use of Polymyxin B (PMB), an antibiotic with a high affinity for endotoxins, is a suitable alternative to endotoxin adsorbent. Polystyrene-divinylbenzene based adsorbents with different pore sizes and clinically approved cytokine adsorbents were studied regarding their efficiency and biocompatibility. In addition to plasma and blood compatibility, the adsorptive removal of selected antibiotics by liver support methods was investigated. Also, it was tested whether the use of regional citrate anticoagulation instead of heparin increases blood compatibility in extracorporeal blood purification processes.

The pores of adsorbents act as molecular sieves and prevent the passage of molecules larger than the pore diameter. The results of this study show that polystyrene-divinylbenzene copolymer-based adsorbents with 15 nm pores are most suitable for cytokine removal. On the other hand, pores between 30 and 40 nm are more effective for the removal of albumin-bound toxins from plasma. Pores with larger diameters reduce the selective effect of adsorbents and lead to uncontrolled adsorption of all plasma proteins. As a result, they are not plasma-compatible and therefore do not meet the requirements for use in extracorporeal blood purification systems. The blood tolerance, selectivity, and efficiency of adsorbents used in blood purification can thus be improved by the right choice of pore distribution. The adsorbent CG161 showed the highest efficiency in the adsorptive removal of cytokines from human plasma. Also, this adsorbent was able to remove a large part of TNF-α in contrast to the adsorbents used in the clinic. In separate *in vitro* experiments, we were able to show that adsorbent used in blood purification reduce the serum levels of selected antibiotics. For better and safer antibiotic monitoring in the extracorporeal treatment of liver failure and sepsis, further investigations must be carried out to determine the clearance of antibiotics during extracorporeal therapy.

We could show in adsorption and filtration experiments with human blood that citrate anticoagulation significantly improves the biocompatibility of materials used in blood purification systems compared to heparin.

Among all endotoxin adsorbents which were studied for endotoxin removal, DEAE sepharose was the most effective one. However, DEAE sepharose cannot be used in

blood and plasma due to its poor biocompatibility. Only PMB-based adsorbents were able to reduce endotoxin levels. However, it could be shown that the decrease in LPS activity was caused by desorbed PMB which binds endotoxins and forms a LPS-PMB complex. When endotoxins are presented in the form of the LPS-PMB complex, they have a lower immune stimulating effect in blood and lead to a strongly reduced cytokine secretion by leukocytes. Based on *in vitro* results, we were able to show that the ideal PMB serum level for LPS inactivation is between 100 and 200 ng/ml. Additionally we coated the CG161 adsorbent with a defined amount of PMB by hydrophobic interaction. In blood and plasma, an equilibration between the free and bound form of PMB leads to a constant PMB level in plasma. Our *in vitro* experiments showed that the combination of cytokine removal and controlled PMB release by the same adsorbent can be realized.

A new option for extracorporeal therapy in gram-negative sepsis could be realized by a combination of existing and newly developed procedures. These are:

- A cytokine adsorbent that removes protein-bound toxins from the blood.
- A process that inactivates the immunostimulatory properties of endotoxins. This can be achieved by continuous Polymyxin infusion or by a Polymyxin coated adsorbent.
- A high cut-off filter that efficiently removes uremic toxins and small to middle molecular weighted inflammation mediators.
- Regional citrate anticoagulation that improves plasma and blood tolerance of materials.

Further in vitro tests are necessary to ensure a safe testing in the clinic.

1. Grundlagen

1.1 Klinische Bedeutung der extrakorporalen Blutreinigung

Extrakorporale Blutreinigungsverfahren sind Systeme, bei denen Blut außerhalb des Patienten behandelt wird, um schädliche Stoffe zu eliminieren. Sie finden in der Medizin überall dort Anwendung, wo es im Organismus zur Bildung beziehungsweise Anreicherung von Substanzen im Blut kommt, die toxisch wirken. Solche Effekte können auf verschiedenste Weise hervorgerufen werden.

Bekanntestes Beispiel ist die chronische Niereninsuffizienz oder Urämie, die wegen der fehlenden exkretorischen Aktivität eine Anreicherung von Stoffwechselendprodukten oder auch Intermediaten wie Harnstoff, Kreatinin, Phosphat und β2–Mikroglobulin hervorruft. Der vollständig anurämische Patient kann über die Niere auch kein überschüssiges Körperwasser ausscheiden, das daher entfernt werden muss. Weitere Beispiele in denen extrakorporale Blutreinigungssysteme eine Rolle spielen sind Stoffwechselstörungen auf Grund von Fehlfunktionen anderer Organe, bei denen unter anderem Intermediate nicht weiter metabolisiert werden, wie beispielweise unkonjugiertes Bilirubin beim Leberversagen. Bei der familiären Hypercholesterinämie kommt es zur extremen Anreicherung von Low-Density-Lipoproteinen (LDL). Bei Autoimmunerkrankungen führt eine fehlgeleitete Immunabwehr zur Produktion von Autoantikörpern und Immunkomplexen.

Eine andere Ursache für das Auftreten von Giftstoffen in der Blutbahn sind Intoxikationen (Vergiftungen) durch exogene Toxine. Dabei können nicht nur chemische Substanzen, sondern auch Keime und deren Abbauprodukte (Endotoxine, Exotoxine und andere) in die Blutbahn eingeschwemmt werden, wie es beim Multiorganversagen durch Permeabilitätsstörungen im Intestinaltrakt vorkommt.

Zusammenfassend lässt sich feststellen, dass immer, wenn die Eliminierung von solchen Substanzen sinnvoll erscheint, extrakorporale Blutreinigungsverfahren grundsätzlich anwendbar sind. Es muss betont werden, dass allerdings für viele Bereiche auch alternative (medikamentöse) Verfahren zur Verfügung stehen und auch bei weitem nicht für alle Indikationen entsprechende Blutreinigungsverfahren zur Verfügung stehen.

1.2 Leberversagen

Die Leber erfüllt im Organismus eine Reihe von wesentlichen Aufgaben, die allerdings nur zum Teil der extrakorporalen Therapie zugänglich sind. Die Funktionen lassen sich in folgende Gruppen unterteilen: 1. Metabolische Aktivitäten (Katabolismus und Anabolismus), 2. Speicherfunktion (Glykogen) sowie 3. Immunologische Funktionen (Kupffersche Sternzellen). Beim Leberversagen sind diese Funktionen je nach Schweregrad mehr oder weniger eingeschränkt. Eine wesentliche Aufgabe ist die Hydrophilisierung von lipophilen Stoffwechselendprodukten, wodurch sie erst nierengängig gemacht werden. Der Ausfall dieser Funktion führt zur Anreicherung dieser Stoffe im Blut, wo sie an Transportproteine gebunden vorliegen. Der bekannteste Vertreter ist unkonjugiertes Bilirubin, Abbauprodukt des Hämoglobins, das generell als Markersubstanz für das Leberversagen gilt, auch wenn seine Toxizität in den üblicherweise vorliegenden Konzentrationen als gering eingestuft wird. Bei Leberversagen

reichert sich das unkonjugierte Bilirubin im Blutkreislauf an, wird dort an Albumin gebunden und verursacht Gelbsucht [1, 2].

Weiteres findet man beim Leberversagen vermehrt Gallensäuren und Phenole im Blut sowie eine Verschiebung des Aminosäureprofils in Richtung aromatische Aminosäuren (Fischer Index), da diese vorwiegend in der Leber abgebaut werden. Beim Abbau von Aminosäuren entsteht Ammoniak, das sofort an Transportproteine gebunden wird und von der Leber im Rahmen der Harnstoff- und Glutaminsynthese entfernt wird. Freier Ammoniak ist in der Lage, die Blut-Gehirnschranke zu überwinden und wirkt dort neurotoxisch.

Die Anzahl der Todesfälle durch Leberkrankheiten und -zirrhose pro 100.000 Einwohner liegt in den USA bei 9,3, in Deutschland bei 12,9 und in Österreich bei 13,8. Wenn bei Patienten mit normaler Leberfunktion plötzlich ein Leberversagen eintritt, spricht man von einem akuten Leberversagen. Als akut-auf-chronisches Leberversagen bezeichnet man eine akute Verschlechterung der Leberfunktionen bei chronischen Lebererkrankungen. Verursacht wird ein akut-auf chronisches Leberversagen oftmals durch eine bakterielle Entzündung, Sepsis, Blutung oder einer Störung der Hämostase und weist eine hohe Mortalität auf [3]. Leberersatzverfahren kommen vor allem bei akutem Leberversagen zum Einsatz und haben dort die Aufgabe die Leberfunktion vorübergehend zu unterstützen. Die Entscheidung die Leberersatztherapie einzusetzen wird durch die Tatsache erschwert den natürlichen Erkrankungsverlauf oftmals nicht vorhersagen zu können. Gleichzeitig erschwert dieser Unsicherheitsfaktor auch die Festlegung des richtigen Zeitpunktes für eine Lebertransplantation. In den meisten Kliniken werden daher die Kings-College-Kriterien für eine Prognoseabschätzung des akuten Leberversagens verwendet [4]. Anders als bei akutem Leberversagen ist für chronische Lebererkrankungen die Lebertransplantation die einzige kurative Therapieoption. Bei chronischen Lebererkrankungen werden daher die Leberersatztherapien verwendet um die Wartezeit zu überstehen, bis ein geeignetes Transplantat zur Verfügung steht (sogenanntes „Bridging to Transplantation“).

1.2.1 Leberersatztherapie

Die unzureichende Verfügbarkeit von geeigneten Leberorgane für eine notwendige Transplantation führten in der Vergangenheit zur Entwicklung neuer chirurgischer Verfahren wie die Leberlebendspende, die Teillebertransplantation sowie die auxiliäre partielle orthotope Lebertransplantation (APOLT). Zusätzlich zu den chirurgischen Verfahren wurden Alternativen wie die extrakorporale Leberersatztherapie entwickelt. Die hepatische Entgiftungsfunktion stellt den primären Ansatzpunkt der extrakorporalen Leberersatzverfahren dar. Die Elimination der toxischen Substanzgruppen ist mit adsorptiven Blutreinigungsverfahren sowie mit Plasmaaustausch möglich [5-8]. Komplexere Aufgaben, wie die Übernahme von Synthesefunktionen (Proteine, Gerinnungsfaktoren), können nur mit Hilfe von Bioreaktoren übernommen werden, bei denen immobilisierte humane oder porzine Hepatozyten extrakorporal perfundiert werden [6, 9, 10].

Im Wesentlichen gibt es bei Leberunterstützungsverfahren drei Strategien, die verfolgt werden: extrakorporale artifizielle und bioartifizielle Leberunterstützungssysteme und extrakorporale Leberperfusion als biologisches Verfahren (Tabelle 1). Wenn die Leberfunktion nachlässt reichern sich sowohl wasserlösliche (z. B. Ammonium,

Mercaptane) als auch Albumin-gebundene, nicht wasserlöslicher Substanzen (z. B. Bilirubin, Gallensäuren, Fettsäuren und aromatische Aminosäuren) im Blut und Gewebe an. In der Klinik konnte man bis jetzt den Zusammenhang zwischen der Anreicherung sogenannter Lebertoxinen und der Dysfunktion von anderen Organen beobachtet werden. Aus diesem Grund wurden Systeme entwickelt, um die im Blut der Patienten angesammelten Toxine zu entfernen. Systeme die die extrakorporale Entgiftung der geschädigten Leber übernehmen werden als artifizielle Leberunterstützungssysteme bezeichnet. Andere wichtige Aufgaben der Leber wie die Regulation komplexer Prozesse und Synthese von Wachstums- und Gerinnungsfaktoren sind durch solche Systeme nicht zu ersetzen. Um neben der Detoxifikation auch andere Aufgaben zu bewältigen, werden bioartifizielle Leberunterstützungssysteme erprobt, welche vitale Leberzellen in einem Bioreaktor inkludieren.

Tabelle 1: Die extrakorporalen Leberunterstützungssysteme lassen sich in die rein maschinellen (artifiziellen) Verfahren und die Bioreaktoren, welche mit Hepatozyten bestückt sind, (bioartifiziellen Verfahren) unterscheiden. Zusätzlich gibt es noch verschiedene Arten der extrakorporalen Leberperfusion.

Artifizielle Verfahren	
Klassische Dialyseverfahren	Hämodialyse, Hämofiltration, Plasmapherese, Hämoadsorption
SPAD	Dialyse mit 5 %iger Albuminlösung als Dialysat
MARS	Dialyse gegen Kreislauf mit 20 %iger Albuminlösung (Regenerierung von Albumin: Adsorber + Low-Flux-Dialyse)
FPSA, Prometheus-Verfahren	Plasmaseparation + direkte adsorptive Albuminaufreinigung im Sekundärkreislauf + High-Flux-Dialyse
Bioartifizielle Verfahren	
ELAD	HepG2-Zellen (C3A) + Ultrafiltration
HepatAssist	Porcine Hepatozyten + Hämadsorption
BAL	Porcine Hepatozyten + Hämadsorption
BLSS	Porcine Hepatozyten
MELS	Humane Hepatozyten + SPAD + High-Flux-Dialyse
Extrakorporale Leberperfusion (ECLP)	
Patientenblut wird durch eine explantierte (humane oder xenogene) Leber in einer sterilen Kammer geleitet und dort aufgereinigt.	
SPAD: *Single Pass Albumin Dialysis*, MARS: *Molecular Adsorbent Recirculating System*, FPSA: *Fractionated Plasma Separation and Adsorption*, ELAD: *Extracorporeal Liver Assist Device*, BAL: *Bioartificial Liver,* BLSS*: Bioartificial Liver Support System*, MELS: *Modular Extracorporeal Liver Support System*	

1.2.1.1 Klassische Dialyseverfahren

Zu den klassischen Leberersatzsystemen gehören die verschiedenen Filtrationsverfahren mit rein entgiftender Funktion wie Hämodialyse, Hämofiltration, Hämodiafiltration und Plasmaaustausch. Dabei werden klein- bis mittelmolekulare Substanzen, welche sich während der Lebererkrankung im Blut ansammeln, durch Dialyse und Filtration entfernt. Beim Plasmaaustausch können zusätzlich durch die Gabe von Spenderplasma oder Albuminlösung wichtige, von der Leber nicht mehr synthetisierte Substanzen zugeführt werden. Es gab etliche klinische Studien mit teilweise erfolgreichen Einzelverläufen, jedoch konnte bis heute die Wirksamkeit der Filtrationsverfahren als Leberersatztherapie nicht bewiesen werden. Oftmals konnte man eine vorübergehende Besserung der

hepatischen Enzephalopathie und eine Verlängerung der Überlebensdauer beobachten, jedoch kam es zu keiner relevanten Steigerung der Überlebensrate [11-13]. Als Ursache wird die geringe Clearancerate von albumingebundenen Toxinen und die teilweise sehr selektive Eliminierung einzelner klein- bis mittelmolekularer Toxine vermutet.

1.2.1.2 Weiterentwickelte Dialyseverfahren

a) Single-Pass Albumin Dialysis (SPAD)

Neben den klassischen Dialyseverfahren wurden Therapien entwickelt um neben den wasserlöslichen Substanzen auch proteingebundene Toxine aus dem Blut abzureichern.

Eines der ersten Verfahren die dies ermöglichte war die Single-Pass-Albumindialyse (SPAD). Bei der SPAD wird das Patientenblut über eine nicht Albumin-permeable Membran gegen Albumin im Sekundärkreislauf dialysiert (siehe Abbildung 1) [14]. In Einzelfällen und kleinen klinischen Studien konnte durch die SPAD-Behandlung eine Reduktion von Lebertoxinen und eine Verbesserung klinischer Parameter festgestellt werden [15, 16]. Eine große randomisierte Studie, in welcher die SPAD-Behandlung inkludiert ist, gibt es bis heute nicht.

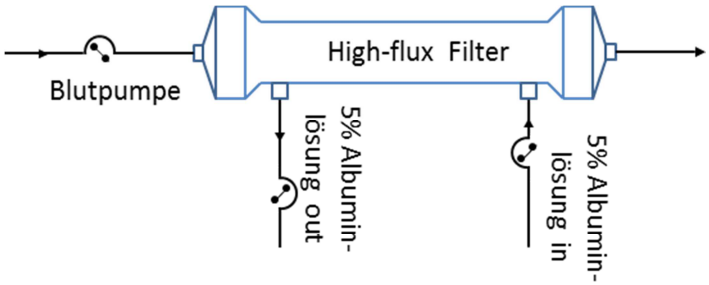

Abbildung 1: Schematische Darstellung der Single-Pass Albumin Dialyse (SPAD).

b) Molecular Adsorbent Recirculating System (MARS)

Eine Weiterentwicklung von SPAD ist das MARS-Verfahren. Um Unterschied zur SPAD-Methode wird das verbrauchte Albumin nicht verworfen, sondern in einem geschlossenen Dialysatkreislauf „recycelt“. Die Aufreinigung vom Albumin-Molekül erfolgt über 2 Adsorber und einer Low-Flux-Dialyse. Die Adsorber bestehen aus Aktivkohle und aus einem anionischen Neutralharz (siehe Abbildung 2). In mehreren kleineren klinischen Studien konnte eine Besserung der hämodynamischen Parameter nachgewiesen werden [17, 18]. Bei einigen behandelten Patienten konnte sogar eine vollständige Regeneration der Leberfunktion beobachtet werden [19, 20]. Bislang sind 2 kontrollierte Studien mit kleinen Fallzahlen (13 bzw. 24 Patienten) und eine größere mit 189 Patienten (MARS RELIEF Studie) zum MARS-Einsatz bei akut-auf-chronischem Leberversagen publiziert [21-23]. Die überwiegende Mehrzahl an MARS-Behandlungen wurde an Patienten mit akut-auf-chronisch und nicht mit akutem Leberversagen durchgeführt [24]. Während erste kleinere Studien zu MARS bei fortgeschrittenem Leberversagen auf ein verbessertes Überleben hindeuten, zeigten die Ergebnisse aus der randomisierten MARS RELIEF Studie keinen Überlebensvorteil bei Patienten mit akut-auf chronischem Leberversagen nach 28 Tagen.

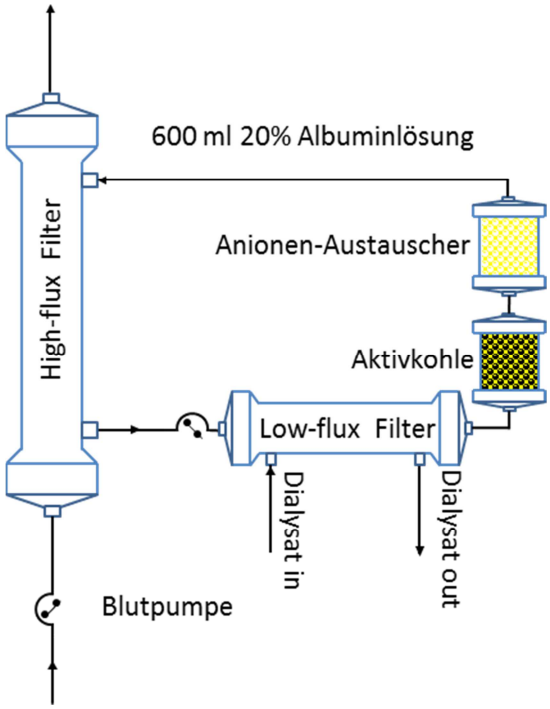

Abbildung 2: Schematische Darstellung des Molecular Adsorbent Recirculating System (MARS).

c) Fractionated Plasma Separation and Adsorption (FPSA, Prometheus)

Ein weiteres in der Klinik eingesetztes Gerät ist das Prometheus System, das auf dem sogenannten FPSA-Verfahren basiert. Dabei wird das Plasma durch einen Albumin-durchlässigen Filter (Albuflow Filter) in einen Sekundärkreislauf separiert und dort mit Hilfe von zwei Adsorberkartuschen gereinigt (siehe Abbildung 3). Eine Adsorberkartusche ist mit Neutralharz und die zweite mit einem Anionen-Austauscher befüllt. Klein- bis mittelmolekulare, nicht proteingebundene Toxine werden mit einer integrierten konventionellen High-Flux-Hämodialyse entfernt [5]. Im Unterschied zum MARS-Verfahren kommt es beim FPSA-Verfahren zu einem direkten Kontakt zwischen dem mit Toxinen beladenen Albuminmolekül und der Adsorberoberfläche. Diese kann zu einer verbesserten Detoxifikation aber auch gleichzeitig zu einer Verschlechterung der Blutverträglichkeit des Verfahrens führen. Eine kleine klinische Studie an 11 Patienten mit akut-auf-chronischem Leberversagen zeigte nach 2 Behandlungen Erfolge bei der Detoxifikation [25].

Es gibt eine große randomisierte Studie mit 145 Patienten in der die Standardtherapie mit der FPSA-Therapie bei Akut auf chronischem Leberversagen verglichen wurde. Bei einigen klinischen Parametern wie zum Beispiel bei der Entfernung von proteingebundenen Toxinen konnte die FPSA-Therapie eine deutliche Verbesserung im Vergleich zur Standardtherapie bewirken. Eine signifikante Verbesserung der Überlebensrate nach 28 Tagen konnte jedoch nicht erzielt werden [26, 27].

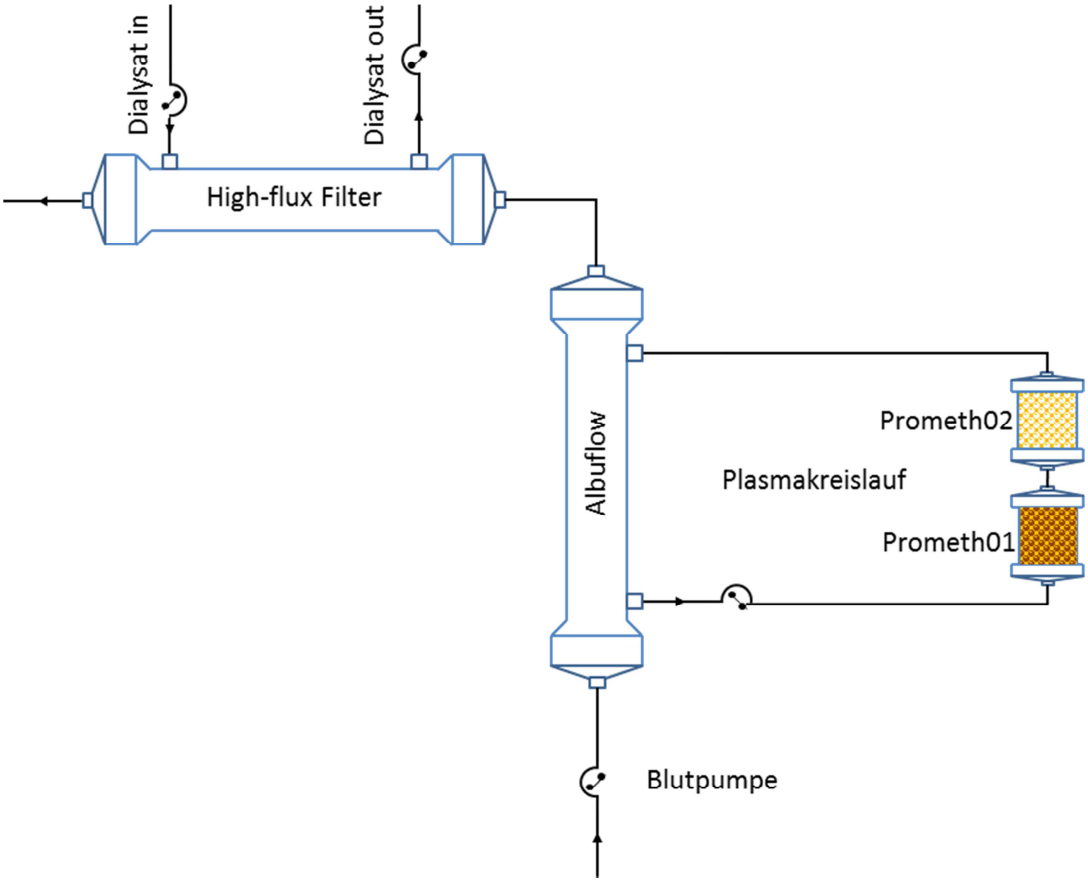

Abbildung 3: Schematische Darstellung des Prometheus-Systems (Fractionated Plasma Separation and Adsorption, FPSA).

1.2.1.3 Bioartifizielle Verfahren

Im Gegensatz zu den artifiziellen Leberunterstützungssystemen soll bei bioartifiziellen Systemen neben der Detoxifikation auch andere Leberfunktionen unterstützt werden. Um dies zu gewährleisten, muss eine ausreichende Menge an vitalen Hepatozyten zur Verfügung stehen. Die Zellen müssen solange aktiv bleiben, bis das Leberversagen überstanden ist, oder ein geeignetes Transplantat zur Verfügung steht („Bridging“). Die bioartifiziellen Leberunterstützungs-Verfahren sind momentan nicht von klinischer Relevanz und werden hier nur der Vollständigkeit halber erwähnt. Die Bioreaktoren der bioartifiziellen Verfahren können mit humanen (allogene), porcinen (xenogene) oder immortalisierten Hepatozyten bestückt sein. Für eine gute Funktionstüchtigkeit des Bioreaktors ist ein guter Stoffaustausch von Nährstoffen, Metaboliten und Toxinen zwischen Patientenblut und Zellen Voraussetzung.

a) Extracorporeal Liver Assist Device (ELAD)

Beim Extracorporeal Liver Assist Device befinden sich humane C3A-Zellen (Klon der HepG2-Zell-Linie) im Dialysatraum von insgesamt vier modifizierten Dialysefiltern (Bioreaktoren). Das ELAD System wurde bereits an 5 Patienten erfolgreich zum Bridging zur Transplantation eingesetzt. Eine kontrollierte Studie an 24 Patienten erbrachte jedoch keinen Überlebensvorteil [28, 29].

b) HepatAssist

Der HepatAssist wird mit 5-7 x 10^9 Schweineleberzellen bestückt, welche bis zur klinischen Anwendung kryokonserviert werden. Die Zellen befinden sich im extrakapillaren Raum eines modifizierten Dialysefilters. Das Plasma des Patienten wird durch den Bioreaktor (Dialysefilter) geleitet, nachdem es einen Aktivkohlefilter und einen Oxygenator durchlaufen hat. Die bisher größte prospektive, randomisierte, kontrollierte Studie umfasste 171 Patienten mit akutem/subakutem Leberversagen. 86 Patienten wurden der Kontrollgruppe zugeordnet, die eine medizinische Standardbehandlung erhielt, und 85 Patienten wurden zusätzlich mit HepatAssist behandelt. Es gab eine signifikante Verlängerung der Überlebenszeit bei Patienten, die mit dem HepatAssist behandelt wurden, jedoch keine signifikante Verbesserung der 30-Tage-Überlebensrate [30].

c) Modular Extracorporeal Liver Support System (MELS)

Eine Kombination verschiedener Verfahren wird beim Modular Extracorporeal Liver Support System (MELS) genutzt: ein Zellmodul mit humanen Hepatozyten, eine Single-Pass-Albumindialyse zur Entfernung von Albumin-gebundenen Toxinen sowie ein Dialysemodul zur Entfernung von wasserlöslichen Substanzen. Das MELS beruht auf das Berlin Extracorporeal Liver Support System (BELS), welches auf porzinen Hepatozyten basierte [9]. Klinisch getestet wurde das MELS bei 8 Patienten mit Leberversagen [31, 32]. Alle Patienten wurden für eine Transplantation mit hoher Dringlichkeit aufgelistet. Bei allen 8 Patienten war das Bridging zur Transplantation erfolgreich. Bei keinem der Patienten konnte eine Infektion mit endogenen Retroviren (PERV) vom Schwein nachgewiesen werden [33].

d) Academic Medical Center Bioartificial Liver (AMC-BAL)

Im Gegensatz zu allen anderen bioartifiziellen Systemen, trennt die bioartifizielle Leber des Academic Medical Center Amsterdam (AMC-BAL) die Zellen vom Plasma des Patienten nicht durch Kapillarmembranen. Eine spiralförmig gewickelte Matte aus Polyestervliesfasern in einem Gehäuse bietet den Leberzellen eine Matrix. Die Sauerstoffkapillaren werden in die Matrix eingearbeitet, um eine lokale Sauerstoffanreicherung zu gewährleisten. 10 x 10^9 primäre Schweineleberzellen werden in die Matrix gesät und adhärieren dort. Während der Therapie wird das Plasma des Patienten direkt durch die Matrix perfundiert, so dass dieses System im Gegensatz zu den meisten anderen bioartifiziellen Verfahren einen direkten Zell-Plasma-Kontakt ermöglicht. In einer klinischen Phase-I-Studie wurden zwölf Patienten, die für eine Transplantation mit hoher Dringlichkeit vorgesehen waren, mit dem AMC-BAL behandelt. Elf Patienten wurden erfolgreich zur Transplantation überbrückt, ein Patient zeigte nach zwei Behandlungen eine verbesserte Leberfunktion und musste nicht transplantiert werden. Im Allgemeinen zeigte die Behandlung keine Nebenwirkungen [34, 35].

1.2.1.4 Extrakorporale Leberperfusion (extracorporeal liver perfusion, ECLP)

Eines der ersten Konzepte für die biologische Leberunterstützung basierte auf extrakorporaler Leberperfusion. Bei der ECLP wird das Blut des Patienten in einer sterilen Kammer durch eine extransplantierte menschliche oder xenogene Leber geleitet. ECLP Behandlungen wurden in den letzten Jahrzenten nur sporadisch bei insgesamt 270 Patienten durchgeführt. Ein Durchbruch in der extrakorporalen Leberersatztherapie konnte mit ECLP nicht erzielt werden da eine Meta-Analyse aller publizierten Fälle gegenüber der konventionellen Therapie keine Vorteile zeigt [36].

1.2.2 Grenzen derzeitiger Leberunterstützungsverfahren

Die bisherigen klinischen Anwendungen extrakorporaler Leberunterstützungssysteme haben gezeigt, dass die Konzepte auch bei schwerkranken Patienten mit Leberversagen sicher angewendet werden können. Klinische Studien zeigen nachweisbare positive Effekte und einen, wenn auch bescheidenen, Überlebensvorteil von Patienten, die mit extrakorporalen Leberunterstützungssystemen behandelt wurden. Allerdings ist die Leistungsfähigkeit extrakorporaler Systeme im Vergleich zur Lebertransplantation derzeit noch zu gering. Bei der alleinigen Verwendung der extrakorporalen Verfahren werden nur entgiftende Leistungen erbracht, wenn keine biologische Komponente vorhanden ist. Ein vollständiger Ersatz aller leberrelevanten Aufgaben kann nur durch die Verwendung von intakten Leberzellen erfolgen (Abbildung 4). Problematisch bei bioartifiziellen Verfahren ist die zu geringe Verfügbarkeit von humanen Hepatozyten. Xenogene Hepatozyten sind von der Funktion betrachtet eine gute Alternative, können jedoch Immunantworten auf Fremdantigene hervorrufen. Zusätzlich können porzine endogene Retroviren (PERV) übertragen werden. Der Nachteil von immortalisierten Zelllinien besteht in deren verringerten leberspezifischen Funktionen. Auch beherbergt die Verwendung von immortalisierten Zellen die Gefahr, dass tumorigene Zellen aus dem extrakorporalen Bioreaktor in das Patientenblut gelangen können.

Bisherige Untersuchungen von bioartifiziellen Leberverfahren konnten zeigen, dass die Hepatozyten in den verschiedenen Bioreaktoren funktionstüchtig sind (4). Für einen klinischen Routine-Einsatz müssen jedoch noch viele logistische Probleme gelöst werden. Zum einen kann die Nachfrage an primären humanen Hepatozyten für bioartifizielle Verfahren nicht abgedeckt werden, zum anderen ist der Transport und die Lagerung von primären Zellen in derartig großen Mengen ohne Verluste noch nicht möglich.

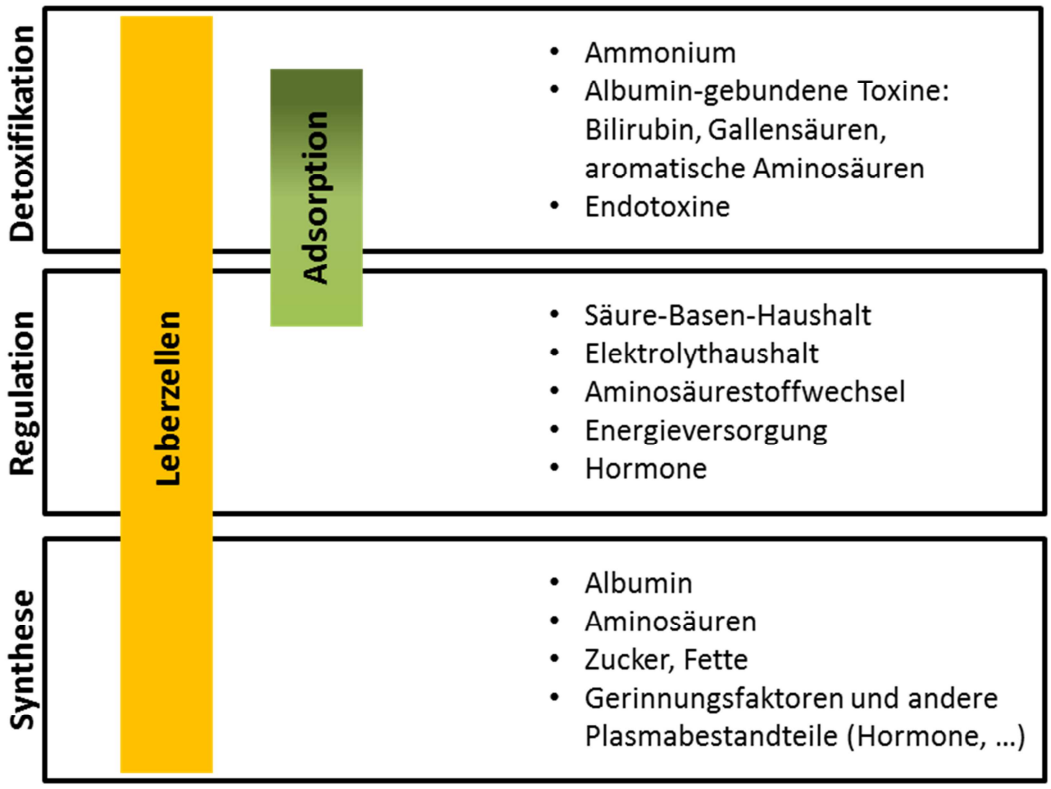

Abbildung 4: Anforderungen an Leberunterstützungssysteme: Regulation und Detoxifikation können durch Adsorber- und Dialysetechniken großteils ersetzt werden. Komplexe regulatorische Prozesse und Synthese können dagegen nur Leberzellen leisten.

1.3 Sepsis

Mit Sepsis wird die systemische Entzündungsreaktion bezeichnet, welche im Gegensatz zu anderen Erkrankungen ein sehr komplexes Krankheitsbild darstellt.

Die mittlerweile veraltete Definition [37] unterscheidet zwischen Systemischem Inflammatorischem Response-Syndrom (SIRS) mit nachgewiesener oder vermuteter Infektion, Schwerer Sepsis (Sepsis mit akuter Organschädigung), sowie Septischem Schock (Schwere Sepsis mit therapierefraktärer Hypotonie, meist kombiniert mit Multiorganversagen). Neue Erkenntnisse zur Pathophysiologie machten in den letzten Jahren eine Überarbeitung erforderlich. Die beiden Fachgesellschaften, *European Society of Intensive Care Medicine* und *Society of Critical Care Medicine*, beauftragte eine Task Force, die eine neue Definition erarbeitete, welche als „Sepsis 3“ bezeichnet wird [38].

Die Sepsis wird jetzt als „lebensbedrohliche Organdysfunktion aufgrund einer fehlregulierten Immunantwort auf eine Infektion“ definiert. Da die SIRS-Kriterien zu unspezifisch sind und schon bei üblichen Infektionen erfüllt werden, kommt SIRS in der neuen Definition nicht mehr vor. Eine Sepsis liegt dann vor, wenn sich der SOFA-Score („Sequential (Sepsis-Related) Organ Failure Assessment Score“) des Patienten akut um mehr als zwei Punkte verschlechtert, da dies mit einem Sterberisiko von ungefähr 10 Prozent verbunden ist. Der SOFA-Score, welcher anhand von sechs Kriterien (Herzkreislauffunktion, Leberfunktion, Atmung, Koagulation, Nierenfunktion und Glasgow Coma-Scale) ermittelt wird, rückt ganz eindeutig die Organfunktion in den Mittelpunkt.

Der septische Schock ist definitionsgemäß ein Sonderfall der Sepsis, bei dem sowohl die zellulären und metabolischen Veränderungen als auch die Kreislaufreaktion so schwerwiegend sind, dass das Sterberisiko deutlich erhöht ist.

Die Letalität schwankt in der Literatur zwischen 38 % und 59 % [37, 39]. Die Letalität von Sepsispatienten in Deutschland beträgt 55 % laut einer 2007 veröffentlichten Epidemiologie-Studie [40]. Dieser Studie zufolge erkranken in Deutschland jährlich über 150.000 Menschen an einer Sepsis, 75.000 davon haben schwere Sepsis oder septischen Schock. Mit ungefähr 60.000 Todesfällen pro Jahr stellt Sepsis die dritthäufigste Todesursache in Deutschland dar. In den USA erkranken jährlich etwa 750.000 Personen an Sepsis, von denen etwa 215.000 versterben [37]. Der in den letzten Jahren abnehmenden Mortalitätsrate bei Sepsis steht ein stetiger Anstieg der Zahl der Sepsisfälle um ca. 8 % pro Jahr gegenüber, der unter anderem durch zunehmenden Einsatz invasiver Verfahren der Intensivmedizin sowie durch zunehmende Antibiotikaresistenzen begründet werden kann. Nach Herzinsuffizienz und Krebserkrankungen ist Sepsis die dritthäufigste Todesursache in Krankenhäusern.

Die ökonomische Belastung, die durch die Sepsis verursacht wird, ist beträchtlich. Die direkten Behandlungskosten betragen in Österreich und der Schweiz etwa 28.000 € pro Patient; dazu kommen indirekte Kosten wie etwa Produktivitätsverlust von etwa 70.000 € pro Patient. Sepsis stellt somit sowohl aus medizinischer als auch aus ökonomischer Sicht ein schwerwiegendes Problem dar.

1.3.1 Pathophysiologie der Sepsis

Das angeborene Immunsystem, der älteste Mechanismus zum Schutz des Körpers vor pathogenen Mikroorganismen, spielt eine wichtige Rolle bei der Entstehung von Sepsis. Das Prinzip der angeborenen Immunität besteht in der Erkennung charakteristischer, in der Evolution hochkonservierter Strukturen (pathogen-assoziierte molekulare Muster; pathogen-associated molecular patterns, PAMPS) [41, 42]. Diese chemisch sehr unterschiedlichen Strukturen werden von pathogenen Mikroorganismen, nicht jedoch vom Wirtsorganismus gebildet und werden von diesem daher als fremd erkannt. Es kann sich dabei entweder um Oberflächenstrukturen von Mikroorganismen handeln (Komponenten der bakteriellen Zellwand wie Lipopolysaccharide, Peptidoglykane, Lipoteichonsäuren, Mannane), oder aber um interne Motive, die bei der Lyse von Mikroorganismen oder absterbenden Zellen freigesetzt werden (bakterielle DNA und doppelsträngige RNA; danger-associated molecular patterns, wie z.B. HMGB-1). Die Bindung dieser Strukturen an entsprechende Rezeptoren an den Zelloberflächen (mustererkennende Rezeptoren; pattern recognition receptors, PRR) löst die angeborene Immunantwort aus. Die wichtigsten mustererkennenden Rezeptoren im Zusammenhang mit der Entstehung von Sepsis sind die Toll-like Rezeptoren (TLRs). TLR-2 ist der Rezeptor für Peptidoglykane (Gram-positive Bakterien), während TLR-4 als Rezeptor für Lipopolysaccharid (Gram-negative Bakterien) fungiert. Die Erkennung der pathogen-assoziierten molekularen Muster durch die entsprechenden Toll-like Rezeptoren führt zur Aktivierung zahlreicher Signaltransduktionswege im Zellinneren und schließlich zur Aktivierung des Transkriptionsfaktors nuclear factor kappa-B (NF-kappaB). Dies führt zur verstärkten Expression der Gene für proinflammatorische Zytokine und zur Bildung einer Reihe teils sekretorischer, teils oberflächengebundener Proteine (Zytokine, Chemokine, Adhäsionsmoleküle, Komplement- und Gerinnungsfaktoren). Diese Proteine aktivieren das Endothel sowie Blutzellen, woraus ein generalisierter Entzündungszustand mit Aktivierung der Blutgerinnung und Abfall des Blutdruckes resultiert (siehe Abbildung 5), der letztlich in Septischen Schock und Multiorganversagen mündet [43, 44].

Neben der Hyperinflammation („Zytokine storm“) in der proinflammatorischen Phase, kommt es im Verlauf der Sepsis auch zu einer antiinflammatorischen Reaktion durch die Bildung antiinflammatorischer Substanzen sowie durch Apoptose von Immunzellen, insbesondere Lymphozyten. Dies bewirkt eine starke Dämpfung der Immunreaktion (Immunparalyse). Die meisten Patienten versterben nicht in der frühen, proinflammatorischen Phase der Sepsis, sondern durch Sekundärinfektionen in der Phase der Immunparalyse [45]. Dieses Phasenmodell der Sepsis (Abbildung 6) hat auch wesentliche Implikationen für die Therapieentwicklung: es ist davon auszugehen, dass eine Therapie, die auf der Modulation oder Entfernung proinflammatorischer Faktoren beruht, in einer frühen Phase der Sepsis erfolgen muss. Andererseits sind jene Faktoren potentiell besonders als therapeutische Ziele interessant, die als „late mediators“ erst in einer späteren Phase wirksam sind.

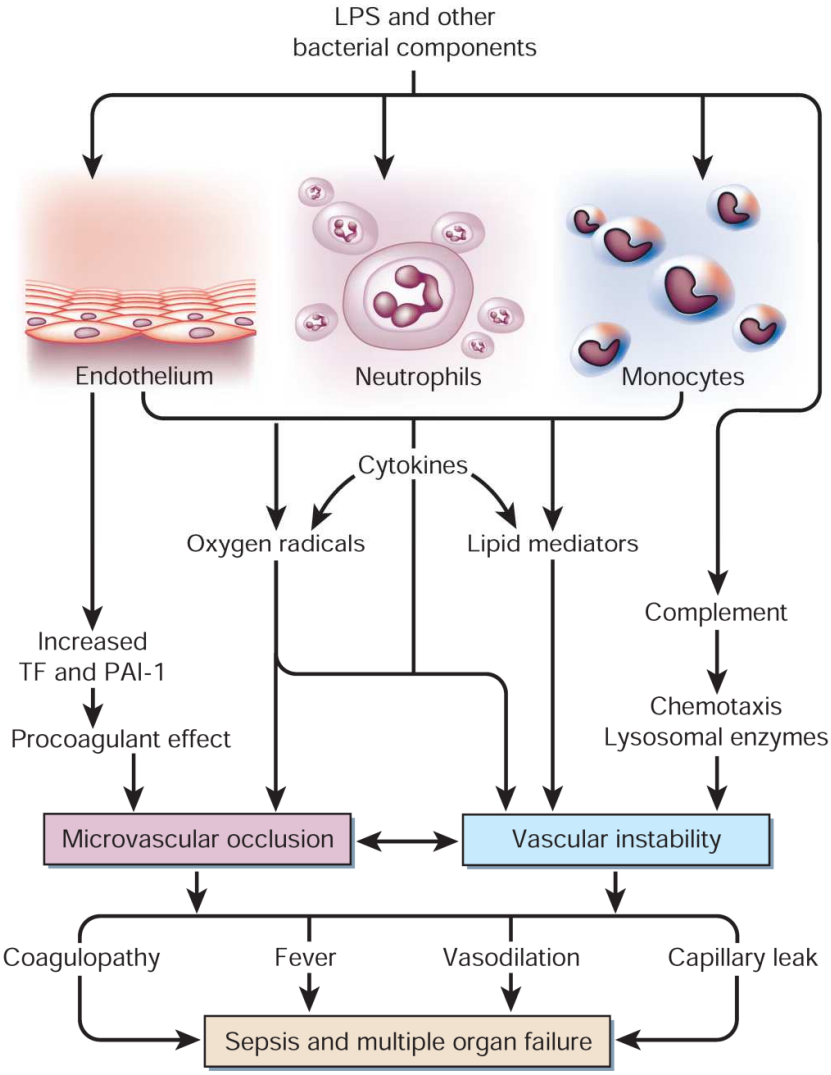

Abbildung 5: Durch die Freisetzung von Mediatormolekülen als Reaktion auf die Aktivierung von NF-kB kommt, es zur Aktivierung von Endothelzellen, Neutrophilen und Monozyten, die ihrerseits wieder verschiedenste Mediator- und Effektormoleküle freisetzen[46].

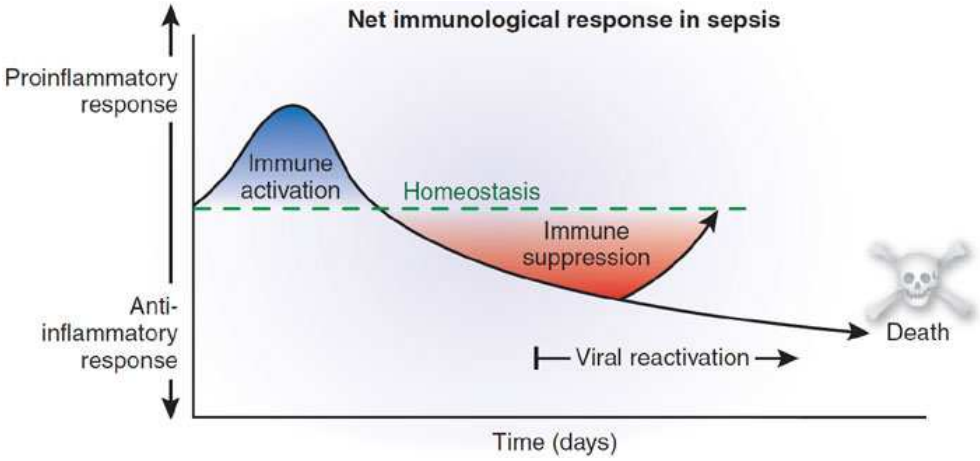

Abbildung 6: Obwohl pro- und antiinflammatorische Immunreaktion zu Beginn einer Sepsis aktiviert werden, dominiert zuerst die proinflammatorische Reaktion. Im weiteren Verlauf wird die antiinflammatorische Reaktion immer stärker. Während dieser späten Phase der Sepsis können Sekundärinfektionen und virale Reaktionen auftreten, welche häufig zum Tod führen [45].

1.3.2 Derzeitige Behandlungsansätze für Sepsis

Das Hauptproblem für eine gezielte Antibiotikatherapie von Sepsis Patienten ist die Unkenntnis des verursachenden Erregers. Die Behandlung mit einer inadäquaten Antibiotikatherapie führt zu einer erhöhten Sterblichkeit an Sepsis [47, 48]. Im Gegensatz dazu ist bei einer frühzeitigen antimikrobiellen Therapie eine Reduktion der Letalität bei Patienten mit grammnegativer und grampositiver Bakteriämie zu beobachten. Diese frühzeitige gezielte Therapie ist, solange noch keine mikrobiologischen Befunde vorliegen, am individuellen Risikoprofil des Patienten und am spezifischen mikrobiologischen Resistenzmuster der Intensivstation ausgerichtet [49-51].

Je früher die Ursache der Sepsis erkannt wird, je früher kann man auch mit einer gezielten Therapie beginnen. Die sogenannte „Golden Hour“ beschreibt dass die Therapie innerhalb von einer Stunde nach der Diagnose erfolgen sollte um effektiv zu sein. Die vielzitierte retrospektive Studie von Kumar [52] mit über 2.700 Patienten im septischen Schock zeigte, dass eine verzögerte Antibiotikatherapie großen Einfluss auf die Letalität der Patienten hat. Pro stündlicher Verzögerung der Antibiotikagabe nimmt die Sterblichkeit um etwa 7 % zu. Die daraus abgeleitete Empfehlung ist folglich, diagnostische Prozeduren, mit Ausnahme der Abnahme von Blutkulturen und der Probengewinnung, hinten anzustellen und möglichst früh nach Verdacht auf eine Infektion nach dem Motto “hit hard and early” mit der Antibiotikatherapie zu beginnen. Jedoch zeigte eine andere Studie [53], welche an hämodynamisch stabilen Patienten auf einer chirurgischen Intensivstation durchgeführt wurde, dass eine ganz gezielte antimikrobielle Behandlung nach Vorliegen des mikrobiologischen Befundes gegenüber einer aggressiven kalkulierten Therapie bei Verdacht auf eine Infektion mit einer verbesserten Überlebensrate assoziiert war. Momentan wird an der Entwicklung von schnellen und zuverlässigen Diagnoseverfahren gearbeitet, welche einerseits durch Kombination mehrerer Biomarker eine beginnende Sepsis zuverlässig erkennen und andererseits den verursachenden Keim für eine gezielte Antibiotikatherapie identifizieren soll. Biomarker könnten in der Sepsis-Diagnostik einen wichtigen Platz einnehmen, da sie nicht nur eine Aussage über den Schweregrad einer vorhandenen Sepsis erlauben, sondern auch zwischen einer bakteriellen, viralen oder von Pilzen ausgelösten Infektion unterscheiden können. Zusätzlich würde eine gute, auf Biomarkern aufgebaute Diagnostik zwischen einer lokalen und einer systemischen Infektion unterscheiden können und über eventuell vorliegende Organdysfunktionen (Herz-, Nieren-, Leber- oder Multiorganversagen) Aussagen treffen können. Daraus würde resultieren, dass schnellstmöglich eine gezielte Behandlung eingeleitet werden kann [54]. C-reaktives Protein (CRP), ein Akut-Phasen-Protein wird seit vielen Jahren als unspezifischer Entzündungsmarker für den Schweregrad entzündlicher Erkrankungen herangezogen [55, 56]. Procalcitonin (PCT), eine Vorstufe des Hormons Calcitonin, welches in der Schilddrüse produziert wird, gilt in der Klinik als zuverlässigerer Biomarker für die Risikoabschätzung von Sepsispatienten. PCT wurde 2005 von der Food and Drug Administration in Verbindung mit weiteren Laborwerten als „Sepsismarker“ zugelassen. Zur Zeit ist es mit den derzeitigen Biomarkern sehr schwierig, zwischen Sepsis und einer nicht infektiösen systemischen inflammatorischen Immunreaktion zu unterscheiden. Dies verdeutlicht, wie wichtig es ist, weiter nach besseren und vor allem spezifischeren Biomarkern zu forschen.

Im letzten Jahrzehnt gab es einen enormen Wissenszuwachs über die komplexe Pathophysiologie der Sepsis [57-59], dies schlug sich jedoch bislang kaum in der Entwicklung erfolgreicher Therapien nieder. Als einziges Medikament wurde bisher rekombinantes aktiviertes Protein C (Handelsname: Xigris™) von der Food and Drug Administration sowie von der Europäischen Arzneimittelagentur für die Behandlung von schwerer Sepsis zugelassen [59]. Es besitzt neben der gerinnungshemmenden auch entzündungshemmende und anti-apoptotische Wirkung, greift also auf mehreren Ebenen in den septischen Prozess ein. Auf Grund schwerwiegender Nebenwirkungen und nicht ausreichend gesicherter Wirksamkeit in Nachfolgestudien wurde Xigris™ Ende 2011 wieder vom Markt genommen.

Zahlreiche klinische Studien wurden mit systemisch verabreichten Antagonisten (meist Antikörpern) gegen Lipopolysaccharid oder proinflammatorische Zytokine (in erster Linie Tumornekrosefaktor-alpha, TNF-α) durchgeführt, mit dem Ziel, die massive Entzündungsreaktion während der Sepsis zu unterbinden bzw. einzudämmen. Keine dieser Studien führte jedoch zu einer signifikanten Senkung der Mortalitätsrate. Nach heutigem Wissen über die Pathomechanismen der Sepsis kann auf Grund der Vielfalt der am Krankheitsablauf beteiligten Mediatoren eine auf die Blockade einer einzelnen Substanz gerichtete Therapie nicht erfolgreich sein [60]. Weiteres sind viele der an der Pathogenese beteiligten Faktoren in geringen Konzentrationen für die Bekämpfung der Entzündung notwendig und dürfen somit nicht völlig entfernt oder inaktiviert werden. Ein dritter wesentlicher Faktor ist der Behandlungszeitpunkt, da etwa eine Entfernung proinflammatorischer Faktoren in der Phase der Immunparalyse kontraproduktiv ist.

Unter diesem Aspekt gewinnen therapeutische Strategien wie die extrakorporale Blutreinigung an Bedeutung, da sie, anders als systemisch verabreichte Inhibitoren, die Modulation von Mediatoren erlauben und nur die in der Zirkulation vorhandenen Pools beeinflussen, ohne jedoch zu einer völligen Blockade der Entzündungsmediatoren im Gewebe zu führen. Weiteres sind diese Therapien flexibel und kontrollierbar, was den Behandlungszeitpunkt betrifft.

1.3.3 Konzept der extrakorporalen Blutreinigung in der Sepsistherapie

Es besteht Konsens darüber, dass die Anwendung extrakorporaler Blutreinigungsmethoden zur unterstützenden Therapie der Sepsis sinnvoll ist, auch wenn große kontrollierte, randomisierte klinische Studien noch fehlen. Blutreinigungssysteme ermöglichen eine unspezifische Entfernung von inflammatorischen Mediatoren und/oder mikrobiologische Toxinen, was die immunologische Hämostase wiederherstellen könnte. Es gibt dazu fünf Hypothesen, welche die Verwendung von Blutreinigungssystemen zur Sepsis Therapie untermauern:

a) Hypothese der Spitzenkonzentration (cytokine peak concentration hypothesis)

Diese wurde von Ronco et al publiziert [61] und beschreibt, dass in der frühen proinflammatorischen Phase die Peaks der Zytokinkonzentrationen eliminiert werden sollen, um die inflammatorische Phase zu stoppen. Dies würde in weiterer Folge zu geringeren Organschäden führen und somit die Inzidenz von Multiorganversagen reduzieren.

b) Immunmodulations-Schwellen Hypothese (threshold immunomodulatin hypothesis)

Diese Hypothese wird auch Honoré Konzept [62] genannt und beschreibt das Gleichgewicht von Entzündungsmediatoren zwischen Blut und Gewebe. Dadurch, dass bei den einzelnen Mediatoren ein Gleichgewicht zwischen Blut, interstitieller Flüssigkeit und Gewebe vorherrscht, wird durch die Entfernung von Mediatoren aus dem Blut auch die Gewebskonzentration dieser Mediatoren herabgesetzt. Unterschreitet die Absenkung einen bestimmten Schwellenwert, so können biochemische Prozesse und Kaskaden gestoppt werden.

c) Hypothese der Mediatorfreisetzung (mediator delivery hypothesis)

Diese Hypothese, aufgestellt von Di Carlo und Alexander [63], beschreibt das bessere Ausschwemmen von Entzündungsmediatoren durch die Verwendung von Hochvolumen-Hämofiltration (HVHF) mit 3 bis 5 Liter Substituatlösung pro Stunde. Durch dieses Blutreinigungsverfahren konnte eine 20- bis 40-fache Steigerung des lymphatischen Flusses beobachtet werden, was dazu führt, dass inflammatorische Mediatoren schneller in die Blutbahn gelangen und somit besser extrakorporal entfernt werden können.

d) Hypothese der Reaktivierung von Immunzellen (cellular level theory)

Peng et al [64] beschreibt, dass nach Blutreinigungstherapien bei Sepsispatienten ein positiver Einfluss auf zellulärer Ebene beobachtet werden kann. Die Reaktivierung des Immunsystems nach der Immunparalyse ist nach der Blutreinigung durch vermehrte Leukozyten-Rekrutierung, oxidativen Burst, steigende Phagozytose und Leukozytenaktivität messbar.

e) Zytokinetisches Modell (cytokinetic model)

Diese Hypothese wird von Rimmelé und Kellum [65] vorgeschlagen und beschreibt die Wiederherstellung der Zytokingradienten zum Entzündungsherd nach Entfernung dieser aus dem Blut. Chemokine sind eine Gruppe der Zytokine, also kleine Signalproteine, die bei Zellen eine Wanderungsbewegung (Chemotaxis) auslösen. Die Zellen bewegen sich dabei entlang eines Konzentrationsgradienten zum Ort der höchsten Chemokinkonzentration. Chemokine spielen eine zentrale Rolle bei der Migration von Immunzellen im Gewebe und bei deren Auswanderung aus dem Blut. Ohne die von Chemokinen ausgelöste Zellwanderung könnte das Immunsystem nicht funktionieren. Nach Reduktion der Zytokinkonzentrationen im Blut kann eine gezielte Wanderungsbewegung der Leukozyten in Richtung Entzündungsherd erfolgen.

1.3.4 Verwendete Blutreinigungssysteme in der Sepsistherapie

Prinzipiell zielen Blutreinigungsverfahren auf eine Entfernung von Entzündungsmediatoren aus dem Blut ab. Die Verfahren, welche zum Einsatz kommen, lassen sich in Diffusions- und Konvektionsverfahren und in Adsorptionsverfahren unterteilen.

1.3.4.1 Diffusions- und Konvektionsverfahren

Zu den Diffusions- und Konvektionsverfahren zählen neben Hämodialyse, Hämofiltration und Hämodiafiltration die Hochvolumen Hämofiltration, welche mit dem Ziel entwickelt wurde, die Entfernung von Entzündungsmediatoren zu optimieren [66].

a) High cut off Hämodialyse (HCO-HD)

Hier wird nach dem Prinzip des Konzentrationsausgleichs kleinmolekularer Substanzen zweier Flüssigkeiten verfahren, die durch eine semipermeable Membran getrennt sind (Abbildung 7). Blut und Dialysat sind durch eine Filtermembran getrennt. Die poröse semipermeable Filtermembran besitzt die Eigenschaften kleine, wasserlösliche Moleküle, Elektrolyte und harnpflichtige Substanzen durchzulassen, aber große Moleküle wie Eiweiße und Blutzellen zurückzuhalten. Niedermolekulare Substanzen werden aus dem Blut durch Konzentrationsgradienten (Diffusion) durch die Membran auf die andere Filterseite in die Dialyselösung (Dialysat) gefördert und dadurch entfernt. Da die Zielsubstanzen in der extrakorporalen Sepsistherapie nieder- bis mittelmolekulare Proteine mit einem Molekulargewicht zwischen 5 und 60 kDa sind, ist man dazu übergegangen, großporigere Membranen (high cut off) mit einem molekularen cut off von 40 – 100 kDa zu verwenden [67]. Beispiele für HCO-Filter, welche in der Klinik verwendet werden, sind SepteX von Gambro (Stockholm, Schweden) und EMiC2 von Fresenius Medical Care (Bad Homburg, Deutschland).

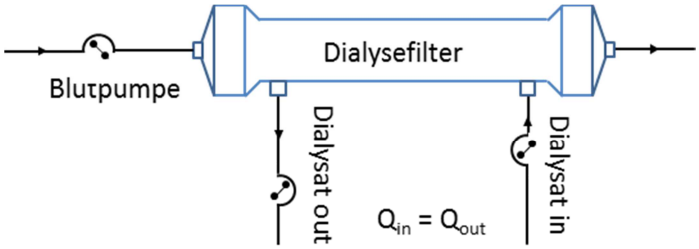

Abbildung 7: Schematischer Aufbau einer Hämodialyse.

b) High cut off Hämofiltration (HCO-HF)

Die Hämofiltration ähnelt vom Aufbau der Hämodialyse. Die Blutreinigung erfolgt nicht wie bei der Hämodialyse über den Stoffaustausch (Diffusion) mit einer Spüllösung (Dialysat) sondern mittels Konvektion. Dabei wird über eine Pumpe ein Druckgradient an der Filtermembran erzeugt, der zu einem Flüssigkeitsentzug (Ultrafiltration) aus dem Blut über die großporige Membran führt. Das heißt bei der Hämofiltration erfolgt die Entfernung von Zielsubstanzen nicht über ein Konzentrationsgefälle (Blut – Dialysat), sondern über ein Druckgefälle. Als Ersatz für die entzogene Flüssigkeit wird dem extrakorporalen Kreislauf eine physiologische Elektrolytlösung (Substituat) zugeführt (Abbildung 8). Die Substitution kann vor oder nach dem Filter erfolgen (Prädilution bzw. Postdilution). Die Hämofiltration weist gewisse Vorteile gegenüber anderen Dialysemethoden auf. Vor allem bei der Entfernung von Zytokinen (8 bis 52 kDa) ist die konvektive Entfernung effizienter als die Diffusion [7, 68]. Außerdem besitzen Hämofilter auch adsorptive Eigenschaften, welche die Entfernung von Mediatoren mit einem größeren Molekulargewicht als der Membran-

Cut-off ermöglicht. Ein Beispiel solch eines Filters ist der von der Firma Gambro entwickelte oXiris™ Dialysator.

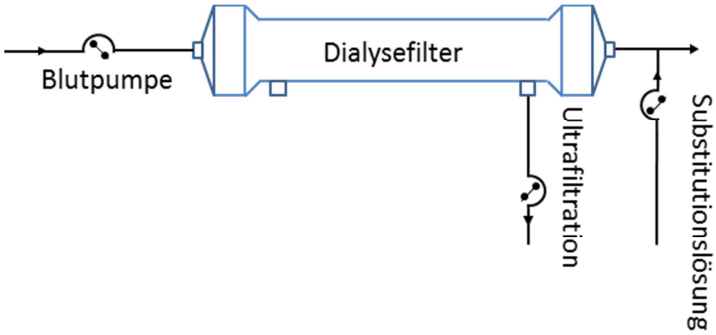

Abbildung 8: Schematischer Aufbau einer Hämofiltration (Postdilution).

c) Hämodiafiltration (HDF)

Die Hämodiafiltration kombiniert die Vorteile der Dialyse mit den Vorteilen der Filtration (Abbildung 7). Bei diesem Verfahren wird ein Dialysatfluss erzeugt, der dafür verantwortlich ist, niedermolekulare Substanzen (Ammonium, Harnstoff, Kreatinin) durch Diffusion rasch aus dem Blut zu entfernen, wogegen die Ultrafiltration für die Entfernung von Molekülen mit mittlerer Molekülmasse verantwortlich ist.

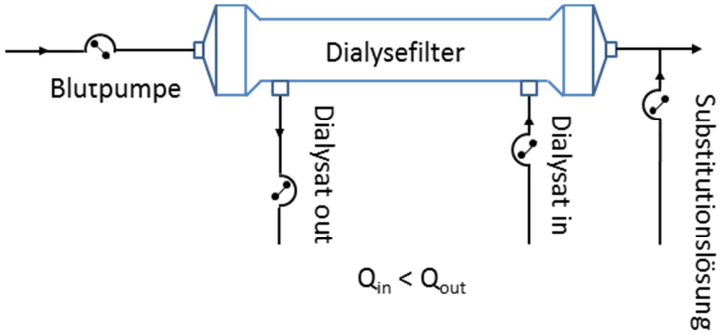

Abbildung 9: Schematischer Aufbau einer Hämodiafiltration.

d) Hoch Volumen Hämofiltration (HVHF)

Diese Art von Hämofiltration wurde 2002 von der Acute Dialysis Quality Initiative Arbeitsgruppe definiert [69]. Demzufolge ist von einer Hochvolumen Hämofiltration die Rede, wenn die Ultrafiltrationsrate größer als 35 ml/kg/h ist. Im klinischen Alltag betragen die Filtrationsraten jedoch bis zu 120 ml/kg/h [70].

1.3.4.2 Adsorptionsverfahren

Mit dem Ziel, eine effizientere Entfernung von Entzündungsmediatoren zu ermöglichen, wurden Adsorptionsverfahren entwickelt, in denen entweder das Blut direkt (Hämosorption) oder das Plasma nach Abtrennung der Blutzellen (Plasmasorption) mit Adsorbermaterialien in Kontakt gebracht wird. Je nach Art der verwendeten Adsorber lassen sich selektive und spezifische Verfahren unterscheiden. Während erstere eine bestimmte Stoffgruppe auf Grund ähnlicher physikochemischer Eigenschaften entfernen

(z.B. Hydrophobizität), zielen die spezifischen Verfahren auf die Abtrennung definierter einzelner Substanzen ab (meist durch Bindung der Zielmoleküle an Antikörper, die auf Trägerpolymeren immobilisiert sind). Folgende Adsorptionsverfahren zur Sepsistherapie werden bereits klinisch verwendet bzw. wurden in Pilotstudien getestet:

a) Toraymyxin®

Toraymyxin® (Toray Industries, Tokyo, Japan) besteht aus einer Hämoperfusionskapsel, welche mit Polymyxin B (PMB)-immobilisierten Fasern bestückt ist (Abbildung 10**).** Polymyxin, ein zyklisches kationisches Peptid, ist in der Lage, Endotoxine spezifisch zu binden und zu inaktivieren. Die Toraymyxin-Kapsel wird mit einem Blutfluss von 80 bis 120 ml/min durchflossen. Die im Blut befindlichen Endotoxine sollen durch das immobilisierte Polymyxin B gebunden und somit aus der Blutbahn entfernt werden. Als Antikoagulans dient Heparin oder Nafamostat. Ältere klinische Studien [71, 72], welche vor 2015 publiziert wurden, zeigten eine verbesserte Überlebensrate von Sepsis-Patienten nach zwei Toraymyxin-Behandlungen. Dieses positive Ergebnis konnte durch eine großangelegte randomisierte klinische Studie (232 Patienten) [73], bei welcher Toraymyxin® gegen Peritonitis induzierten septischen Shock eingesetzt wurde, nicht bestätigt werden. 2014 wurde von unserer Arbeitsgruppe eine *in vitro* Studie veröffentlicht [74], welche zeigte, dass die Endotoxine im Blut durch die Toraymyxin-Behandlung nicht adsorbiert, sondern durch einen Polymyxin-Release von den immobilisierten Fasern inaktiviert werden. Wenn man Toraymyxin-Fasern mit Blut oder Plasma inkubiert, kann man einen Polymyxin-Serumspiegel von 200 ng/ml nachweisen. Diese Konzentration reicht aus, um die im Blut befindlichen Endotoxine in ihrer immunstimulierenden Aktivität stark zu reduzieren [51].

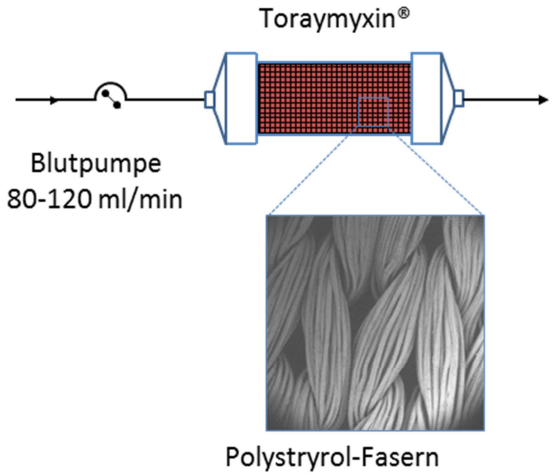

Abbildung 10: Schematische Darstellung der Toraymyxin-Behandlung.

b) Alteco® LPS Adsorber

Der Alteco® LPS Adsorber ist ein Medizinprodukt zur extrakorporalen Entfernung von Endotoxinen aus Vollblut und besteht aus einem Gehäuse, welches mit porösen Polyethylen Platten bestückt ist. Diese sind mit einem synthetischen Peptid beschichtet, welches hohe Affinität zu Endotoxinen besitzt, und haben die Aufgabe, während der Behandlung Endotoxine aus dem Blut adsorptiv zu entfernen. In einigen Fallberichten und

Serien von Patienten mit septischem Schock konnte eine Endotoxinreduktion durch die Adsorberbehandlung erreicht werden [75, 76], jedoch gibt es keine randomisierte kontrollierte Studie zu diesem Medizinprodukt. In einer *in vitro* Studie, in der verschiedene Endotoxinadsorber charakterisiert und verglichen wurden, konnte keine Endotoxinreduktion in Plasma und Vollblut durch die Verwendung von Alteco® LPS Adsorber nachgewiesen werden [74].

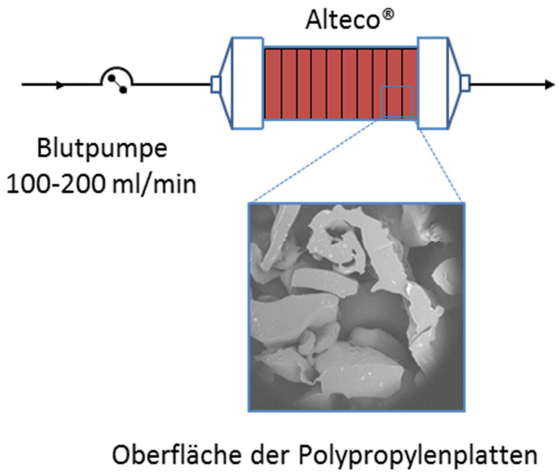

Abbildung 11: Schematische Darstellung der Alteco® LPS Adsorberkartusche. Vollblut wird durch Polypropylenplatten, auf welchen ein synthetisches Peptid immobilisiert ist, gepumpt.

c) Coupled Plasma Filtration and Adsorption (CPFA)

Eine Technik, die Plasmapherese und Nierenersatztherapie kombiniert, ist das Coupled Plasma Filtration Adsorption Verfahren (CPFA). Beim CPFA Verfahren wird das vom Blut abgetrennte Plasma durch eine Adsorberkartusche gepumpt. Der sphärische Adsorber besteht aus Polystyrol-Divinylbenzol und hat eine durchschnittliche Porengröße von 30 nm. Die Aufgabe des porösen Neutralharzes ist es, Entzündungsmediatoren wie TNF-α und Interleukine aus dem Blut adsorptiv zu entfernen. Die nachgeschaltete Hämofiltration ermöglicht eine zusätzliche Entfernung von urämischen Toxinen, welche nicht durch Adsorption entfernt werden. Die empfohlene Behandlungszeit liegt bei 10 Stunden. Als Antikoagulans kann Heparin oder Zitrat verwendet werden. Bis heute wurden einige Pilotstudien mit CPFA bei Sepsispatienten durchgeführt. Diese zeigten zum Teil eine Verbesserung der hämodynamischen Parameter, jedoch konnte die Mortalität durch diese Behandlung nicht gesenkt werden [77, 78].

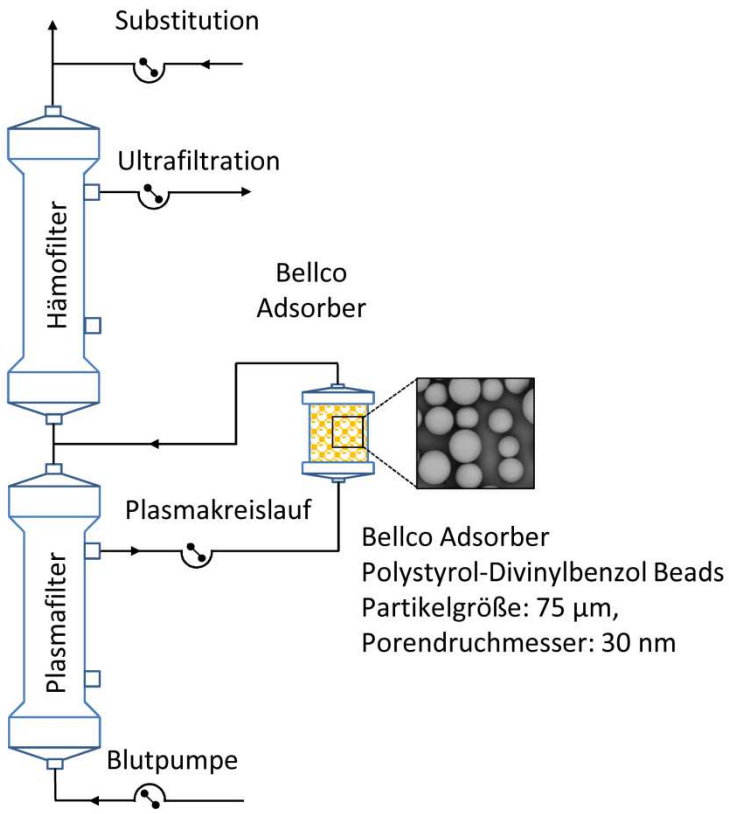

Abbildung 12: Schematische Darstellung des Coupled Plasma Filtration and Adsorption Verfahrens (CPFA). Das Verfahren separiert Plasma und leitet dieses durch eine Adsorptionskartusche. Bevor das Blut in den Patienten gelangt, erfolgt zusätzlich eine Hämofiltration, um urämische Toxine zu entfernen.

d) Cytosorb™

Die CytoSorb™ Technologie (CytoSorbents Corporation, Monmouth Junction, NJ, USA) besteht aus einer 300 ml Adsorberkartusche, welche mit einem Polystyrol-Divinylbenzol-basierten Adsorber befüllt ist. Durch die Beschichtung des Adsorbers mit Polyvinylpyrrolidon ist dieser Adsorber im Vollblut einsetzbar (Abbildung 13). Die Behandlung kann auf jedem Dialysegerät adaptiert und mit konventionellen Blutreinigungsverfahren (Dialyse, Filtration) kombiniert werden. Die Technologie zielt auf eine rasche Eliminierung von pro- und antiinflammatorischen Zytokinen [79] im Blut des Sepsispatienten ab. Eine kontrollierte randomisierte multizentrische Studie wurde 2013 publiziert [80]. Ventilierte Patienten, die die Kriterien für schwere Sepsis und akutes Lungenversagen erfüllten, wurden in diese Studie aufgenommen, in der der Behandlungsstandard mit oder ohne Hämoperfusionstherapie verglichen wurde. Dreiundvierzig Patienten (18 behandelte Patienten, 25 Kontrollgruppe) wurden in diese Studie aufgenommen. Durch die Cytosorb™ Behandlung konnten die Plasmakonzentrationen von IL-6, IL-1ra, IL-8 und von monocyte chemoattractant protein (MCP) signifikant reduziert werden. Die 28- und 60-Tage-Mortalität unterschieden sich nicht signifikant zwischen den beiden untersuchten Gruppen. Eine zweite randomisierte multizentrische Studie für das Indikationsgebiet Sepsis wurde Ende 2016 abgeschlossen und ist noch nicht publiziert.

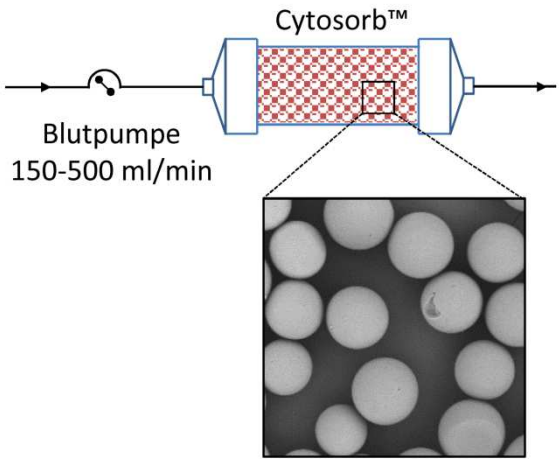

Abbildung 13: Schematische Darstellung der Cytosorb™ Behandlung.

1.3.5 Derzeitige Rolle der extrakorporalen Blutreinigung bei der Sepsis-Behandlung

Die geeignetste und universell einsetzbare Blutreinigungsstrategie für die Behandlung der Sepsis ist trotz großer klinischer Anstrengungen noch nicht gefunden. Ein besseres Verständnis der Wirkungsweise dieser Therapien durch Modulation der zytotoxischen und zytokinetischen Effekte von Entzündungsmediatoren ist unerlässlich. Konvektion, Diffusion und Adsorption sind bei der Entfernung von Zieltargets in der Sepsistherapie nicht als konkurrierende, sondern als komplementäre Mechanismen in der Blutreinigung zu sehen. Viele experimentelle und klinische Studien haben vielversprechende Ergebnisse gezeigt, nämlich dass Blutreinigungs-Therapien gut verträglich sind, Entzündungsmediatoren und/oder Endotoxine aus dem Plasma entfernt werden können und für die Verbesserung verschiedener physiologischer Parameter (Hämodynamik und Oxygenierung) verantwortlich sind. Wichtige Fragen wie Zeitpunkt, Dauer und Häufigkeit dieser Therapien im klinischen Umfeld bleiben jedoch unbeantwortet. Große multizentrische Studien, in denen die Verwendung dieser extrakorporalen Therapien zur Verbesserung der klinischen Ergebnisse (d.h. der Mortalität oder des Organversagens) untersucht werden, anstatt sich auf Surrogat Marker wie die Zytokin-Clearance oder die vorübergehende Verbesserung physiologischer Variablen zu konzentrieren, sind erforderlich, um die genaue Rolle der Blutreinigung bei der Behandlung von Sepsis zu definieren.

2. Publikationen

2.1 Peer reviewed Publikationen

2.1.1 Endotoxin-Adsorber in der extrakorporalen Blutreinigung: Erfüllen sie die Erwartungen?

Stephan Harm, Dieter Falkenhagen, Jens Hartmann: *Endotoxin adsorbents in extracorporeal blood purification: Do they fulfill expectations?* Int J Artif Organs. 2014; 37(3):222-32, DOI:10.5301/ijao.5000304

Kurzfassung:

Einleitung/Ziel: Lipopolysaccharide (LPS), auch Endotoxine genannt, bilden die äußere Membran Gram-negativer Bakterien. Im Blut kann LPS schon in sehr geringen Konzentrationen das Immunsystem aktivieren und ist an der Pathogenese von Sepsis und septischem Schock beteiligt. Aufgrund der starken Immun-stimulierenden Eigenschaft von Endotoxinen ist eine effiziente und rasche Entfernung aus dem Blut des Patienten von großer Bedeutung. Ziel dieser Studie war es, kommerziell erhältliche Endotoxin-Adsorber für die Endotoxinentfernung in Pufferlösung, Proteinlösung, Serum und heparinisiertem Plasma zu testen. LPS-Adsorber, welche für diese Studie ausgewählt wurden, waren Toraymyxin® PMX-20R, Alteco® LPS Adsorber, DEAE-Sepharose, Polymyxin B-Agarose und EndoTrap® red. Zusätzlich wurden einige Adsorber im Vollblut getestet.

Material & Methoden: Die Endotoxinentfernung in wässriger Puffer- und Proteinlösung wurde mit Hilfe von Fluoreszenz-markiertem LPS (100 ng/ml) überprüft. Die adsorptive Endotoxinreduktion in Serum, Plasma und Vollblut wurde durch Batchtests mit dem limulus amebocyte lysate (LAL) Test bestimmt.

Ergebnisse: Von den untersuchten Materialien zeigte die DEAE-Sepharose die effektivste Endotoxin-Adsorption. DEAE-Sepharose kann jedoch durch ihre geringe Bioverträglichkeit in Blut und Plasma nicht eingesetzt werden. Von den übrigen Adsorbern bewirkten nur PMB-basierende Adsorber eine Endotoxin Reduktion. Jedoch konnte gezeigt werden, dass die Abnahme der LPS-Aktivität durch desorbiertes PMB verursacht wurde, welches Endotoxine bindet und inaktiviert.

Schlussfolgerungen: Keiner der in dieser *in vitro*-Studie untersuchten Adsorber zeigte vielversprechende Eigenschaften, die für einen möglichen Einsatz in der extrakorporalen Blutreinigung sprechen.

Mein Beitrag für diese Publikation:

- Planung der Versuche
- Verfassen der Publikation
- Durchführung der Tests
- Analytik: Fluoreszenzmessungen, Polymyxin-Analytik mittels HPLC
- Datenauswertung

Int J Artif Organs 2014; 37 (00): 000-000 DOI: 10.5301/ijao.5000304

ORIGINAL ARTICLE

Endotoxin adsorbents in extracorporeal blood purification: Do they fulfill expectations?

Stephan Harm, Dieter Falkenhagen, Jens Hartmann

Department for Health Sciences and Biomedicine, Danube University Krems, Krems - Austria

Introduction: *Lipopolysaccharides (LPS) are extremely strong stimulators of inflammatory reactions, act at very low concentrations, and are involved in the pathogenesis of sepsis and septic shock. Because of its toxicity, the efficient removal of endotoxin from patients' blood is very important in clinical medicine. The purpose of this study was to determine the endotoxin adsorption capacities of commercial endotoxin adsorbers for endotoxin removal in buffer solution, protein solution, serum and heparinized plasma; some of these were also characterized in whole blood. The tested LPS adsorbers were Toraymyxin® PMX-20R, Alteco® LPS Adsorber, DEAE-Sepharose, Polymyxin B-Agarose, and EndoTrap® red.*

Methods: *The adsorber materials were tested in buffer and protein solutions spiked with fluorescently labeled LPS (100 ng/ml). Additionally, batch tests with LPS-spiked serum, heparinized plasma and whole blood were performed with an LPS concentration of 5 ng/ml. Additionally, the washing solutions of the two tested Polymyxin B (PMB)-based adsorbers were analyzed for PMB release to determine if the resulting LPS inactivation was caused by PMB leakage.*

Results: *The results show that DEAE-Sepharose was most effective in LPS adsorption. Of the other tested endotoxin removal materials, only the PMB-based adsorbers were able to reduce the LPS activity. However, we were able to show that the reduction in LPS activity was caused by desorbed PMB, which inactivates endotoxins.*

Conclusions: *None of the adsorbents that were tested in this study showed promising results for potential use in extracorporeal blood purification.*

Keywords: *Polymyxin B, Toraymyxin®, Alteco® LPS Adsorber, EndoTrap®, Lipopolysaccharide*

Accepted: January 2, 2014

INTRODUCTION

In recent years, endotoxins, also known as lipopolysaccharides (LPS), have become one of the main targets in diseases that are treated with extracorporeal blood purification. LPS is a major constituent of the outer cell wall of Gram-negative bacteria in particular and it is extremely toxic. Intravenous doses as low as 1 ng/kg body weight per h cause an inflammatory response in humans (1). The LPS molecule typically consists of a hydrophobic domain known as lipid A, a non-repeating "core" oligosaccharide and a distal polysaccharide. LPS is released from the cell wall of growing bacteria or when antibiotics or the complement system destroy bacteria (2). Endotoxins that enter the circulatory system bind to the soluble lipopolysaccharide binding protein (LBP). This complex initiates the inflammatory response by binding to the CD14 membrane protein of monocytes and macrophages, subsequently triggering the production of cytokines via toll-like-receptors (TLRs). LPS activation of TLR4 triggers the biosynthesis of diverse mediators of inflammation, such as TNF-α and IL1-β (3), and activates the production of co-stimulatory molecules

required for the adaptive immune response (4). In mononuclear and endothelial cells, LPS also stimulates tissue factor production (5). As long as this process is limited to a local increase of pro- or anti-inflammatory cytokines, it is a normal response of the patient's immune reaction to pathogens. However, in severe cases, the production of cytokines gets out of control, leading to a more severe inflammatory response, such as SIRS (systemic inflammatory response syndrome) or sepsis. When overproduced systemically in the setting of severe sepsis, various mediators and clotting factors can damage small blood vessels and precipitate Gram-negative septic shock, accompanied by disseminated intravascular coagulation and multiple organ failure (6). In Gram-negative sepsis, the effective removal of endotoxins is essential in order to reduce cytokine production.

Furthermore, although the healthy human body is able to handle large quantities of gut-derived LPS by the reticuloendothelial system (RES), endotoxins play an important role in liver failure. In patients with impaired liver function, endotoxins originating from the patients' intestines can pass the liver due to reduced endotoxin removal via the RES, which can lead to endotoxemia and finally to the symptoms described above (7, 8). Adsorbent-based blood purification includes plasmasorption and hemadsorption or hemoperfusion. Adsorption is defined as the removal of molecules by binding on the surface of a material. The binding of molecules on the surface of adsorbent material occurs by different processes, including hydrophobic interactions, hydrogen binding, electrostatic interactions, covalent bonds, Van der Waals forces or chemical conversion. Because of these often coexistent binding processes, adsorbing materials can target molecules in a non-selective, selective or specific way.

Typical adsorbents used in extracorporeal blood purification devices consist of activated charcoal and charged (ionic) or uncharged resins (hydrophobic resins) (2). There are several vendors who claim that their commercially available products remove endotoxins by adsorption. Some of these are based on immobilized Polymyxin B (PMB). PMB is an antibiotic primarily used for infections caused by multidrug resistant Gram-negative bacteria and is commercially available for parenteral use as the sulphate salt. It is characterized by a heptapeptide ring, a tripeptide side chain and a fatty acid tail. This cyclic, highly cationic decapeptide contains six diaminobutyric acid (Dab) residues and an acyl chain coupled to the N terminus. It is derived from the bacterium Bacillus polymyxa (9). The bactericidal activity of the antibiotic PMB against Gram-negative bacteria is based on its ability to destabilize the outer bacterial wall by direct interaction with the lipid A component of LPS. PMB is also able to break down endotoxin aggregates (9). The model for the action of PMB involves interaction of the positively charged PMB Dab residues and the negatively charged lipid A phosphate groups. This initial electrostatic interaction temporarily stabilizes the LPS-PMB complex and brings the N-terminal fatty acyl chain of the PMB molecule into proximity with the lipid A fatty acyl chains (10). The LPS-PMB complex is very stable and has an association constant (Kb) according to the type of LPS between 1.8×10^{-6} and 2.3×10^{-6} M (11). Knowledge on the pharmacokinetics and pharmacodynamics of Polymyxins is very limited due to the lack of intravenous use in the past 50 years. Clinical use has been limited due to nephrotoxicity and neurotoxicity, but because of increasing numbers of multidrug-resistant Gram-negative pathogens and limited development of newer antimicrobials, PMB is now a therapeutic option for Gram-negative infection (12).

The application of affinity chromatographic sorbents based on PMB ligands is described as a method to remove endotoxins from protein solutions without denaturation and loss of products (13). There are several products available based on PMB immobilized on agarose gel: Detoxi-Gel™ Endotoxin Removing Gel (Thermo Fisher Scientific, Waltham, MA, USA), AffiPrep® Polymyxin Matrix (BioRad, Hercules, California, USA), Polymyxin B agarose (Sigma-Aldrich, St Louis, MO, USA), Endotoxin Affisorbent™ (bioWORLD, Atlanta, Georgia, USA) and others. The manufacturers of these adsorbers claim that the immobilized PMB gel is a stable affinity matrix that resists leaching of the ligand into the medium. *In vitro* animal trials with rats showed that PMB-Sepharose columns removed 94% of a challenge dose of 5 µg of endotoxin (14).

Since 1994, a PMB-based extracorporeal hemoperfusion device, called Toraymyxin® (Toray Medical Co., Chiba, Japan), has been commercially available and it now is approved as a therapeutic device by the health insurance system in Japan (15). It is designed for selective blood purification of endotoxins via direct hemoperfusion. Toraymyxin® is made up of polystyrene-derivative fibers, to which the peptide is covalently bound on the surface of an insoluble carrier material inside the cartridge. PMB is covalently bound at a weight ratio of 0.5% w/w (16). Direct hemoperfusion using such a PMB-immobilized fiber column

(PMX-F) has been tested and used for about 15 years for the treatment of septic shock (17). Direct hemoperfusion with PMX-F can be applied to patients with endotoxemia or suspected Gram-negative infection who fulfil the conditions of systemic inflammatory response syndrome and have septic shock requiring vasoactive agents. Several studies have demonstrated efficient removal of endotoxin with PMX-F as well as suppression of *Staphylococcus aureus* lipoteichoic acid-induced TNF-α production. Since 1994, adsorption techniques using Toraymyxin® cartridges have been successfully used in clinical practice in Japan in more than 60 000 patients with severe sepsis (18).

Since endotoxins are negatively charged, anion exchangers are used for their adsorption. Diethylaminoethyl-Sepharose (DEAE-S) is a positively charged resin based on tertiary amine functional groups and is used in ion exchange chromatography. Through its positively charged side groups, it is able to bind negatively charged molecules like nucleic acids, proteins and others. Boos et al reported that the DEAE-cellulose adsorber (H.E.L.P. Heparin Adsorber, B. Braun Melsungen, Melsungen, Germany) used in the H.E.L.P. system also binds LPS in plasma with high affinity and capacity at physiological pH (19). Clinical results of a pilot study where patients with an endotoxin level ≥0.3 EU/ml were treated for 13 days with the DEAE-cellulose adsorber showed a slow reduction in LPS and interleukin-6 levels (20).

Launched in 2006, the Alteco® LPS adsorber (CE 0088; Alteco Medical AB, Lund, Sweden) is an adsorption column consisting of 20 porous polyethylene slabs with a total surface area of 3.3 m^2 and with a priming volume of 20 mL. On these slabs, a special cationic peptide, HAE 27, is immobilized, which selectively binds and adsorbs LPS in a recommended single two-hour treatment with a blood flow of 100 ml/min to 200 ml/min (21). The adsorption capacity of the adsorber is 7 mg to 8 mg LPS, corresponding to 10 times the amount of LPS in the plasma of a patient with a LPS concentration of 1 EU/ml (22). *In vitro* tests have shown that the adsorber reduces LPS levels from 1 EU/ml in 5000 mL bovine blood to less than 0.05 EU/ml during recirculation for 60 min at a flow rate of 100 ml/min (23). In an initial case study of severe abdominal sepsis, blood endotoxin was almost eliminated, i.e. from 1.44 EU/ml before treatment to 0.03 EU/ml post treatment, with a concurrent reduction in inflammatory cytokines (22). However, no clinical studies with sufficient patient numbers have yet been performed in order to provide clear and convincing data regarding removal of endotoxins or reduction of mortality.

EndoTrap®, developed by Profos AG (Regensburg, Germany), is an affinity chromatography resin which is intended for the efficient removal of bacterial endotoxins from aqueous solutions containing low- or high-molecular weight substances such as proteins and nucleic acids. The EndoTrap® adsorber consists of a special bacteriophage protein immobilized on spherical Sepharose beads with a mean diameter of 90 µm. This bacteriophage protein has a high affinity for lipopolysaccharide molecules. EndoTrap® can be applied either in column or batch mode, by gravity flow or on fully automated liquid chromatography systems. The binding capacity of EndoTrap® is approximately 2×10^6 EU per mL (data from the manufacturer).

The present study was conducted to evaluate the efficacy of different commercial endotoxin adsorbers for endotoxin removal in buffer solution, protein solution, serum, and heparinized plasma. Some of these were further studied using whole blood.

MATERIALS AND METHODS

A Toraymyxin® PMX-20R cartridge was purchased from GEPA-MED Medizintechnik GmbH (Vienna, Austria). The Alteco® LPS Adsorber was obtained from Blood Interventional Technologies MEDICAL (Vienna, Austria). Endotoxin removal EndoTrap® red columns were purchased from Hyglos GmbH (Bernried, Germany). DEAE-Sepharose with a molecular cutoff of 4000 kDa, Polymyxin B-Agarose, lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* and fluorescein isothiocyanate-labeled LPS (FITC-LPS) from *Escherichia coli* O55:B5 were acquired from Sigma-Aldrich (St Louis, MO, USA). Human serum albumin solution (200 g/l) was purchased from Pharmosan (Vienna, Austria). A porous adsorber (CG161) consisting of polystyrene divinylbenzene was obtained from Rohm and Haas (Philadelphia, PA, USA).

Adsorber washing

All washing steps were done under sterile conditions and only sterile wash solutions were used. DEAE-cellulose and EndoTrap® red adsorber were washed three times with 0.9% sodium chloride solution (10 mL saline solution per gram of adsorber). The EndoTrap® red adsorber was additionally washed three times with an equilibration buffer, which was included in the EndoTrap® red adsorber kit, based on

HEPES buffer at pH 7.5. After washing, the adsorbers were stored in 50% suspension (v/v) in 0.9% NaCl solution at 4°C before they were used for the following batch test. The Toraymyxin® fibers were aliquoted under sterile conditions into 2 g pieces and stored in 15 mL sterile tubes (Greiner). To ensure that all non-covalently bound Polymyxin B was removed from the PMB-based adsorbers (Toray fibers, PMB-Agarose), they were washed as followed: ten times with saline solution, followed by five times with 0.1 N hydrochloric acid, twice with phosphate buffer (pH 7.2) and finally once with saline solution. Each washing step was performed with 10 mL of washing solution per gram of fiber or per mL of PMB-Agarose beads for 10 min on a shaker. The supernatant after each washing step was collected for PMB quantification via the HPLC method. The Alteco® LPS Adsorber was cut into small strips using a scalpel and washed three times with saline solution before use.

LPS adsorption batch test in buffer and protein solution

Fluorescently-labeled LPS was incubated with the five endotoxin adsorbers as follows: FITC-LPS was diluted in 10 mM PBS buffer (pH 7.2) or 4% HSA solution in 10 mM PBS buffer to yield a final FITC-LPS concentration of 100 ng/ml. To this, 100 µL of PMB-Agarose, EndoTrap® red adsorber or DEAE-Sepharose beads or in the case of Alteco® LPS Adsorber and Toraymyxin® fibers 100 mg (dry weight) was added to each 1 mL aliquot and samples of each adsorber were placed on a shaker shielded from light for 1 h. At the end of the incubation period, beads were removed from the corresponding sample and the FITC-LPS concentration of each sample was determined by a spectrofluorometric detector (RF-551; Shimadzu, Kyoto, Japan) working with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. A control without the adsorber and a standard curve of FITC-LPS in water and in a protein solution were required for FITC-LPS quantification.

LPS adsorption batch test in serum, plasma and blood

To assess the efficiency of the different LPS adsorbers for clinical use, the same batch tests described above, with some changes, were performed using fresh human plasma and serum (to avoid the influence of anticoagulants on LPS adsorption). Each adsorber material (200 µL DEAE-cellulose, EndoTrap® red-adsorber or PMB-Agarose beads, 200 mg dry weight of the Alteco® LPS Adsorber or Toraymyxin® fibers) was incubated with 2 mL of heparinized plasma (10 IU) or serum, then spiked with LPS from *Pseudomonas aeruginosa* to yield a final LPS concentration of 5 ng/ml.
To circumvent LPS inactivation by desorbed PMB from the PMB-based adsorbers, a batch test was done where 1% (v/v) porous polystyrene divinylbenzene beads (CG161) were added. The CG161 adsorber material is able to adsorb PMB very efficiently, but not LPS.
To verify LPS inactivation by PMB release from the PMB-based adsorber materials, an additional batch test was done in which 10% (w/v) of the different washed Toray fibers or 10% (v/v) of different washed PMB-Agarose was incubated in LPS spiked serum for 60 min. Additionally, LPS-free serum, incubated with Toray fibers for 60 min, was spiked with 5 ng/ml LPS after the fibers were removed. As control, LPS-spiked serum was used which had not been in contact with any adsorber material.
Only the adsorbents which offer blood compatibility, namely the two hemoperfusion adsorbers (Alteco® LPS Adsorber, Toraymyxin®), were tested in whole blood (50 mg adsorber per mL blood) with a final LPS concentration of 3 ng/ml.
The incubation of all batch tests was performed on a roller mixer at 37°C for 1 h. To determine the amount of adsorbed LPS, a Limulus Amoebocyte Lysate test (LAL) from Charles River (Wilmington, MA, USA) for endotoxin quantification was used and PMB quantification in serum and plasma was done by a PMB ELISA. To verify the PMB concentration required for LPS inactivation, fresh human heparinized plasma with 5 ng/ml or 0.5 ng/ml LPS from *Pseudomonas aeruginosa* was incubated with different PMB concentrations (0, 10, 100, 250, 500 and 1000 ng/ml) for 60 min at 37°C. The batch test was performed in pyrogen-free 3 mL glass vials and the LPS activity was measured using the LAL test.

PMB-Quantification

The concentration of PMB in the aqueous washing solutions was determined by a sensitive, high-performance liquid chromatographic method (24), with minor changes. 2-mL samples (washing solution) were transferred to a solid-phase extraction C18 cartridge (Sep-Pak, Waters). After the cartridge was washed with 500 µL of carbonate buffer (1%, w/w, pH 10), 110 µL of 9-fluorenylmethyl

chloroformate (FMOC-Cl, Sigma) solution (containing 30 µL of 100 mM FMOC-Cl in acetonitrile and 80 µL of methanol) was added. Following 10 min of reaction in the dark, the PMB derivatives were eluted with 900 µL of acetone. The eluate was mixed with 600 µL of boric acid (0.20 M) and 500 µL of acetonitrile. After vortex mixing, 20 µL of the eluate was injected onto the HPLC column (50 × 4.6mm Onyx Monolithic C18 column coupled with a 4 × 3.0 mmC18 guard column; Phenomenex, Torrance, CA, USA). The mobile phase with a flow rate of 1 ml/min was acetonitrile-tetrahydrofuran-water (50:25:25) and the run time was 10 min. Fluorescence detection was performed at an excitation wavelength of 260 nm and an emission wavelength of 315 nm. The concentrations of PMB were calculated on the basis of the sum of the chromatographic peak areas of Polymyxin B1 and B2 in the HPLC assay. The limit of quantification was 0.025 mg/l. The quantification of PMB in blood, plasma and serum was measured using a competitive enzyme immunoassay kit for the analysis of Polymyxin B and E (Kwinbon Biotech; Beijing; China) with a detection limit of 1 ng/ml. To obtain a signal in the standard range between 0 and 81 ng/ml, the samples were diluted with dilution reagent, which was provided with the ELISA kit.

Statistics

All experiments were done thrice and data are expressed as mean ± SD which was calculated using Microsoft Excel 2010.

RESULTS

PMB quantification in washing solutions

To remove the non-covalently bound PMB from the Toray fibers and PMB-Agarose beads, we incubated them ten times in 0.9% NaCl followed by five times in 0.1 N HCl solution. After washing the PMB-based adsorbers, the washing solutions were collected and stored at 4°C until we performed PMB quantification by HPLC. Following frequent washing of the Toray fibers with physiological sodium chloride solution, we detected 102 ± 8 ng PMB/ml in the first and 4 ± 0.5 ng PMB/ml in the fourth collected sample. Using 0.1 N HCl solution, we found 215 ± 22 ng PMB/ml after the first and 42 ± 12 ng PMB/ml after the fifth washing step. By quantification of the washing solutions with which PMB-Agarose beads were treated, in saline solution, we found 2534 ± 139 ng/ml in the first and 20 ± 6 ng/ml in the third wash solution. In 0.1 N HCl solution, we found 329 ± 149 ng/ml in the first and 27 ± 6 ng/ml in the fourth washing solution (Fig. 1).

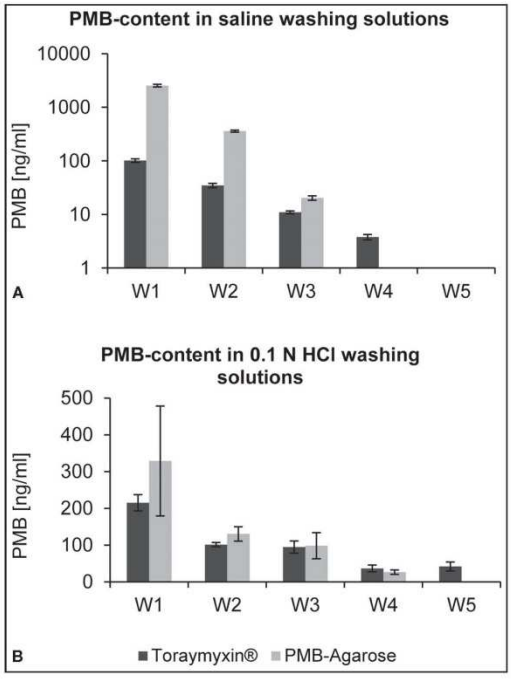

Fig. 1 - *PMB content in washing solutions of PMB-based adsorbers measured by HPLC. W1 - W5 = washing solutions 1 to 5. Adsorbers were first washed with 0.9% NaCl solution* ***(A)*** *and then with 0.1 N HCl solution* ***(B)****.*

LPS adsorption in buffer and protein solution

To determine the applicability of the endotoxin adsorbers for LPS reduction in buffer and protein solution, a batch test with 100 ng/ml of fluorescein-labeled LPS was prepared in phosphate buffer (10 mM, pH 7.2) and 4% HSA solution buffered with PBS (10 mM) to pH 7.2. After 60 min of incubation time, the samples were analyzed using a fluorescence detector. The results show (Fig. 2) that the ionic-based adsorber, DEAE-Sepharose, had the greatest effect on LPS

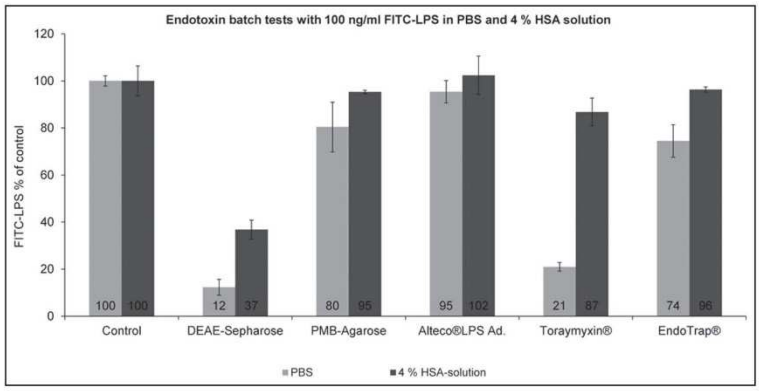

Fig. 2 - *Direct comparison of fluorescently-labeled LPS adsorption by endotoxin adsorbers in 10 mM PBS buffer and in 4% (w/v) HSA solution. The batch tests were performed using 10% adsorber in a 100 ng FITC-LPS per mL solution.*

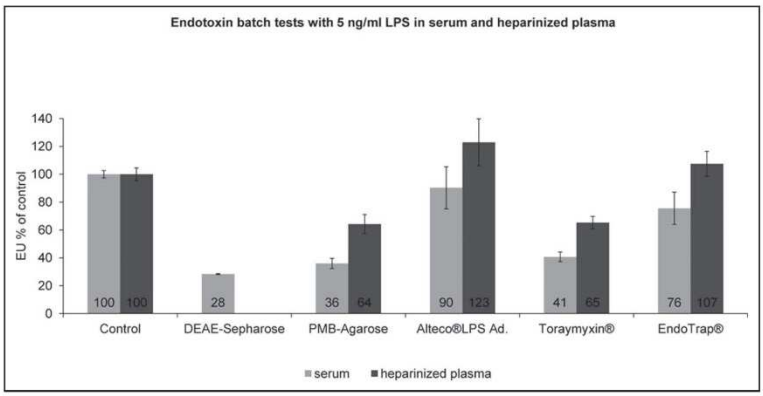

Fig. 3 - *Reduction of LPS activity by endotoxin adsorber in serum and heparinized plasma. The batch tests were performed using 10% adsorber in 5 ng LPS (Pseudomonas aeruginosa) per mL of spiked serum or plasma.*

reduction. Using DEAE-Sepharose, the LPS level decreased in the buffer solution to 18 ± 8.5% and in the HSA-solution to 37 ± 4% related to the control without the adsorber. Of the other tested adsorbers, only the Toray fibers were able to reduce LPS activity in the buffer solution to 21 ± 2% and in the HSA solution to 87 ± 6% in comparison to the control without the adsorber.

LPS adsorption batch test in serum, plasma and blood

The LPS adsorption ability of the different adsorbents was assessed by incubating the adsorbents in freshly drawn plasma and serum. Serum was used to exclude the effect of anticoagulants on LPS adsorption since especially DEAE-Sepharose effectively binds heparin, which leads to plasma clotting. After 1 h of incubation, we compared the LAL activity of plasma and serum with the adsorber to plasma or serum without the adsorber, which was our positive control. The results (Fig. 3) show that DEAE-Sepharose reduced the LPS level in serum most efficiently (28 ± 0.8%) compared to the control. With Toray fibers, we could decrease the LAL activity to 41 ± 3.5%, with PMB-Agarose to 36 ± 3.6% and with the EndoTrap® red adsorber to 76 ± 11.5% related to the control serum without an adsorber. The Alteco® LPS Adsorber showed poor LPS reduction in our tests (90 ± 15%). When we tested the LPS adsorption ability in heparinized plasma, the two PMB-based adsorbers showed similar activity. The Toray fibers reduced the LPS level to 65 ± 4.5% and the PMB-Agarose beads to 64 ± 6.8%. Alteco® LPS Adsorber and EndoTrap® red were not able to decrease the LPS concentration in heparinized plasma. DEAE-Sepharose

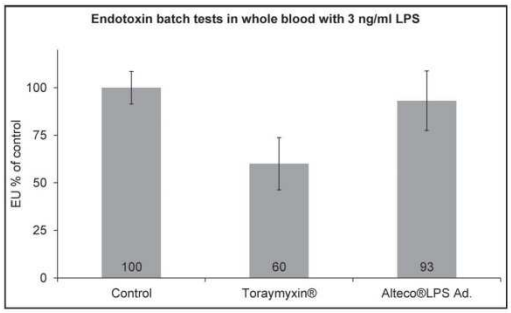

Fig. 4 - *Comparison of the two hemoperfusion adsorbers (Toraymyxin® and Alteco® LPS Adsorber) in whole blood via batch test using 5% (w/v) adsorber with a final LPS concentration of 3 ng/ml.*

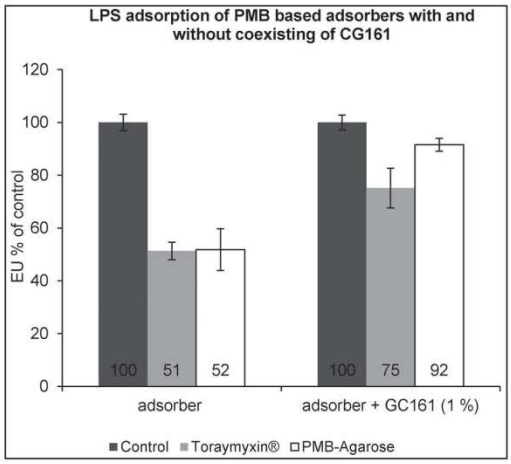

Fig. 5 - *To prove if LPS inactivation is a function of PMB release from Toray fibers and PMB-agarose beads, additional batch tests in LPS spiked plasma with and without the presence of 1% CG161 were performed. CG161, a porous polystyrene divinylbenzene-based adsorber, is able to adsorb PMB by hydrophobic interactions. The influence on PMB desorption was measured by the LAL test.*

was not tested in heparinized plasma because heparin is removed by ionic interaction of the anion exchanger and causes plasma clotting.

After testing the two hemoperfusion adsorbers, Toraymyxin® and Alteco® LPS Adsorber, in whole blood, only the Toray fibers showed an acceptable reduction in LPS activity to 60 ± 14% (Fig. 4).

LPS inactivation by PMB release from the PMB-based adsorbers

Since the PMB molecule has amphiphilic properties, it can be bound by hydrophobic forces onto hydrophobic surfaces like polystyrene matrix. One percent (v/v) of a polystyrene divinylbenzene adsorber (CG161) with an average particle size of 120 µm was added to the PMB-based adsorber in a batch test to capture the released PMB and to show if LPS inactivation was mainly mediated by actual adsorption to the Toray fibers and PMB agarose or was instead a function of PMB release. The results show (Fig. 5) that the LPS adsorption by the PMB-based adsorber decreased after adding a hydrophobic adsorber material which eliminates free PMB molecules in plasma. The LAL activity increased from 51 ± 3.5 to 75 ± 7.5% by adding CG161 to the Toray fibers and from 52 ± 8 to 92 ± 2.5% for the PMB-Agarose compared to the control without an adsorber. CG161 itself did not have an effect on the LPS reduction in plasma, which was separately tested (data are not shown).

To verify LPS inactivation by PMB release from the PMB-based adsorbers, a separate batch test was performed. In this test, in addition to the adsorbers which were tested in LPS spiked serum, a serum extract of the two PMB-based adsorbers was prepared. Serum was incubated with the adsorber material for 60 min. After removing the adsorber, the serum was spiked with 5 ng/ml LPS and LAL activity was compared to LPS spiked serum which was not in contact with the adsorber. The results (Fig. 6) show that serum which was exposed to the PMB-based adsorber had a decreased LPS level. The serum extract of Toray fibers reduced the endotoxin level to 81 ± 3% and the serum extract of PMB-Agarose to 52 ± 12% compared to the control. Additional washing of the adsorbers with 0.1 N HCl caused a reduction in LPS inactivation from 67± 8% to 86 ± 9%, but only in the case of Toray fibers.

LPS inactivation as a function of PMB concentration

To assess which PMB concentration in plasma is required to decrease the LAL activity to a certain point, LPS inactivation in plasma was measured as a function of the PMB concentration. To set the LAL activity to half

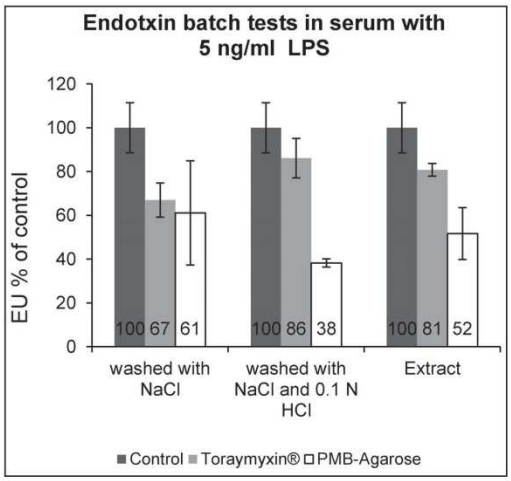

Fig. 6 - *Direct comparison of LPS inactivation in serum by different washed PMB-based adsorbers and an extract of these.*

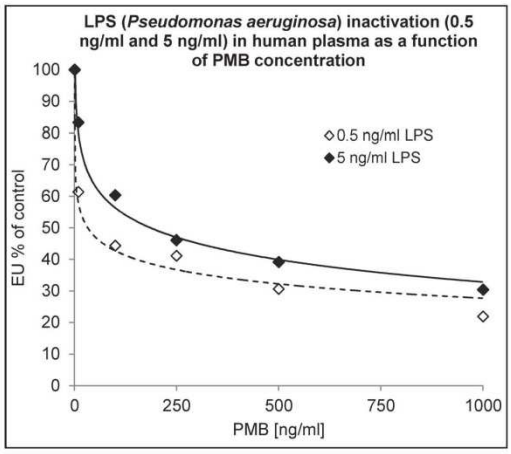

Fig. 7 - *Plasma with 5 ng/ml (open squares) and 0.5 ng/ml (**filled squares**) LPS was incubated with different PMB concentrations ranging from 10 to 1000 ng/ml to verify the effect of LPS inactivation as a function of the PMB content.*

of the control level (LPS without PMB), a PMB dose of 100 ng/ml for 0.5 ng/ml LPS and 250 ng/ml for 5 ng/ml LPS from *Pseudomonas aeruginosa* was required (Fig. 7). These two PMB concentrations are in the range of PMB release of the two tested PMB-based adsorbers in plasma.

PMB quantification in batch test serum

To verify the amount of desorbed PMB in serum, a competitive enzyme immunoassay kit (Beijing Kwinbon Biotechnology, Zhongguancun , China) was used for the analysis of Polymyxin B and E with a detection limit of 1 ng/ml.

The desorption batch tests in serum were carried out with different washed Toray fibers and PMB-Agarose. They were tested untreated, washed with physiological sodium chloride solution and additionally washed with 0.1 N hydrochloric acid solution. Serum which was incubated with the unwashed adsorber lead to 918 ± 154 ng/ml PMB release using Toray fibers and 1891 ± 29 ng/ml PMB using PMB-Agarose. When serum was exposed to the washed adsorbers, the PMB release decreased to 225 ± 23 ng/ml for Toray fibers and 168 ± 11 ng/ml for PMB-Agarose. The PMB quantification showed that further washing steps of Toray fibers with 0.1 N HCl solution resulted in lower PMB release (118 ± 6 ng/ml), but preventing PMB desorption into serum could not be achieved by these washing procedures (Fig. 8).

By coincubating a PMB-based adsorber and 1% (v/v) hydrophobic CG161 adsorber in LPS spiked plasma, we could observe lower PMB concentrations in the plasma samples. In the case of Toray fibers, the coexisting CG161 reduced the PMB content in plasma from 109 ± 45 ng/ml to 20 ± 3 ng/ml and with PMB-Agarose from 165 ± 8.5 ng/ml to 12.5 ± 6 ng/ml (Fig. 9).

DISCUSSION

For endotoxin removal from protein-free solutions, anion exchangers are used, such as anion exchange polymeric matrices, since endotoxins exhibit net-negative charges because of their phosphate groups originating from lipid A (26). The adsorbing capacity of diethylaminoethyl (DEAE)-Sepharose was the best compared to the other tested adsorber materials. The disadvantage of using DEAE functionalized polymers is the non-specific removal of all negatively charged proteins and molecules like serum albumin, protein C, heparin and others. Using DEAE-Sepharose in

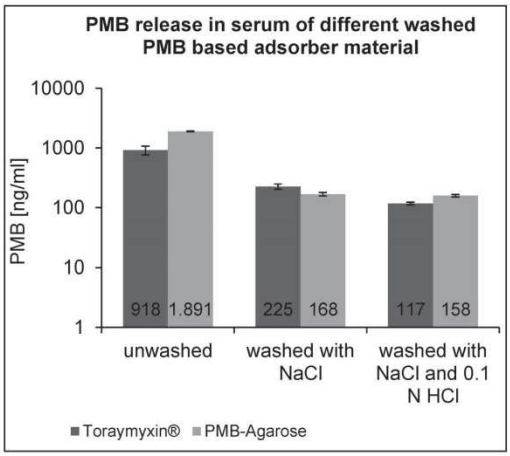

Fig. 8 - *Determination of desorbed PMB in serum from PMB-based adsorbers. PMB release was tested with unwashed material, material washed with physiological sodium chloride solution, and additionally with 0.1 N hydrochloric acid solution. The PMB content in serum was quantified using a competitive enzyme immunoassay kit for the analysis of Polymyxin B and E with a detection limit of 1 ng/ml.*

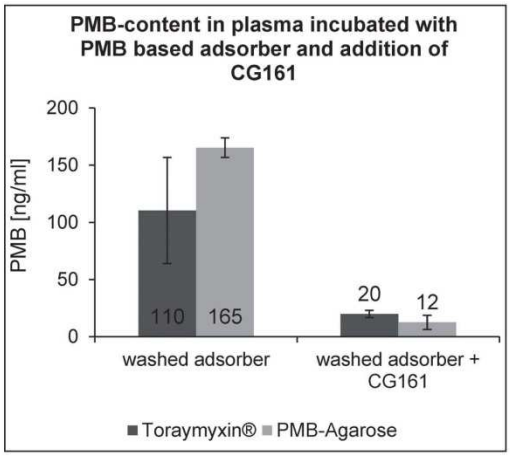

Fig. 9 - *Effect of PMB content in plasma which was incubated with PMB-based adsorbers with and without the presence of CG161, a porous polystyrene divinylbenzene-based adsorber which is able to adsorb PMB via hydrophobic interactions.*

heparinized plasma leads to clotting by eliminating heparin via adsorption onto the cationic surface. This was the reason why we could not test the LPS reduction in plasma by DEAE-Sepharose. Furthermore, it is well-known that natural polymers like Sepharose and cellulose, carrying surface hydroxyl groups, strongly activate the complement system through the alternative pathway (25). As a result, anion-exchangers, such as DEAE-Sepharose, are effective LPS adsorbers but are not able to selectively adsorb endotoxins from acidic protein-containing solutions. Therefore, they are not applicable for endotoxin removal in extracorporeal blood purification because of poor biocompatibility.

Of the two tested PMB-based adsorbers, only Toray fibers showed efficient reduction of LPS activity in PBS buffer. This can be explained by the strong hydrophobic interactions between LPS and the hydrophobic resin in aqueous solutions. The Toray fibers showed only low affinity for LPS, when using 100 ng/ml FITC labeled LPS in a protein solution containing 4 mg/ml human serum albumin. The PMB-based adsorbers were able to reduce the LPS activity in plasma to about 60% of the starting concentration. However, we showed that preliminary treatment of Toray fibers with 0.1 N HCl solution led to lower PMB release and decreased LPS inactivation. Furthermore, we were able to show that a serum extract of the two PMB-based adsorber materials had a similar effect on LPS reduction compared to serum incubation with these adsorbers. By determining the PMB concentration-dependent decrease in LPS activity, in plasma we found that a PMB dose of 100 ng/ml for 0.5 ng/ml LPS and a dose of 250 ng/ml for 5 ng/ml of LPS from *Pseudomonas aeruginosa* was required to set the LAL activity to half the level seen with the control serum without PMB. These PMB concentrations were in the range of PMB release by Toray fibers and PMB-Agarose. Following coincubation with 1% polystyrene particles (CG161) and PMB-based adsorbers in LPS spiked plasma, the PMB level in plasma was much lower due to the fact that released PMB was captured by the polystyrene matrix via hydrophobic forces. However, the decrease in PMB in plasma led to an increase in the LPS activity shown by the LAL assay. Taken together, these results suggest that the main effect of the tested PMB-based adsorbers is LPS inactivation caused by PMB release into the sample solution. Therefore, LPS inactivation depends on the amount of PMB that is desorbed from the adsorber material.

The structure of PMB in aqueous solution is well known, both in the free and in LPS-bound states, using nuclear magnetic resonance (NMR) spectroscopy (10). On the

basis of the NMR structure of PMB when bound to LPS, a molecular model of the PMB-LPS complex was constructed. The modeling process took into account the electrostatic interactions between the Dab side chains and lipid A phosphates and maximized the reduction of solvent-exposed hydrophobic area on both molecules. The model implied that the complex is stabilized by a combination of electrostatic and hydrophobic interactions (10). A simple reason why covalently immobilized PMB is not as effective as free PMB could be that this conformational change in PMB cannot take place due to steric hindrance.

Furthermore, it is known that LPS molecules form large aggregates, also called micelles, which have high molar masses of more than 10^6 Da in aqueous environments. The size of these micelles depends on conditions such as temperature, pH, hydrophilicity and the presence of mono- or divalent cations (27). The LPS molecule inserts into the micelle via integration of the acyl chains into the micelle interior, and the polysaccharide portions are fully exposed to the aqueous compartment (27). As a result, the area of LPS that binds to PMB, the lipid A part, is shielded by the polysaccharide chains of LPS which face outwards from the micelle. This is an additional explanation as to why immobilized PMB is not able to come into contact with the lipid A of LPS, so it has no LPS inhibition or LPS adsorption effect.

When using EndoTrap® red in batch mode, according to the manufacturer's instructions, we could only see an effect on endotoxin removal in buffer solution. In human serum albumin solution, human serum and heparinized plasma, we did not observe a reduction in endotoxin levels. A possible explanation could be that this adsorber works only in solutions with endotoxin levels above 100 EU/ml, which was not tested in this work.

The Alteco® LPS adsorber consists of a cartridge filled with porous plates of polyethylene. A tailor-made, non-toxic, non-drug peptide with high affinity for endotoxin is bound to the surface of the porous plates. Although it is claimed that, during hemoperfusion with this adsorber, the cationic part of the peptides captures the negatively charged endotoxin molecules (28), we did not observe endotoxin adsorption by this adsorber in any medium. Moreover, clinical experience with Alteco® LPS adsorber is scarce.

The tests for LPS adsorption were conducted with endotoxins from *E. coli* and *P. aeruginosa*. Most of the vendors of the tested adsorbents claim that the target domain for LPS adsorption is the Lipid A, which is the conserved region of the endotoxin molecule. Therefore, similar results can be expected for LPS from other bacteria.

CONCLUSIONS

It is well-known that endotoxins induce a strong host immune response. Although there is an urgent need for endotoxin elimination not only for the treatment of sepsis, but also for liver failure, endotoxin neutralization studies have been disappointing. The adsorbents we tested in this study did not show effective elimination of LPS in endotoxin containing buffer, protein solution, serum, plasma, and blood. The best quantitative removal of LPS was achieved by DEAE-Sepharose. However, this adsorbent is not applicable for its use in extracorporeal blood purification due to its poor biocompatibility and its strong heparin adsorption. The results of the PMB-based adsorbers, Toraymyxin® and PMB-Agarose, show that the main effect on LPS inactivation of these adsorbers is achieved by the release of PMB, which neutralizes the biological activity of LPS. In reality, there is no effective technology currently available for the removal of endotoxin from human blood. Further activities in the challenging field of endotoxin adsorbent development could focus on the use of endotoxin-binding substances such as lactoferrin or synthetic peptides with endotoxin binding domains which have recently showed promising results (29).

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Conflict of Interest: None.

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2.1.2 Porengröße - eine Schlüsselparameter zur selektiven Toxinentfernung bei der Blutreinigung

Stephan Harm, Dieter Falkenhagen, Jens Hartmann: *Pore size – A key property for selective toxin removal in blood purification*. Int J Artif Organs. 2014; 37(9):668-78, DOI:10.5301/ijao.5000354

Kurzfassung:

Einleitung: Bei akutem Leberversagen kommen Membran- und Adsorptionsbasierte extrakorporale Blutreinigungssysteme zum Einsatz, um Toxine aus dem Blut zu entfernen. Bei Sepsis-Patienten werden solche Systeme verwendet, um Entzündungsmediatoren wie Interleukine und TNF-α aus dem Blut zu reduzieren. Neben den chemischen Eigenschaften spielt vor allem die Porengröße für die Selektivität von Adsorbern eine große Rolle.

Material & Methoden: In dieser Studie wurden drei Adsorber, welche sich nur in der Porengröße unterscheiden, hinsichtlich Adsorption von albumingebundener Lebertoxine, Zytokine und Gerinnungsfaktoren verglichen. Die Adsorber bestehen aus einer hydrophoben Poly(styrol-divinylbenzol)-Copolymer Matrix und haben mittlere Porengrößen von 15, 30 und 100 nm.

Ergebnisse: Poren auf der Adsorberoberfläche wirken als Molekularsieb und verhindern das Durchdringen von Molekülen, die größer als der Porendurchmesser sind. Die Ergebnisse dieser Studie zeigen, dass bei Polystyrol-Divinylbenzol-Copolymer basierten Adsorbern 15 nm Poren für die Zytokin-Entfernung am geeignetsten sind. Hingegen sind für die Entfernung von albumingebundenen Toxinen aus dem Blut Poren zwischen 30 - 40 nm am effektivsten. Sehr große Poren verringern die selektive Wirkung von Adsorbern und führen zu einer unkontrollierten Adsorption von diversen Plasmaproteinen. Dadurch sind diese nicht plasmakompatibel und erfüllen somit nicht die Anforderungen für die Anwendung in der extrakorporalen Blutreinigung.

Schlussfolgerung: Die Blutverträglichkeit, Selektivität und Effizienz von Adsorbern, welche in der Blutreinigung eingesetzt werden, kann durch die richtige Wahl der Porengröße und Porenverteilung verbessert werden.

Mein Beitrag für diese Publikation:

- Planung der Versuche
- Verfassen der Publikation
- Durchführung der Tests
- Analytik: Größenausschlusschromatographie, Messung der Größenverteilung
- Datenauswertung

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ORIGINAL ARTICLE

Pore size – a key property for selective toxin removal in blood purification

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Purpose: *Extracorporeal blood purification systems based on combined membrane/adsorption technologies are used in acute liver failure to replace detoxification as well as to remove inflammatory mediators in sepsis patients. In addition to coating and chemical modification of the surface, pore size significantly controls the selectivity of adsorption materials.*
Methods: *This study addresses the adsorption of albumin bound liver toxins, cytokines, and representative plasma compounds on three adsorbents which differ only in pore size distribution. All three adsorbents are based on hydrophobic poly(styrene-divinylbenzene) copolymer matrices and have mean pore sizes of 15, 30, and 100 nm.*
Results: *The pores of adsorbents act as molecular sieves and prevent the entry of molecules that are larger than their molecular cut-off. The results of this study reveal that adsorbents based on styrene-divinylbenzene polymers with 15 nm pores are suitable for cytokine removal, and the same adsorbents with 30-40 nm pores are the best choice for the removal of albumin-bound toxins in the case of liver failure. Adsorbents with very large pores lack selectivity which leads to uncontrolled adsorption of all plasma proteins. Therefore, hydrophobic adsorbents with large pores offer inadequate plasma compatibility and do not fulfill the requirements for blood purification.*
Conclusions: *Biocompatibility and efficiency of adsorbents used for blood purification can improved by fine tuning of adsorbent surface pore distributions.*

Keywords: *Adsorption, Blood purification, Pore size, Liver failure, Sepsis, Cytokines*

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INTRODUCTION

Blood purification based on adsorption includes plasmasorption and hemoadsorption. Adsorption is defined as the removal of molecules by binding to the surface of a material. The binding of molecules to the surface of adsorbent material occurs by various processes, including hydrophobic interactions, hydrogen binding, electrostatic interactions, covalent binding, or Van der Waals forces. Because these binding processes can occur simultaneously, adsorbing materials can target molecules in a non-selective, selective, or specific way. The selective adsorption of pathological substances by the use of adsorbents using plasma adsorption or direct hemoperfusion has already been applied clinically (1-3). Typical adsorbents used in extracorporeal blood purification devices consist of activated charcoal and charged (ionic) or uncharged (non-ionic) resins (2). In acute liver failure, a range of potentially toxic substances accumulate in the blood stream of the patient. Metabolites associated with liver failure include hydrophobic substances like unconjugated bilirubin, bile acids, phenols, aromatic amino acids, fatty acids, and water-soluble compounds of low molecular mass like ammonium. In the case of hydrophobically bound metabolites, many of these are bound strongly to albumin, which is the predominant carrier protein in plasma. Therefore, the aim of adsorption technologies in liver support is to remove these albumin-bound substances.

Based on their large size, the protein-bound toxins are not able to cross the typical dialysis membranes. Consequently, the removal of protein-bound liver toxins by conventional dialysis techniques is inefficient (4-6). Two current devices in which both water-soluble toxins and protein-bound toxins are removed are the Molecular Adsorbents Recirculating System (MARS®; Gambro, Lund, Sweden) and the Prometheus System (Fresenius Medical Care, Bad Homburg, Germany). MARS uses an intermediate albumin dialysis step to transfer toxins from albumin in the plasma to solid adsorbents (7), while Prometheus directly contacts the adsorbents with fractionated plasma in a closed-loop system (8).

Another promising field of application for adsorption-based technology is the management of sepsis or systemic inflammatory response by removing inflammatory cytokines that have been implicated in their pathogenesis (9). A hemoperfusion cartridge that is used for cytokine removal in intensive care medicine is the CytoSorb™ cartridge (MedaSorb Technologies, Princeton, NJ, USA) which is filled with 300 mL hemoadsorption beads (10). CytoSorb™ hemoadsorption beads are polystyrene-divinylbenzene porous particles (450 µm average particle diameter, 0.8-5 nm pore diameter, 850 m^2/g surface area) with a biocompatible polyvinyl-pyrrolidone coating (11). Another device for cytokine removal is the Coupled Plasma Filtration Adsorption (CPFA®; Bellco, Mirandola, Italy). CPFA® is an extracorporeal therapy that Bellco developed and patented for the treatment of patients with multiorgan failure (MOF) or sepsis. CPFA® combines plasmasorption and hemofiltration for cytokine elimination in patients' blood. The non-selective removal of inflammatory mediators is achieved by a hydrophobic styrene resin with average pore size of 30 nm, which has a high affinity and capacity for many cytokines and mediators (12).

Adsorption to the internal pore surface is accomplished by combination of nonspecific hydrophobic interactions, and size exclusion of larger molecular weight solutes than the target substances. According to the International Union of Pure Applied Chemistry (IUPAC) classification, pores are divided into three groups according to their size of entry: micropores (less than 2 nm); mesopores (2-50 nm); and macropores (over 50 nm). For medical applications, mesopores become relevant due to the fact that target substances with high and middle molecular mass and low molecular protein-bound substances cannot be removed by micropores because they cannot enter these pores (4). The removal efficiency of cytokines was studied on mesoporous carbons and it was confirmed that the removal of cytokines is affected by the relationship between adsorbate critical molecular size and pore size, which can be optimized by modulating synthesis parameters (13). But the pore structure also influences the biocompatibility in blood and plasma because of clotting factor adsorption (14). In this paper, we investigated the effect of the pore structure of styrene-divinylbenzene polymers on the removal of liver toxins and cytokines from blood plasma and the influence of the pore structure on the biocompatibility of the adsorbent material. The aim was to give a suggestion as to which pores sizes of styrene-divinylbenzene based adsorbents are optimal in liver support devices and for sepsis treatment.

MATERIALS

The polymeric adsorbents based on poly(styrene-divinylbenzene) copolymer (PS-DVB) used in this study were provided by Dow Chemical (Philadelphia, PA, USA). Phosphate-buffered saline, bilirubin, cholic acid, phenol, and tryptophan were purchased from Sigma-Aldrich (St Louis, MO, USA), and methanol was obtained from VWR (Vienna, Austria). Human serum albumin was purchased from Octapharma (Vienna, Austria). Human plasma was obtained from a local plasma donation centre (fresh-frozen plasma) and cytokines (TNF-α, IL-1β, IL-6, IL-8 and IL-10) were purchased from R&D Systems (Minneapolis, MN).

METHODS

Adsorbent characterization

Particle size distributions of the microspheres were measured by using a laser light scattering particle size analyzer (Mastersizer 2000; Malvern Instruments, Malvern, UK). About 500 µL of microspheres were suspended in 100 mL of distilled water and stirred under sonication to avoid agglomeration of particles during measurements. The particle size distribution results are volume based.

The dry weight from wet adsorbent materials was determined gravimetrically. A volume of 1 mL of wet adsorbent, hydrated in deionized water was dried in a compartment

drier at 100°C for 24 h (n = 6) and weighed after cooling to room temperature. The percentage of dry weight per milliliter of adsorbent material was calculated using:

$$\%\ Weight\ [w/v] = \frac{dry\ weight\ [g]}{volume\ [ml]} \times 100 \quad [1]$$

The structural characteristics and accessible pore size of each adsorbent were determined by scanning electron microscopy (SEM) and inverse size exclusion chromatography (iSEC). SEM images were obtained by washing the adsorbent particle with pure ethanol and drying these in a heating cabinet at 100°C for 12 h. The particles were than sputtered with gold (Q150R ES, QUORUM) and imaged by SEM (TM-1000 Table Microscope; Hitachi, Tokyo, Japan).

Inverse size-exclusion chromatography (iSEC) was used to determine the accessible pore size and intraparticle porosity of each adsorbent from the retention of glucose and dextran standards with molecular masses between 6 kDa and 5 000 kDa. For this purpose, each adsorbent was flow packed in 0.46 x 15 cm HPLC columns from Grace Davison Discovery Sciences (GRACE, Columbia, MD, USA). As mobile phase for the strongly hydrophobic materials, 15 % (v/v) isopropyl alcohol in PBS buffer was used to prevent dextran adsorption. A Waters HPLC System (Milford, CT, USA) with a Bischoff model 8110 refractive index detector was used to determine the retention volume of individual dextrans for 30 µL injections of 10 mg/ml solutions with a flow rate of 0.8 ml/min. The retention volume of each dextran standard was experimentally determined and characterized in terms of the well-known SEC distribution coefficient:

$$K_d = \frac{V_R - V_0}{V_T - V_0} \quad [2]$$

where V_R is the retention volume, V_0 is the interparticle void volume and V_T is the total mobile phase volume. K_d values range between 0 for a totally excluded compound (dextran with a molecular mass of 5,000 kDa) and 1 for compounds able to permeate and access the total pore volume (glucose with a molecular mass of 180 Da). Since (V_T – V_0) represents the intraparticle mobile phase volume, K_d represents the extent of permeation for molecules into the pore volume of the stationary phase material. The molecular size of dextrans is given by the viscosity radius R_S and was estimated by the following correlation (15):

$$R_S = 0.0271 \times M_r^{0.498} \quad [3]$$

where M_r is the dextran molecular mass.

The adsorbent porosity ε_P, was calculated using (16, 17):

$$\varepsilon_P = \frac{V_T - V_0}{V_B - V_0}; \quad [4]$$

where V_B is the column bed volume.

The pore volume of the adsorbent materials was calculated using:

$$V_P = V_T - V_0 \quad [5]$$

Adsorption studies in batch

Adsorbent pre-treatment

Prior to use for adsorption experiments, the adsorbents were washed with ethanol, water, and 0.9% saline solution for 60 min each, using a ratio of one volume part adsorbent and four volume parts liquid for each washing step. HSA coating of the adsorbent was performed by incubation of one milliliter of adsorbent with 2.5 mL of 0.5% (w/v) HSA solution for 12 h at room temperature. After incubation, the coated adsorbent was washed three times with 0.9% saline. After the last washing step, a 50% (vol/vol) suspension of adsorbent in 0.9% saline was prepared and stored at 4°C before they were used for adsorption experiments.

Human Serum Albumin (HSA) adsorption capacity

Adsorption studies were performed in solutions containing the most prevalent plasma protein HSA diluted with PBS puffer (pH 7.2). The adsorption isotherms of HSA with the three adsorbents were obtained by equilibrating a known mass (about 250 mg) of conditioned particles with 1000 µL solutions of varying protein concentration in closed 1.5 mL microcentrifuge tubes. The adsorbent suspensions were incubated on a roller mixer for 12 h at room temperature (n = 6) and protein quantification was done using an automated analyzer (Hitachi 902). Albumin was quantified with the ALBplus reagent set from Roche (Penzberg, Germany), which is based on the bromcresol green method. Before starting the experiment, the resin samples were centrifuged at 15 000 g for 10 min, and the supernatant was removed to achieve a defined conditioned adsorbent bed.

Adsorption studies of liver toxins in human plasma by batch experiments

For adsorption studies of liver toxins, human plasma was spiked with the following liver toxins: bilirubin (300 µM), cholic acid (100 µM), phenol (2 mM), and tryptophan (100 µM). These substances were then dissolved in a small volume of 0.3 M NaOH, and the solution was added to the plasma and stirred for 60 min at room temperature to allow for the binding of the substances to albumin. Finally, an equivalent volume of 0.3 M HCl was added to neutralize the NaOH. Aliquots of 1 mL of adsorbent (wet pellet volume after centrifugation at 3,000 g) were incubated with 9 mL of spiked plasma for 60 min at 37°C with constant shaking. Samples for the quantification of bilirubin, cholic acid, tryptophan and phenol were drawn at 0, 10, 20, 30, and 60 min and stored at -80°C until quantification of albumin, bilirubin, cholic acid, tryptophan, and phenol was performed. The preparation of the spiked plasma and the subsequent batch experiments were performed in tubes protected from light, since bilirubin is light sensitive. Total bilirubin and cholic acid were quantified on an automated analyzer device (Hitachi 902), where bilirubin was determined with a reagent set from Roche (Penzberg, Germany) and Cholic acid was quantified with an enzymatic method using a reagent set from Trinity Biotech (Wicklow, Ireland). Prior to quantification of tryptophan and phenol, plasma proteins were precipitated by the addition of a 10-fold excess of methanol to the plasma samples and incubation for 20 min at -70°C. Precipitated protein was removed by centrifugation, and tryptophan and phenol were quantified in the supernatant by reversed-phase HPLC on a Nucleosil C18 column (150 × 4.6 mm; Varian, Darmstadt, Germany).

Aliquots of 0.5 mL of HSA and non-HSA coated adsorbent (wet pellet volume after centrifugation at 3000 g) were incubated with 4.5 mL of fresh citrated plasma for 60 min at 37°C with constant shaking. Protein C and Fibrinogen quantification were analyzed from batch samples at time 0 and 60 min with an automated coagulation analyzer (Sysmex CA-500; Kobe, Japan). Protein C was determined using the Berichrom® Protein C reagent (Siemens Healthcare Diagnostics, Austria). Protein C levels are given as activity (%), with a range for normal plasma of 70-149%. Fibrinogen was analyzed using the Dade® Thrombin Reagent (Siemens Healthcare Diagnostics, Vienna, Austria).

Adsorption studies of cytokines in human plasma by batch experiments

For testing the ability of cytokine adsorption, human plasma was spiked with the recombinant cytokines: TNF-α, IL-1β, IL-6, IL-8, and IL-10 at a target concentration of 500 pg/ml each. Batch tests in triplicates were conducted by incubating aliquots of 100 µL HSA and non-HSA coated adsorbent with 900 µL of spiked plasma. Spiked plasma without adsorbents served as a control. After 60 min of incubation at 37°C on a roller mixer, samples were centrifuged and the supernatants were collected and stored at -80°C until quantification using the Bio-Plex cytokine array (Biorad, Vienna, Austria).

RESULTS

Adsorbent characterization

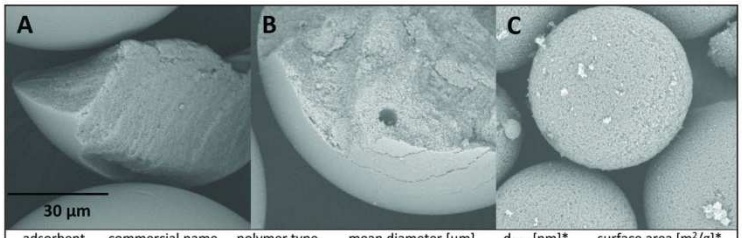
Representative SEM images for adsorbents A, B, and C are shown in Figure 1. In these images, the cracked particles illustrate that the outer thin shell around the adsorbent particles acts as a molecular sieve for entering the inner surface, which is the adsorbent surface for the target molecules. The dry weight of wet adsorbents was between 17% w/v and 18% w/v. The sizes of the beads were similar to the manufacturer dates as summarized in Figure 1.

The K_D values obtained for glucose and dextran probes from the iSEC experiments are shown in Table I. When at a certain viscosity radius R_S value the K_D value reaches zero, the complete molecular exclusion is achieved. As shown in this table, K_D approaches zero for adsorbent A between 8.4 nm and 10.3 nm, for adsorbent B between 11.8 nm and 18.6 nm and for adsorbent C between 37.2 nm and 58.8 nm. The porosity ε_P (see Tab. I) of the three tested adsorbent particles was different. Adsorbent B had the highest porosity value with 0.82, followed by adsorbent C with 0.78, and the lowest was adsorbent A with 0.69.

Adsorption studies in batch

Human Serum Albumin (HSA) adsorption capacity

Adsorption isotherms for HSA are shown in Figure 2. The lines shown are based on the Langmuir isotherm defined as follows:



adsorbent	commercial name	polymer type	mean diameter [µm]	d_{pore} [nm]*	surface area [m^2/g]*
A	CG161c	PS-DVB	121	15	900
B	CG300c	PS-DVB	128	30	700
C	CG1000s	PS-DVB	37	100	200

* manufacturer dates

Fig. 1 - *SEM images of adsorbents **A**, **B**, and **C** at 2000 magnification, laser-light scattering determination of particle size distribution, and manufacturer data of the tested adsorbents.*

TABLE I - SUMMARY OF INVERSE SIZE EXCLUSION CHROMATOGRAPHY AND DRY WEIGHT DATA OF WET ADSORBENT MATERIAL

M_r	RS [nm]	K_D adsorbent	K_D adsorbent	K_D adsorbent
		A	B	C
180	0.36	1.00	1.00	1.00
6 000	2.06	0.41	0.69	0.99
10 000	2.66	0.12	0.59	0.94
20 000	3.76	0.07	.045	0.89
40 000	5.31	0.04	.021	0.82
100 000	8.37	0.03	0.08	0.81
150 000	10.25	0.00	0.08	0.74
200 000	11.83	0.00	0.08	0.66
500 000	18.67	0.00	0.00	0.49
2 000 000	37.23	0.00	0.00	0.12
5 000 000	58.76	0.00	0.00	0.00
		A	B	C
$V_{T\,(Glucose)}$ [mL]		1.522	1.632	1.611
$V_{0\,(Dextran\ 5000\ kDa)}$ [mL]		0.929	0.922	0.989
$V_{B\,(column)}$ [mL]		1.792	1.792	1.792
V_P [mL]		2.115	2.341	2.232
ε_P		0.687	0.816	0.775
iSEC pore radius r [nm]		8.4 < r < 10.3	11.8 < r < 18.7	37.5 < r < 58.8
Percentage dry weight [w/v] (n = 6)		17.6 ± 0.4%	17.2 ± 0.1%	18.3 ± 0.1%

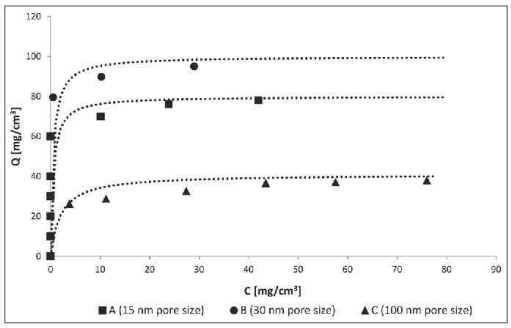


Fig. 2 - *Adsorption isotherms for HSA after equilibration time. Dotted lines are from Langmuir model [Eq. (6)].*

$$Q = \frac{Q_{max} KC}{1 + KC} \quad (6)$$

with Q_{max} (Langmuir isotherm adsorption capacity) and K (Langmuir iosotherm equilibrium constant) determined by data fitting. The adsorbent with the lowest surface area also has the lowest adsorption capacity for HSA (less than 50 mg/ml). On adsorbent A, which has the highest surface area, lower HSA adsorption was observed (less than 80 mg/ml) than on adsorbent B (less than 100 mg/ml). A reason for this phenomenon is probably that the smaller pores of adsorbent A prevent the diffusion of HSA into parts of the inner surface area. These results suggest that adsorbent B is the most efficient one in removing HSA-bound toxins.

Adsorption studies of plasma proteins by batch experiments

The adsorption of individual proteins that play important roles in the regulation of coagulation was also investigated. In particular, the adsorption of protein C and fibrinogen was tested. The results for protein C adsorption on the tested polymers are shown in Figure 3. Under physiological conditions, 85% to 90% of circulating protein C is present as a pro-enzyme with a molecular mass of 62 kDa, which is similar to that of albumin (66 kDa). As shown in Figure 3, protein C was markedly adsorbed by the uncoated adsorbents B and C, and albumin coating strongly decreased its adsorption. Thus, albumin coating of adsorbents, based on PS-DVB, improves their blood compatibility. Adsorbent A did not substantially influence

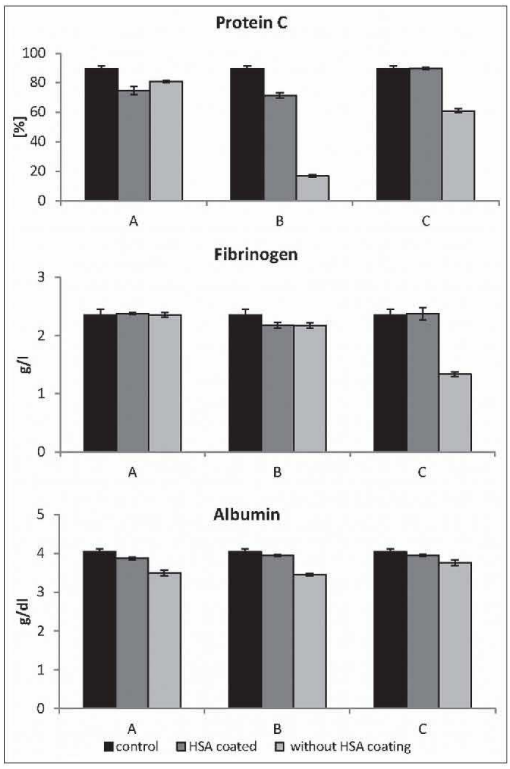


Fig. 3 - *The final plasma concentration (mean ± SD) of protein C, fibrinogen and albumin after 60 min incubation with uncoated and HSA-coated adsorbents. The adsorbent to plasma ratio was 1:9 (v/v).*

the protein C level in plasma because of its small pore structure, which causes a barrier between protein C and the inner surface of the adsorbent beads. In addition to protein C, the adsorption of fibrinogen was assessed. Because of its high molecular mass and very large viscosity radius, it is excluded from the pores of adsorbent A and B. Only adsorbent C, which has the largest pores, was able to reduce the fibrinogen level from 2.2 g/L to 1.2 g/l. The amount of albumin removal from plasma was also monitored. Under the experimental conditions applied, 13%, 14%, and 7% of albumin were removed by adsorbents A, B and C, respectively. As shown in Figure 3, albumin adsorption of all adsorbents and fibrinogen adsorption of adsorbent C was prevented from plasma using HSA-coated material.

Adsorption studies of liver toxins in human plasma by batch experiments

Adsorption of toxins related to liver failure was assessed with *in vitro* batch experiments using human plasma spiked with bilirubin (300 µM), cholic acid (100 µM), L-tryptophan (100 µM), and phenol (2 mM). These spike concentrations of bilirubin, tryprophan, and cholic acid are in the range of toxin levels usually found in patients with liver failure (26). The phenol concentration used in this study was significantly higher than that reported from patients with liver failure and was chosen as a marker for weakly bound aromatic compounds. Bilirubin, which is strongly albumin-bound with an association constant of 9.5×10^7 M^{-1}, was only efficiently adsorbed by adsorbent B, which reduced the bilirubin level from 17.3 ± 0.7 to 2.9 ± 0.8 mg/dl after 60 min. The bilirubin content after adsorbent A treatment was 7.9 ± 0.2 mg/dl, whereas adsorbent C does not seem to be able to remove bilirubin. Cholic acid (association constant with albumin: 0.33×10^4 M^{-1}) was almost completely removed by adsorbents A and B from 126 ± 2 to 7 ± 1 and 6 ± 1 µM. The plasma treatment with adsorbent C caused a colic acid reduction to 68 ± 2 µM. In principle, tryptophan [(KA) 1×10^4 M^{-1}] showed similar adsorption behavior to cholic acid (preferred binding to polymers with small pores; Fig. 4), but in contrast to cholic acid, tryptophan binding was only moderate. Phenol, which is only loosely associated with albumin, bound equally well to adsorbents A and B, but poorly to adsorbent C. However, tryptophan and phenol can be removed to a large extent by dialysis, which normally is performed during extracorporeal liver support therapy.

Adsorption studies of cytokines in human plasma by batch experiments

The cytokine adsorption properties of the adsorbents were investigated in batch experiments using spiked human plasma. The selected cytokines for these experiments are the pro-inflammatory TNF-α, IL-1β, IL-6, IL-8, and the anti-inflammatory cytokine IL-10. TNF-α has a molecular mass ranging from 17 kDa to 51 kDa depending on whether it is found in the monomeric, dimeric, or trimeric state. Among them, the homotrimer is the most active form of TNF-α, which has the largest dimension of all cytokines as far as the crystal structure and viscosity radius are concerned (see Fig. 5). Because of the large size of the trimeric form, the removal of TNF-α from the bloodstream presents a significant challenge. The best TNF-α adsorption could be observed by adsorbent A (Fig. 6), which removed 83 ± 1%, followed by adsorbent B with a removal rate of 80 ± 2%.

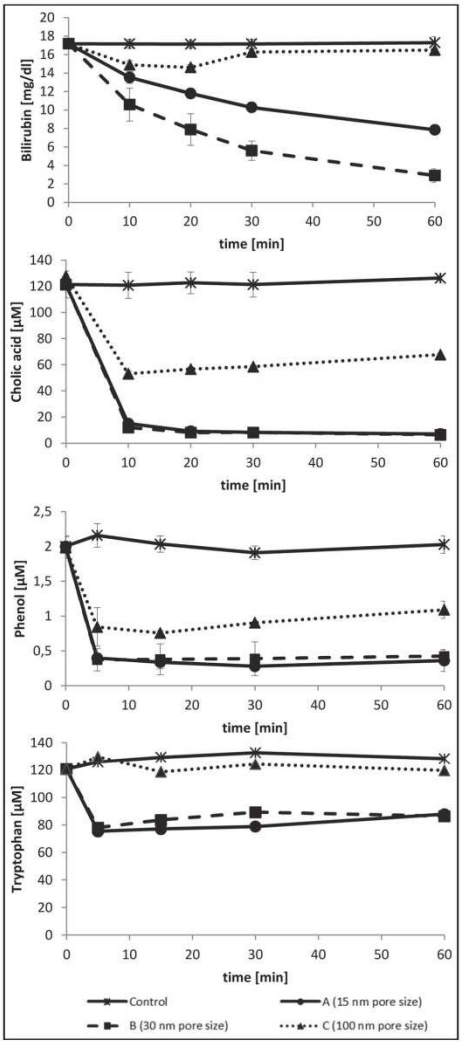


Fig. 4 - *Concentration (mean ± SD) of unconjugated bilirubin, cholic acid, phenol, and tryptophan remaining in spiked plasma samples after incubation with adsorbents A, B, and C at 5, 15, 30, and 60 min. The adsorbent to plasma ratio was 1:9 (v/v).*

Protein	MW [kDa]	Crystal-Dimension [nm] a x b x c	Amino-acids	Viscosity radius [nm]	Diffusion coefficient * 10^{-7} [cm^2/s]
Fibrinogen	*340*	Fibrinogen *17.6 x 94.8 x 20.9*	*~1500*	12.7	1.0
Human Serum Albumin	*66*	HSA *5.5 x 5.5 x 12*	*585*	Monomer: 3.55 Dimer: 4.34 Tetramer: 5.18 Hexamer: 5.85	3.57 2.92 2.45 2.17
Protein C	*62*	Protein C *5.7 x 9 x 10.2*	*419*	3.3	3.84
TNFα-Trimer	*57*	TNFα *9.5 x 9.5 x 11.7*	*555*	3.6	3.52
Interleukin-10 Homodimer	*37*	IL-6 *7 x 7 x 7.1*	*320*	N/A	N/A
Interleukin-6	*21.6*	IL-10 *5 x 5 x 12.2*	*183*	2	6.34
Interleukin-1ß	*17.4*	IL-1ß *5.5 x 5.5 x 7.5*	*153*	N/A	N/A
Interleukin-8	*8.4*	IL-8 *4 x 4 x 9*	*99*	N/A	N/A

Fig. 5 - *Molecular weight, crystal dimension, viscosity radius, and calculated diffusion coefficient of selected plasma proteins for adsorption in blood purification (18-25).*

The poorest TNF-α adsorption was observed by adsorbent C, with 51 ± 9% reduction after 60 min. The HSA coating of the adsorbents make them more biocompatible, but also lead to lower cytokine removal from plasma. The TNF-α removal rates decreased to 78 ± 1%, 68 ± 6%, and to 32 ± 16% for adsorbents A, B, and C, respectively. The other tested cytokines were nearly removed by adsorbents A and B. Adsorbent C has the poorest cytokine removal properties because of its lower surface area and larger pores.

DISCUSSION

It is well known that biomaterials coated with albumin improve blood compatibility because adsorbed albumin has the ability to reduce platelet and leucocyte adhesion and inhibit thrombus formation (27). As shown in Figure 3, albumin-coated PS-DVB adsorbent material improves

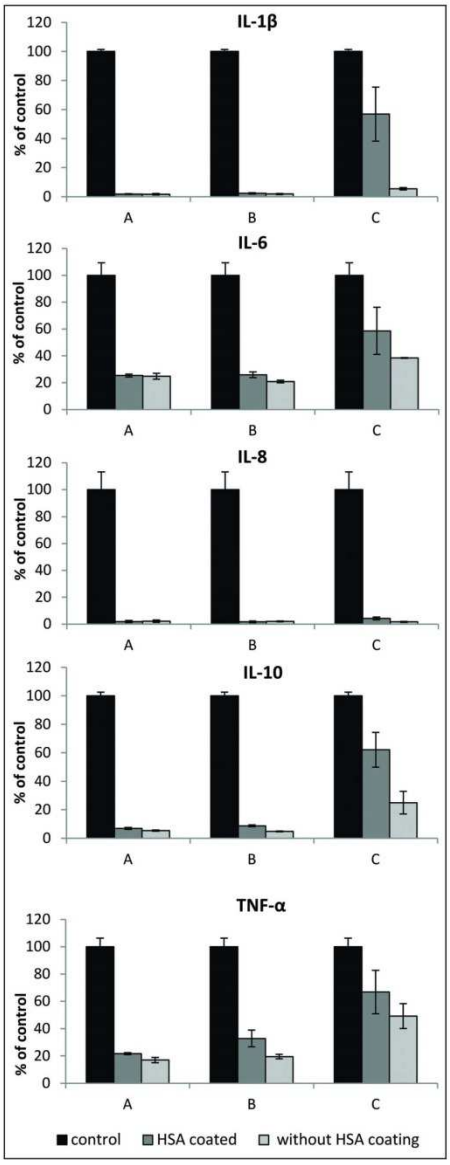


Fig. 6 - *Amounts (mean ± SD) of cytokines (TNF-α, IL-1β, IL-6, IL-8, and IL-10) remaining in spiked human plasma after 60 min of incubation with HSA and non-HSA coated adsorbents. Batch tests were conducted in triplicate using a 10% adsorbent-to-plasma ratio (v/v).*

the blood and plasma biocompatibility by reduced removal of albumin and clotting factors. Protein C, also known as autoprothrombin IIA and blood coagulation factor XIV, is a zymogenic (inactive) protein. The activated form plays an important role in regulating blood clotting, inflammation, cell death, and maintaining the permeability of blood vessel walls. The importance of fibrinogen adsorption comes from the close relationship between fibrinogen and platelets, and the promotion by adsorbed fibrinogen of platelet reactivity, the replacement of adsorbed fibrinogen by high molecular mass kininogen (HMWK), a protein participating in the activation of the intrinsic coagulation, and a possible interaction of fibrinogen with leucocytes (27). Removal of coagulation factors during extracorporeal treatment can be very critical because liver and sepsis patients often have coagulation defects. Consequently a strict pore size distribution together with an albumin coating of the adsorbent material greatly reduce the adsorption of coagulation factors which will lead to higher blood and plasma compatibility of adsorbents.

In the case of liver toxins, the kinetic curve of adsorbent C shows that bilirubin is adsorbed less in the first 20 min and then is displaced from the adsorbent surface by molecules from the large molecular mass plasma fraction, as is described by the Vroman effect. The Vroman effect, named after Leo Vroman, is exhibited by protein adsorption to a surface by blood serum proteins. The small plasma proteins, which have the highest mobility, generally arrive first and are later replaced by larger, less motile proteins that have a higher affinity to the surface. A typical example of this occurs when fibrinogen displaces earlier adsorbed proteins on a biopolymer surface and is later replaced by high molecular weight kininogen (28). Plasma molecules with much higher molecular masses also have a lower diffusion coefficient (D) and as a result they need more time to diffuse in the pores of the adsorbents. A dependence of the diffusion coefficient on molecular size in liquids can be found using Stokes–Einstein equation, which predicts that:

$$D = \frac{k_B T}{6\pi\eta R_0} \tag{7}$$

where k_B is the Bolzmann constant, η is the dynamic viscosity of the fluid, R_0 is the hydrodynamic radius and T is the temperature in kelvins. Adsorbent A has the largest inner surface, but the smallest pores, so the whole inner surface is not accessible for bilirubin-loaded albumin and as a result the bilirubin removal is not as good as with adsorbent B. Therefore, for good bilirubin adsorption good accessibility for albumin into the inner surface of the adsorbent material is required. This criterion is fulfilled by adsorbent B. The reason why cholic acid, phenol, and tryptophan removal of adsorbent C were much lower than the other tested adsorbents is because of the both its smaller surface and its larger pores, which do not act as a barrier for the high molecular plasma fraction. The reason for the efficient TNF-α removal by adsorbent A could be that the pores are optimal for TNF-α entering and additionally prevents the diffusion of larger proteins, which replace TNF-α from the adsorbents' surface according to the Vroman effect (28). Due to the fact that cytokines in contrast to liver toxins are not linked to plasma proteins, adsorbents with smaller pores are suitable for cytokine removal in extracorporeal treatment. In the case of adsorbents A and B, an additional HSA coating can be used for better blood or plasma biocompatibility because the decrease of the cytokine adsorption was only slightly less than adsorbent C.

One negative aspect of the use of adsorbents for extracorporeal purification treatments is the lack of selectivity in their action. In addition to the removal of target substances from the bloodstream, many useful blood and plasma components, for example clotting factors, can also be adsorbed. Especially if adsorbents are to be used continually for the treatment of chronic patients, this may become a problem and optimizing the selectivity of adsorption will be necessary. The most selective adsorbents can be synthesized by the immobilization of bioligands (e.g., antigen-antibody pair) with high affinity toward target molecules. But these adsorbents are currently too expensive. Furthermore, the use of selective adsorbents requires knowledge of what substances have to be removed from patients' blood and this is not always possible. It is also unknown if the cytokines from patients' blood suffering from sepsis should be completely removed or if it is better to modulate the cytokine level to a lower dose. Consequently, nonspecific adsorbents will keep their role in the treatment of diseases with unknown etiology (4).

In addition to coating and chemical modification of the surface, the selectivity of adsorption can be increased by regulation of the pore size. Similar to the pores of membrane filters, the pores of adsorbents act as molecular sieves and prevent the entry of molecules that are larger than the molecular cut-off of the pores. In case of adsorbents material

with defined pore structure, the viscosity radius of molecules, also known as hydrodynamic radius, is essentially for adsorption because it determines if the molecule can come in contact with the inner adsorbents surface or not (see Fig. 6).

CONCLUSIONS

The outcomes of this study reveal that adsorbents based on styrene-divinylbenzene polymers with 15 nm pores are suitable for cytokine removal, and the same adsorbents with 30 nm to 40 nm are better for the removal of albumin-bound toxins in the case of liver failure. This knowledge can be used for the design of new selective adsorbents for liver support systems and for sepsis treatment; additionally, it will make the therapy more efficient and more plasma- or blood-compatible. For example, the CPFA® cartridge, which is filled with an Amberchrom type of resin with an average pore diameter of 30 nm (29), has optimal pore structure for removal of albumin-bound liver toxins, but it is used for cytokine removal in sepsis treatment. For this application, the CG161c, which has 15 nm pores would be more suitable. By increasing pore size, adsorbents will lose their selectivity and biocompatibility because they will also remove high molecular weight substances such as immunoglobulins, fibrinogen, and other clotting factors from the plasma. This makes adsorbents with large pore sizes inapplicable for the use in extracorporeal treatment, especially in sepsis and liver treatment, in which patients often have coagulation disorders. But the efficiency of this approach requires further research, favorable in the fine tuning of pore distributions of adsorbents as well as in dynamic experiments. Finally the promising outcome from the *in vitro* experiments has to be validated *in vivo*.

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Conflict of Interest: None.

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2.1.3 Adsorption des Entzündungsmediators High-Mobility Group Box 1 durch unterschiedliche Polymere

Carla Tripisciano, Tanja Eichhorn, Stephan Harm, Viktoria Weber: *Adsorption of the Inflammatory Mediator High-Mobility Group Box 1 by Polymers with Different Charge and Porosity*. Biomed Res Int. 2014; 2381602014(1):238160, DOI:10.1155/2014/238160

Kurzfassung:

Einleitung: Das High-Mobility Group Box 1 Protein (HMGB1) ist ein konserviertes Protein mit einer Vielzahl biologischer Funktionen innerhalb und außerhalb der Zelle. Sobald es von aktivierten Immunzellen freigesetzt wird, wirkt es als proinflammatorischer Entzündungsmediator. Seine im Vergleich zu proinflammatorischen Zytokinen verhältnismäßig späte Freisetzung hat das Interesse an HMGB1 als potenzielle Zielsubstanz in der extrakorporalen Therapie geweckt.

Material & Methoden: Hier untersuchen wir die Adsorption von HMGB1 an anionische Polymere auf Methacrylatbasis sowie an neutrale Polystyrol-Divinylbenzol-Copolymere. Die Adsorptionsversuche wurden in Plasma in Batchtests durchgeführt.

Ergebnisse: Beide Gruppen von Adsorbern binden effizient rekombinantes HMGB1 und natives HMGB1, welches durch Lipopolysaccharid-Stimulation aus peripheren mononukleären Blutzellen gewonnen wurde. Die Adsorptionseigenschaften waren von der Partikelgröße, der Porosität, der Porenzugänglichkeit und der Ladung abhängig. Neben physikalisch-chemischen Parametern der Adsorber beeinflussen auch Modifikationen wie Acetylierung, Phosphorylierung und Oxidation am HMGB1 Molekül die Bindungseigenschaften zwischen Polymer und HMGB1. Weiteres üben Wechselwirkungen zu anderen Plasmaproteinen und die Art des Antikoagulans einen Einfluss auf die Adsorptionseigenschaften von HMGB1 aus.

Schlussfolgerung: Posttranslationale Modifikationen am HMGB1 Molekül beeinflussen die Bindungseigenschaften zu Adsorberoberflächen. Diese Eigenschaft kann genutzt werden, um HMGB1 Moleküle mit einer bestimmten biologischen Funktion aus dem Blut zu entfernen.

Mein Beitrag für diese Publikation:

- Analytik: Rasterelektronenmikroskopische Aufnahmen

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Research Article

Adsorption of the Inflammatory Mediator High-Mobility Group Box 1 by Polymers with Different Charge and Porosity

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High-mobility group box 1 protein (HMGB1) is a conserved protein with a variety of biological functions inside as well as outside the cell. When released by activated immune cells, it acts as a proinflammatory cytokine. Its delayed release has sparked the interest in HMGB1 as a potential therapeutic target. Here, we studied the adsorption of HMGB1 to anionic methacrylate-based polymers as well as to neutral polystyrene-divinylbenzene copolymers. Both groups of adsorbents exhibited efficient binding of recombinant HMGB1 and of HMGB1 derived from lipopolysaccharide-stimulated peripheral blood mononuclear cells. The adsorption characteristics depended on particle size, porosity, accessibility of the pores, and charge of the polymers. In addition to these physicochemical parameters of the adsorbents, modifications of the molecule itself (e.g., acetylation, phosphorylation, and oxidation), interaction with other plasma proteins or anticoagulants (e.g., heparin), or association with extracellular microvesicles may influence the binding of HMGB1 to adsorbents and lead to preferential depletion of HMGB1 subsets with different biological activity.

1. Introduction

High-mobility group box 1 protein (HMGB1) is a ubiquitous nonhistone DNA binding protein with distinct intra- and extracellular functions. It is crucial for nuclear architecture and has been implicated in DNA replication, repair, and transcription. It acts as a sentinel for nucleic acid-mediated immune responses [1, 2] and as a pathogenic inflammatory mediator during sterile and infectious injury [3–6].

Extracellular HMGB1 is either derived from passive release by injured or necrotic cells or derived from active secretion by immune cells, such as monocytes and macrophages [7–9], or natural killer cells [10, 11] after exposure to pathogen-associated molecular patterns including lipopolysaccharide (LPS) and inflammasome agonists [12, 13]. Secretion of HMBG1 from monocytes/macrophages starts 8–12 h after ligation of cell surface receptors, which represents a significantly delayed release as compared to most other inflammatory mediators produced by these cells, fostering interest in HMGB1 as a target for therapy [3, 14, 15].

HMGB1 secretion is regulated by phosphorylation and acetylation of its two nuclear localization sequences (NLS) [8, 16, 17]. Cell stress and inflammation induce NLS acetylation of HMGB1, resulting in its cytoplasmic accumulation, loading into secretory lysosomes, and release by exocytosis [18]. Secreted HMGB1 acts through various pattern-recognition receptors including the receptor for advanced glycation end products (RAGE), toll-like receptors TLR-2, TLR-4, and TLR-9, T-cell immunoglobulin domain and mucin domain 3 (TIM-3), and CXC chemokine receptor type 4 (CXCR-4) [19–24].

While the secretion of HMGB1 is regulated by phosphorylation and acetylation, its extracellular biological activity and interaction with different receptors depend on the redox

state of three conserved cysteine residues at positions 23, 45, and 106. With these residues in a reduced form, HMGB1 induces chemotaxis. With a disulfide bridge between C23 and C45 and a free sulfhydryl at position 106, HMGB1 interacts with toll-like receptor 4 (TLR-4) to stimulate cytokine production, while it loses its biological activity in its completely oxidized form [25, 26].

The depletion of HMGB1 by extracorporeal therapies, such as hemofiltration with porous membranes [27] or hemoperfusion with adsorption columns has been reported [28, 29]. As HMGB1 possesses two DNA binding domains that interact with negatively charged groups, we tested different anionic polymers for their ability and capacity to bind HMGB1, compared their adsorption efficiency to neutral polystyrene divinylbenzene-based polymers, and correlated the binding characteristics to the physicochemical properties of the polymers. We show here that porosity, size distribution, hydrophobicity, and effective charge density as well as the distribution and accessibility of functional groups on the adsorbent surface are critical determinants of the adsorption characteristics. This implies that a given polymer may preferentially bind subsets of molecules with different posttranslational or oxidative modifications and with different biological activity.

2. Materials and Methods

2.1. Chemicals and Reagents. Recombinant human HMGB1 was purchased from R&D Systems (Minneapolis, USA). Cell culture medium 199 (M199), phosphate-buffered saline (PBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), penicillin-streptomycin (PS), and lipopolysaccharide (LPS) from *E. coli* (O55:B5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Unfractionated heparin (5000 IU/mL) was from Baxter (Vienna, Austria).

2.2. Plasma. Venous blood was drawn into tubes containing 3.8% trisodium citrate (Vacuette, Greiner Bio-One, Kremsmuenster, Austria) from healthy adult volunteers after written informed consent. Plasma was obtained by centrifugation of the whole blood at 2000 ×g for 10 min at room temperature.

2.3. Adsorbents. Negatively charged and neutral adsorbents of different particle sizes and porosities were tested in this study. PSDVB-16 and PSDVB-30 (trade names CG161 and CG300; both from Rohm & Haas/Dow Chemical), as well as PVP-PSDVB (trade name Cytosorb; Cytosorbents Corporation), are hydrophobic neutral resins that are under evaluation or already in clinical application as selective cytokine adsorbents in extracorporeal blood purification. DALI (Fresenius Medical Care, Bad Homburg, Germany) and ReliSorb (Resindion S.r.l.) are both methacrylate-based polymers functionalized with polyacrylate. DALI is clinically applied for whole blood lipid apheresis. As a third negatively charged polymer, we used the cellulose-based adsorbent Cellufine sulfate (Chisso Corporation), while the neutral Cellufine GCL-2000 served as negative control. Prior to the adsorption studies, adsorbents were extensively washed with pyrogen-free 0.9% NaCl and stored at 4°C in saline solution until further use.

2.4. Adsorbent Characterization. Particle morphology was analyzed by scanning electron microscopy (SEM) using a TM-1000 Tabletop Microscope (Hitachi, Tokyo, Japan). Samples were washed with 100 vol% ethanol and dried for 12 h at 100°C in a heating cabinet. Adsorbents were subsequently sputter-coated with gold (Q150R ES, Quorum Technologies). Cellulose-based adsorbents were incubated overnight with 2.5 vol% glutaraldehyde, rinsed with dH_2O, and dehydrated with increasing concentrations of ethanol (30 to 100 vol%) before sputter coating.

To determine the specific surface and the pore size distribution, nitrogen adsorption and desorption isotherms were recorded at −196°C and at relative pressures P/P_0 between 0.001 and 1.0 using an ASAP 2010 V2.00 C physisorption analyzer (Micrometrics Instrument Corp., Norcross, USA). The Brunauer-Emmett-Teller (BET) equation [30] was used to calculate the specific surface area (S_{BET}). The micropore volume (pore size < 2 nm) was calculated with the Horvath-Kawazoe (H-K) method [31], whereas the mesopore and macropore volume (2–50 and 50–300 nm, resp.) was obtained via the Barrett-Joyner-Halenda method (BJH) [32]. Assuming cylindrical pore geometry, the average pore diameter d was calculated as $d = 4V/S_{BET}$ (with V = maximum adsorbed nitrogen volume). Values for the average particle diameter and the charge density of the negatively charged adsorbents were provided by the manufacturers.

2.5. Adsorption of Recombinant HMGB1. Adsorption of HMGB1 to the different polymers was studied in batch experiments using adsorbent-to-plasma ratios of 1, 5, and 10 vol%. Plasma was spiked with recombinant human HMGB1 to a target concentration of 200 ng/mL and incubated with the adsorbents at 37°C with gentle shaking. Spiked plasma without adsorbent served as a control. Samples were taken after 15 and 60 min and centrifuged immediately at 4600 ×g for 5 min to remove the adsorbents. Supernatants were collected, aliquoted, and stored at −80°C until further analysis. All experiments were conducted in triplicate. HMGB1 was quantified by enzyme-linked immunosorbent assay (ELISA, Shino-Test Corporation, Kanagawa, Japan) according to instructions of the manufacturer.

2.6. Adsorption of HMGB1 Derived from Stimulation of Peripheral Blood Mononuclear Cells. Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood anticoagulated with heparin (5 IU/mL final concentration) by density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare, Uppsala, Sweden) as described [33]. Cells were suspended in medium M199 supplemented with 10 vol% human plasma, 0.02 M HEPES, and 100 μM PS. Aliquots of 1×10^6 cells per mL of medium were stimulated with LPS (0.01–1000 ng/mL) for 16 h in HydroCell Surface 24-well plates (1 mL/well) in humidified atmosphere (5 vol% CO_2, 37°C). After stimulation, the cells were pelleted by

Table 1: Physicochemical characteristics of the adsorbents used in this study.

Polymer	Core	Ligand	Particle size [μm]	Pore size [nm]	Surface area [m^2/g]	Total V_{pores} [mL/g]
PVP-PSDVB	PS-DVB coated with polyvinylpyrrolidone	None	450	0.8–5	850	1.4
PSDVB-16	Polystyrene-divinylbenzene	None	120	15	900	2.1
PSDVB-30	Polystyrene-divinylbenzene	None	50–100	30	700	1.5
Cellufine	Cross-linked cellulose	None	40–130	n.d.	n.d.	n.d.
Cellufine sulfate	Cross-linked cellulose	Sulfate ester	40–120	n.d.	n.d.	n.d.
DALI	Polymethacrylamide	Polyacrylate	150–230	~180	50	1.4
ReliSorb	Polymethacrylamide	Polyacrylate	150–230	~200	28	1.7

n.d.: not determined; PSDVB: polystyrene-divinylbenzene.

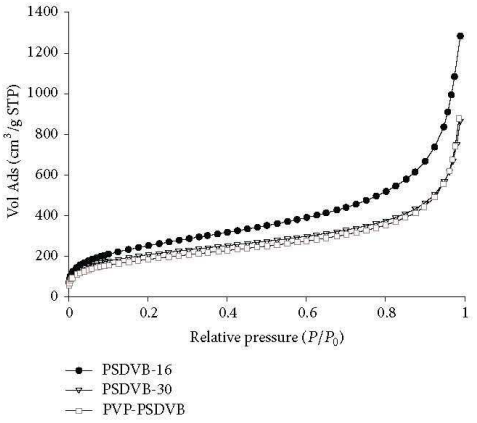


Figure 1: Nitrogen adsorption isotherms of uncharged polymers. Vol Ads: volume of nitrogen adsorbed; STP: standard temperature and pressure.

centrifugation, the supernatant (conditioned medium) was harvested, and the concentration of released HMGB1 was quantified by ELISA. Conditioned medium was incubated for 60 min with Cellufine sulfate or PSDVB-16, respectively, at an adsorbent-to-plasma ratio of 5 vol%. Adsorbents were removed by centrifugation and HMGB1 remaining in the supernatant was quantified by ELISA.

3. Results and Discussion

3.1. Physicochemical Characterization of the Adsorbents. The characteristics of the adsorbents used in this study are summarized in Table 1. The neutral polymers PSDVB-16 and PSDVB-30 are mesoporous polystyrene-divinylbenzene copolymers with average pore sizes of 15 and 30 nm, respectively. The smaller mean pore size of PSDVB-16 is reflected by its higher specific surface area in comparison to PSDVB-30. PVP-PSDVB, which is based on polystyrene-divinylbenzene coated with polyvinylpyrrolidone, exhibited the lowest pore diameter of all hydrophobic resins tested.

The two anionic methacrylate-based adsorbents DALI and ReliSorb differ not only with respect to their surface morphology [34], but also with respect to charge density, which is 530 and 300 μequivalents of COOH per mL of dry adsorbent for DALI and ReliSorb, respectively. Cellufine sulfate, the third anionic polymer used in this study, has an approximately tenfold higher charge density.

Adsorption isotherms were obtained by measuring the amount of N_2 adsorbed across a wide range of relative pressures at a constant temperature. The isotherms resembled type IV according to the classification by Brunauer et al. [35], typically occurring on porous adsorbents with pores in the range of 1.5–100 nm. At lower pressures, the slope of the isotherms is given by micropores, while at higher pressures the slope reflects an increased uptake of adsorbate as pores become filled, with the inflection point typically occurring near the completion of the first monolayer (Figure 1). The neutral polystyrene-divinylbenzene-based adsorbents showed a high S_{BET} resulting from the presence of micro-, meso-, and macropores.

The morphology of the adsorbent particles was characterized by scanning electron microscopy (SEM; Figure 2). DALI and ReliSorb, the two methacrylate-based polymers, exhibited a comparably structured inner surface but showed clear differences with respect to their outer surface, which appeared open and porous on scanning electron micrographs for ReliSorb, while it had a closed and smooth appearance for DALI. The polystyrene-divinylbenzene-based resins all had a smooth outer surface but differed with respect to their porosity, which was highest for PSDVB-30 in accordance with nitrogen adsorption measurements.

3.2. Adsorption of Recombinant HMGB1. HMGB1 adsorption was studied both with recombinant human HMGB1 and with HMGB1 derived from stimulated peripheral blood mononuclear cells. Pathological blood levels of HMGB1 have been reported to range between 10 and 150 ng/mL [36]. Therefore, we used a target concentration of 200 ng/mL of recombinant HMGB1 in the adsorption experiments, which are summarized in Figure 3. HMGB1 was efficiently adsorbed by both neutral and anionic polymers. Binding

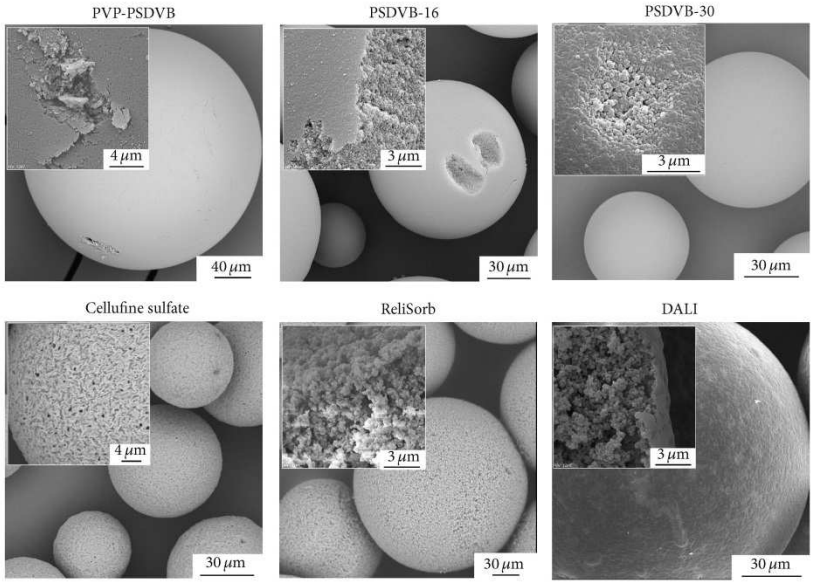


Figure 2: Electron micrographs of adsorbents used in this study.

of HMGB1 to neutral PSDVB beads occurs via hydrophobic interactions between the amphipathic adsorbate and the hydrophobic matrix, as confirmed by the negligible HMGB1 adsorption by neutral hydrophilic cellulose beads. Regarding the polystyrene-divinylbenzene-based polymers, HMGB1 adsorption was higher for PSDVB-30 as compared to PSDVB-16, most likely due to a better accessibility of the pores due to the higher average pore diameter. PVP-PSDVB bound significantly less HMGB1 than the uncoated PSDVB polymers under identical experimental conditions despite an equivalent specific surface area. This lower efficacy can be attributed to a smaller average pore diameter as well as to diminished accessibility of the inner surface due to the coating of the polymer with polyvinylpyrrolidone. Adsorption of albumin, the most abundant plasma protein, was evaluated for the 5 vol% batch experiment. Plasma albumin levels were reduced by 1.4% to 3% for the negatively charged hydrophilic polymers and by 8% to 10% for neutral hydrophobic resins (Table 2).

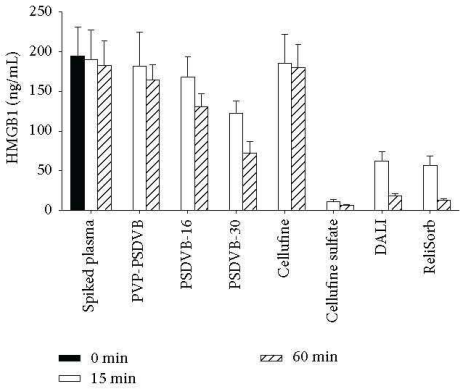
The adsorption of cytokines of higher molecular mass, in particular tumor necrosis factor alpha (TNF-α; 51 kDa), to PVP-PSDVB polymers is considerably reduced due to a lack of accessibility of their inner surface [37, 38]. The same may hold true for HMGB1 (30 kDa), and as discussed further below, its association with a wide range of plasma proteins as well as with microvesicles may additionally influence its binding characteristics. PSDVB-based resins are clinically applied in extracorporeal liver support to remove albumin-bound metabolites such as unconjugated bilirubin [39–41].

Table 2: Albumin adsorption from plasma after 60 min of incubation at an adsorbent-to-plasma ratio of 5 vol%; $n = 3$.

Adsorbent	Albumin adsorbed [mg/mL adsorbent]	Albumin remaining [% of initial concentration]
PSDVB-16	76.6 ± 3.2	90.0 ± 0.8
PSDVB-30	62.7 ± 7.1	91.8 ± 0.5
Cellufine	10.1 ± 11	98.7 ± 1.4
Cellufine sulfate	10.8 ± 3.2	98.6 ± 0.4
DALI	19.0 ± 3.1	97.5 ± 0.3
ReliSorb	24.7 ± 3.1	96.7 ± 0.6

Noteworthy, blocking of HMGB1 activity has been shown to improve hepatocyte regeneration after ischemia/reperfusion injury [2], suggesting that its removal by extracorporeal liver support systems may provide a benefit for liver regeneration.

As a DNA-associated protein, we reasoned that HMGB1 would bind to anionic surfaces via electrostatic interactions. Confirming this assumption, all anionic polymers showed efficient binding of HMGB1, with the highest efficiency for Cellufine sulfate, which has a high negative charge density and a small particle diameter, resulting in a large outer surface. Application of Cellufine sulfate as adsorbent for HMGB1 in rat hemoperfusion models resulted in decreased HMGB1 serum levels and improved survival in rats with ischemia-reperfusion injury [28, 29].



	μg HMGB1 adsorbed/mL adsorbent					
	10 vol% batch experiment		5 vol% batch experiment		1 vol% batch experiment	
Adsorbent	15 min	60 min	15 min	60 min	15 min	60 min
PVP-PSDVB	n.d.	n.d.	n.d.	n.d.	0.8 ± 0.6	1.8 ± 1.6
PSDVB-16	1.1 ± 0.06	1.4 ± 0.02	1.2 ± 0.2	2.0 ± 0.2	2.2 ± 1.1	5.2 ± 1.5
PSDVB-30	1.5 ± 0.003	1.5 ± 0.03	2.2 ± 0.2	2.4 ± 0.2	6.7 ± 2.2	11.0 ± 2.0
Cellufine	Negligible	0.1 ± 0.04	Negligible	Negligible	0.4 ± 0.6	0.3 ± 0.7
Cellufine sulfate	1.5 ± 0.03	1.5 ± 0.04	2.5 ± 0.2	2.3 ± 0.2	17.6 ± 3.5	17.5 ± 3.0
DALI	1.4 ± 0.03	1.4 ± 0.03	2.5 ± 0.2	2.3 ± 0.3	12.6 ± 2.6	16.3 ± 2.8
ReliSorb	1.5 ± 0.02	1.4 ± 0.03	2.5 ± 0.2	2.4 ± 0.2	13.2 ± 3	16.8 ± 2.9

FIGURE 3: Adsorption of recombinant HMGB1. The graph shows the amount of HMGB1 remaining in spiked plasma after 15 and 60 min of incubation at an adsorbent-to-plasma ratio of 1 vol%. The amount of HMGB1 adsorbed to the polymers at adsorbent-to-plasma ratio of 1, 5, and 10 vol% is summarized in the table. Results are expressed as mean values ± standard deviation of three experiments.

The two methacrylate-based adsorbents bound similar amounts of HMGB1 as Cellufine sulfate after 60 min (17.5 ± 3.0 versus 16.3 ± 2.8 versus 16.8 ± 2.9 μg for Cellufine sulfate, DALI, and ReliSorb, resp.). The delayed binding of HMGB1 to DALI and ReliSorb as indicated by the higher remaining concentrations after 15 min may be due to differences in particle size, with the most favourable surface-to-volume ratio for Cellufine sulfate. This indicates rapid binding of HMGB1 to the adsorbent surface, while diffusion and adsorption to functional groups inside the particles occur gradually over time. DALI is clinically applied to remove low-density lipoproteins in patients with familial hypercholesterolemia, and the adsorption of HMBG1 may provide an additional benefit in the setting of atherosclerosis, which has been shown to trigger the release of HMGB1 from macrophages [42].

3.3. Adsorption of HMGB1 Derived from Stimulated Peripheral Blood Mononuclear Cells. Stimulation of PBMCs with LPS resulted in a time-dependent release of HMGB1 reaching a peak after 16 h. PBMCs treated with increasing LPS concentrations (0.01–1000 ng/mL) for 16 h released HMGB1 in a concentration dependent manner (Figure 4(a)). PBMC-derived HMGB1 was efficiently removed by both adsorbents tested (243 versus 113 ng/mL adsorbent for Cellufine sulfate and PSDVB-16, respectively, for an initial concentration of 13.7 ng/mL and an adsorbent-to-medium ratio of 5 vol%, Figure 4(b)).

Next to the physicochemical parameters of the adsorbent polymers, modifications of HMGB1 are likely to influence its binding to different polymers. Acetylation, phosphorylation, and oxidation are critical for the diverse biological activities of HMGB1. In an inflammatory environment, the production of reactive oxygen species induces HMGB1 oxidation, leading to a loss of its biological activity and restricting its proinflammatory role in a temporal and spatial manner. Since acetylation, phosphorylation, and oxidation induce changes in charge and conformation of HMGB1, they might influence its binding to different polymers. It is, thus, conceivable that adsorbents preferentially deplete certain subsets of HMGB1 with different biological activity.

Finally, HMGB1 is associated with microvesicles from both activated and apoptotic cells [43]. It may be embedded

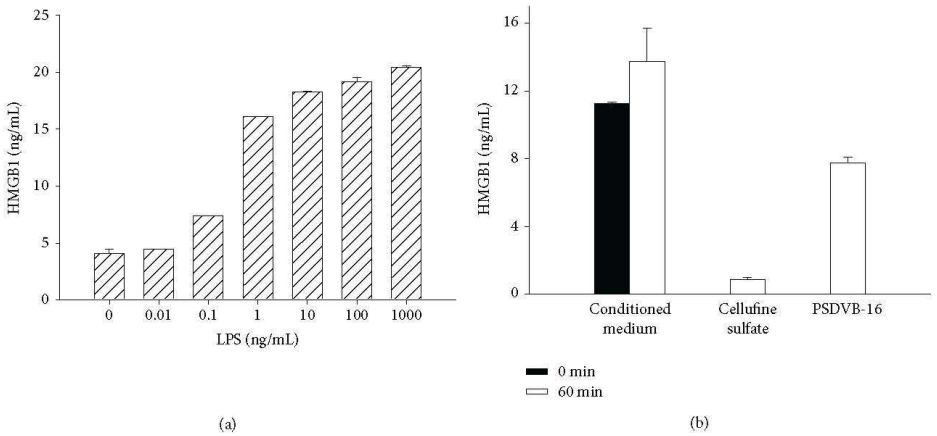


FIGURE 4: (a) Release of HMGB1 after stimulation of PBMCs with increasing concentrations of lipopolysaccharide from *E. coli* for 16 h; (b) PBMC-derived HMGB1 remaining in conditioned medium after 60 min of incubation at an adsorbent-to-medium ratio of 5 vol%; $n = 3$.

into microvesicles or interact with negatively charged phosphatidylserine residues on the microvesicle surface. In any case, this association influences the adsorption of HMGB1 to porous polymers due to increased size of the adsorbate, and different subsets of HMGB1 may preferentially be associated with microvesicles, again leading to selective depletion of HMGB1 variants.

4. Conclusions

Neutral and anionic polymers were tested for their capability to remove recombinant as well as PBMC-derived HMGB1 from human plasma. HMGB1 was efficiently adsorbed by negatively charged beads in a time-dependent manner due to electrostatic forces. It also bound to neutral porous polystyrene-based particles via hydrophobic interactions. Unmodified hydrophilic cellulose adsorbed only negligible amounts of the cytokine, despite its large pores and high surface-to-volume ratio, while Cellulose sulfate bound HMGB1 with high efficiency.

Posttranslational modification and/or oxidation of HMGB1 are critical for the regulation of its biological activity. Since these modifications influence the charge as well as the conformation of HMGB1, subsets with different biological activity may show preferential interaction with adsorbent polymers or with plasma proteins deposited on these polymers, which might open a way to selectively deplete HMGB1 subsets with different biological activity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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2.1.4 Adsorption von ausgewählten Antibiotika an Trägermaterialien, die in der extrakorporalen Blutreinigung eingesetzt werden.

Stephan Harm, Anna Gruber, Franz Gabor, Jens Hartmann: *Adsorption of Selected Antibiotics to Resins in Extracorporeal Blood Purification*. Blood Purif. 2016; 41(1-3):55-63, DOI:10.1159/000440973

Kurzfassung:

Einleitung/Ziel: Extrakorporale Blutreinigungssysteme sind spezifische Adsorber zur Eliminierung von Toxinen und Zytokinen. In dieser Studie sollte geklärt werden, ob Adsorber, welche in Leberunterstützungssystemen eingesetzt werden, gleichzeitig während der Behandlung verabreichte Antibiotika entfernen können.

Material & Methoden: In vitro-Adsorptionsversuche wurden in Humanplasma im Batchtest durchgeführt. Es wurden Adsorber vom Typ Prometheus (Prometh01 und Prometh02) und aus dem Mars System (Dia Mars AC250) untersucht. Die Konzentration der Antibiotika entsprach der empfohlenen intravenösen therapeutischen Dosierung und sie wurden mittels ELISA-Tests und HPLC-Methoden im Plasma quantifiziert.

Ergebnisse: Alle Adsorber bewirkten eine Reduktion der getesteten Antibiotika in Plasma. Dia Mars AC250 reduzierte alle Antibiotika bis nahe an deren Nachweisgrenze.

Schlussfolgerung: Für ein verbessertes und sinnvolles Antibiotika-Monitoring in der extrakorporalen Leber- und Sepsis-Behandlung sind weitere Untersuchungen zur Clearance von Antibiotika unbedingt nötig .

Mein Beitrag für diese Publikation:

- Planung der Versuche
- Verfassen der Publikation
- Analytik: Rasterelektronenmikroskopie
- Datenauswertung

Original Paper

Blood Purification

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Adsorption of Selected Antibiotics to Resins in Extracorporeal Blood Purification

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Key Words
Adsorption · Artificial liver support · Blood purification · Liver failure · Plasmapheresis · Sepsis

Abstract
Background/Aims: Extracorporeal blood purification systems (EBS) use specific adsorbents for the elimination of toxins and cytokines. The aim of this study was to test different adsorbents for their ability to reduce antibiotics in parallel to extracorporeal blood purification therapy. ***Methods:*** The in vitro adsorption experiments were carried out in human plasma with a newly established hydrophobic resin (Amberchrom CG161c) and adsorbents commercially available and approved in the clinics. The concentration of antibiotic was chosen equivalent to the recommended therapeutic dosage applied intravenously and was measured in plasma using ELISA test kits and high-performance liquid chromatography methods. ***Results:*** The adsorbent that reduced all tested antibiotics in plasma close to the detection limit was the dia MARS AC250, which is an activated charcoal involved in the Molecular Adsorbents Recirculation System. ***Conclusion:*** For better antibiotic monitoring in sepsis treatment, further investigations have to be performed to determine the clearance rate of antibiotics by different EBS devices.

Introduction

Extracorporeal blood purification (EBP) is used to 'clean' blood from endogenous and exogenous toxins and waste products by diffusion, convection and/or adsorption using dialysers, hemofilters and adsorbents. The removal of pathological substances by the use of adsorbents, using plasma adsorption or direct hemoperfusion has already been applied clinically. Typical adsorbents used in EBP devices consist of activated charcoal and charged (ionic) or uncharged (non-ionic) resins [1, 2]. A promising field of application for adsorption-based technologies is supportive therapy for sepsis. Sepsis develops when the host immune response to a pathogen or a microbial toxin is accelerated to an inappropriate degree, which often leads to an uncontrolled release of pro-inflammatory cytokines [3–5]. In many cases, progress (the further course) of the disease will lead either to an unbalanced coexistence of pro- and anti-inflammatory mediators (mixed antagonistic response syndrome) or to an excess of anti-inflammatory cytokines, which end up in immunosuppression. Besides pharmacological therapy strategies, EBP techniques were applied to remove endotoxins or to modulate pro- and anti-inflammatory cytokines in septic patients [3]. Two adsorbent-based systems, which are clinically tested, are available

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Table 1. Adsorbents evaluated for their characteristics to remove antibiotics from human plasma

Name	Provider	Characteristics
Prometh01	Fresenius Medical Care Adsorber Tec GmbH, Austria	PS-DVB-based neutral resin, BP[a]
Prometh02	Fresenius Medical Care Adsorber Tec GmbH, Austria	PS-DVB based matrices with quaternary ammonium cations (anion exchanger), BP[a]
dia Mars AC 250	Gambro, Sweden	Activated charcoal, BP[a]
Amberchrom® CG161c	Dow Chemical, US	PS-DVB based neutral resin

[a] BP indicates adsorbents that are commercially used for blood purification.

Table 2. The amount of antibiotic that is generally used in the clinics for one treatment dosage [16]

Name	Amount, g/3,360[a] ml plasma	Concentration, μg/ml	Description	Molecular weight, Da	Protein binding rate, % [8, 17, 18]
Vancomycin	1	298	Glycopeptide antibiotic	1,450	30
Meropenem	2	595	Beta-lactam antibiotic	383	2.4
Piperacillin	4	1,190	Beta-lactam antibiotic	518	20–30
Tazobactam	0.5	149	Beta-lactamase inhibitor	300	20–23
Cilastatin	1	298	Non-antibiotic, enzyme inhibitor	358	41
Imipenem	0.5	149	Beta-lactam antibiotic	299	2
Ciproflaxacin	0.4	119	Fluoroquinolone	331	26.1–31.6
Metronidazole	0.5	149	Nitroimidazole	171	<20
Ofloxacin	0.4	119	Fluoroquinolone	361	32 (manufacturer's data)
Polymyxin B	0.0168	5	Polypeptide antibiotic	1,302	n.a.

[a] The value of 3,360 ml plasma was calculated as the average amount of plasma present in adults (70 kg body weight, 8% blood volume, haematocrit 40%).

for cytokine removal. These are the CytoSorb™ hemoadsorption cartridge and the coupled plasma filtration adsorption (CPFA®) system [4, 5]. Both were capable of decreasing proinflammatory cytokines significantly, but a reduction of mortality in patients with septic shock was not observed [6, 7]. When choosing a combination therapy for the treatment of sepsis, attention has to be paid to the fact that adsorbents do not only bind toxins or other target substances, but also the applied antibiotics [8–10]. Antimicrobial therapy is a very important factor in the treatment of sepsis due to the fact that undesired patient outcomes are highly increased due to inadequate antibiotic therapy [11]. At the beginning of the sepsis treatment, broad spectrum antibiotics against both gram-positive and gram-negative bacteria are administered. They are applied intravenously to ensure quick and efficient action [12]. The factor time is the most important aspect in the therapy of sepsis. The dosage and the too long latency from selection of appropriate antimicrobials to administration lead to higher mortality in patients suffering from severe sepsis and septic shock [13, 14]. For an optimized antibiotic dosage, it would be ideal to know the removal rate of each antibiotic by different EBP procedures. First attempts were started to describe pharmacokinetic characteristics and protein-binding rates, but the rapid development of new membranes hindered the establishment of effective and safe antibiotic therapies [15, 16]. The aim of this study was to evaluate the effect of adsorbents used in the blood purification system on the plasma level of different antibiotics and enzyme inhibitors used in sepsis treatment. The adsorbents and antibiotics that were chosen for the experiments are listed in tables 1 and 2.

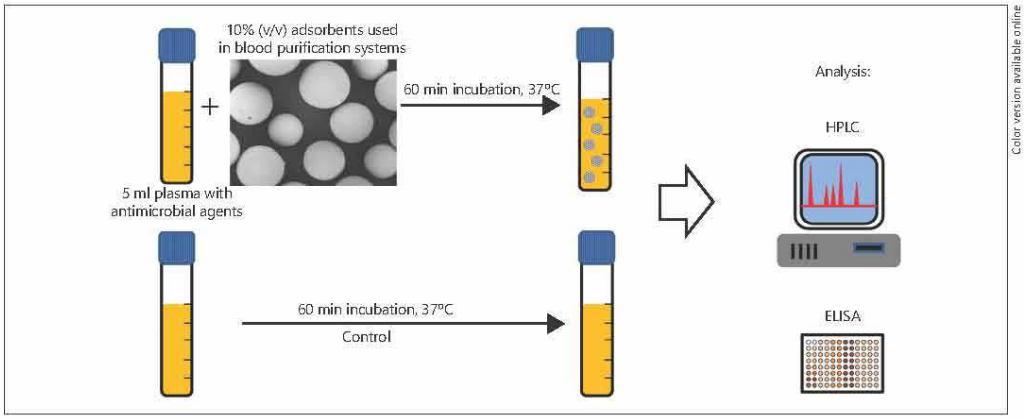


Fig. 1. The setup of the experiments where the adsorption of various antimicrobial agents to certain adsorbents was determined. Five milliliter plasma, spiked with drug was incubated together with 0.5 ml adsorbent material for 60 min at 37°C on a roller mixer. Spiked plasma without adsorbent acts as control. The quantification of each drug was done by ELISA and HPLC.

Materials and Methods

Materials

Acetonitrile (ACN), methanol (both high-performance liquid chromatography (HPLC) grade), acetone, sodium borate, boric acid, MOPS buffer, potassiumdihydrogenphosphate and sodium carbonate were purchased from Sigma Aldrich (St. Louis, Mo., USA). Tetrahydrofuran was purchased from VWR (Pa., USA). Bidistilled water and physiological sodium chloride solution (0.9%) were purchased from Fresenius Kabi, Graz, Austria. All antibiotics and enzyme inhibitors (Vancomycin, Meropenem, Piperacillin, Tazobactam, Cilastatin, Imipenem, Ciprofloxacin, Metronidazole, Ofloxacin, Polymyxin B) were obtained from Sigma Aldrich (St. Louis, Mo., USA). Prometheus® adsorbents Prometh01 (neutral resin) and Prometh02 (anion exchanger) were obtained from Fresenius Adsorber TecKrems, the charcoal used in the Molecular Adsorbents Recirculation System (MARS) system was purchased from Gambro (Gambro Hospal GmbH, Germany) and the CG161c adsorbent, based on hydrophobic poly(styrene-divinylbenzene; PS-DVB) copolymer matrices, was provided from Dow Chemical (Philadelphia, Pa., USA). Fresh frozen citrate-anticoagulated plasma was obtained from a local donation center. Heparin-spiked whole blood was obtained from Rotes Kreuz, Vienna, Austria. The Quinolones ELISA kit by Green Spring, Shenzhen Lvshiyuan Biotechnology Co., Ltd. was purchased via Hölzel Diagnostika, Köln, Germany. The test kit for beta-lactam antibiotics (MaxSignal ELISA kit) was purchased from BIOO scientific, Austin, Tex., USA. The Nitroimidazole ELISA kit was purchased from Beijing Kwinbon Biotechnology Co., Ltd.

Methods

Adsorbent SEM Pictures

The structural characteristics of each adsorbent were determined by scanning electron microscopy (SEM). SEM images were obtained by washing adsorbent particles with pure ethanol and drying them in a heating cabinet at 100°C for 12 h. The particles were than sputtered with gold (Q150RES, QUORUM) and imaged by SEM (TM-1000, Table Microscope, Hitachi).

Particle size distributions of the microspheres were measured by using a laser light-scattering particle size analyser (Mastersizer 2000, Malvern Instruments, Malvern, UK). About 500 µl of microspheres was suspended in 100 ml of distilled water and stirred under sonication to avoid agglomeration of particles during measurements. The particle size distribution results are volume based.

Adsorption Studies in Batch

Batch tests were carried out in human plasma (either freshly prepared plasma or frozen plasma) to determine the amount of antibiotic that is adsorbed by the different adsorbents and compared with each other. A graphical set up of the experiments is shown in figure 1. Prior to the use for adsorption experiments, the adsorbents were washed with ethanol, water and 3 times with 0.9% saline solution for 60 min each using a ratio of one volume part adsorbent and 9 volume parts liquid for each washing step. The batch tests were carried out in a 10% approach which means that 500 µl adsorbent was suspended in 4,500 µl with antibiotic-spiked human plasma. The plasma was spiked with a certain concentration of antibiotic, which is normally used in the clinical formulation (dosage), applied intravenously to the patient. The amount of antibiotic and enzyme inhibitor (table 2) was calculated from the pharmaceutical dosage being applied to a patient [17] and the average plasma amount of adults (3,360 ml plasma/patient). As control, plasma without adsorbents and plasma without antibiotics were included in all batch tests. The samples were incubated for 60 min at 37°C on a roll mixer, and samples were taken after 15 and 60 min. The samples were frozen at –80°C until quantification of antibiotics

Table 3. HPLC settings for quantification of Vancomycin, Meropenem, Cilastatin, Imipenem, Tazobactam and Polymyxin B

	Vancomycin	Meropenem	Cilastatin	Imipenem	Tazobactam	Polymyxin B
Mobile phase	50 mM KH_2PO_4 (pH 3.5):ACN (90:10)	10 mM KH_2PO_4 (pH 3.0):ACN (90:10)	40 mM KH_2PO_4:ACN (88:12)	10 mM borate buffer (pH 7.2):MeOH (95:5)	30 mM KH_2PO_4 (pH 3.0):MeOH (90:10)	ACN:THF:water (50:24:26)
Flow rate, ml/min	1.1	1.0	1.2	1.0	1.0	1.0
Run time, min	10	8	15	8	14	7
Detection wavelength	UV-detector 210 nm	UV-detector 290 nm	UV-detector 216 nm	UV-detector 300 nm	UV-detector 225 nm	Fluorescence detector excitation: 260 nm emission: 315 nm
Injection volume, µl	20	20	20	20	20	20

and enzyme inhibitors was performed by an ELISA kit or HPLC analysis [18, 19].

All batch tests were done in quadruples and the mean results ± SD are given as retaining ratio in percent compared to the control samples without adsorbents; this ratio is calculated as follows:

$$\text{retaining ratio (\%)} = \frac{\text{drug concentration in plasma with adsorbents}}{\text{drug concentration in plasma without adsorbents}} \times 100.$$

Quantification by HPLC

For each antibiotic, a specific HPLC method was applied. For analyses, proteins had to be removed from the plasma samples regarding possible disturbances during the analysis. The protein-removing methods were specific for each antibiotic, depending mostly on the mobile phase, and were carried out either by precipitation with ACN or solid phase extraction (SPE). Protein removing of the Vancomycin samples was carried out with SPE (Sep-Pak® Light C18 cartridges, Waters GmbH, Vienna, Austria) as follows: the SPE columns were equilibrated with 1 ml MeOH and 1 ml distilled water. Then 200 µl samples were transferred to the cartridge. After the cartridge was washed with 500 µl distilled water, the Vancomycin was eluated with 500 µl MeOH:water (50:50). For plasma samples containing Meropenem, Cilastatin or Imipenem, protein elimination was done as follows: in 1.5 ml micro centrifuge tubes, 300 µl plasma was mixed with 300 µl ACN and centrifuged. The supernatant was used for antibiotic quantification. For PMB quantification, 200 µl plasma samples were precipitated with 200 µl ACN. The supernatant was transferred to a solid-phase extraction C18 cartridge (Sep-Pak® Vac RC (100 mg) C18 cartridges, Waters GmbH, Vienna, Austria). After the cartridge was washed with 500 µl of carbonate buffer (1%, w/w, pH 10), 110 µl of 9-fluorenylmethyl chloroformate (FMOC-Cl, Sigma) solution (containing 30 µl of 100 mM FMOC-Cl in ACN and 80 µl of methanol) was added. Following 10 min of reaction in the dark, the PMB derivatives were eluted with 900 µl of acetone. The eluate was mixed with 600 µl of boric acid (0.20 M) and 500 µl of ACN. The HPLC system consisted of a Waters™ 717plus-Autosampler, Waters™ 600-Controller, Waters™ In-Line Degasser, UV-VIS detector (Waters 2487 Dual λ Absorbance Detector) and a fluorescence detector (RF-551 Shimadzu). For all HPLC runs, a C18 column (50 × 4.6 mm Onyx Monolithic C18 column coupled with a 4 × 3.0 mm C18 guard column, Phenomenex) was used with different settings for each antibiotic (table 3).

Quantification by ELISA

For the antibiotics Piperacillin, Ciprofloxacin, Ofloxacin and Metronidazole ELISA kits were available. For each ELISA kit, a different procedure had to be followed and the samples had to be diluted to obtain a concentration within the standard curve of the test kit.

Results and Discussion

Adsorbent Characterisation

SEM Images

Figure 2 SEM images. Electronic microscopy of Prometh01, Prometh02, dia MARS 250 AC and Amberchrom CG161c at 200- and 5,000-fold magnifications was carried out with gold-coated adsorbent samples.

Particle Size Distribution

Figure 3 particle sizes of adsorbents. Size distributions of the tested adsorbents were determined using a laser light-scattering particle size analyser.

Adsorption Studies in Batch

The adsorption of antibiotics onto adsorbent materials is highly dependent on the chemical structure of the specific antibiotic, the chemical structure of the adsorbent and the specific binding forces and interactions.

Prometh01, a hydrophobic (neutral) resin, which is commercially used in extracorporeal liver support for the removal of aromatic amino acids, bile acids and other hy-

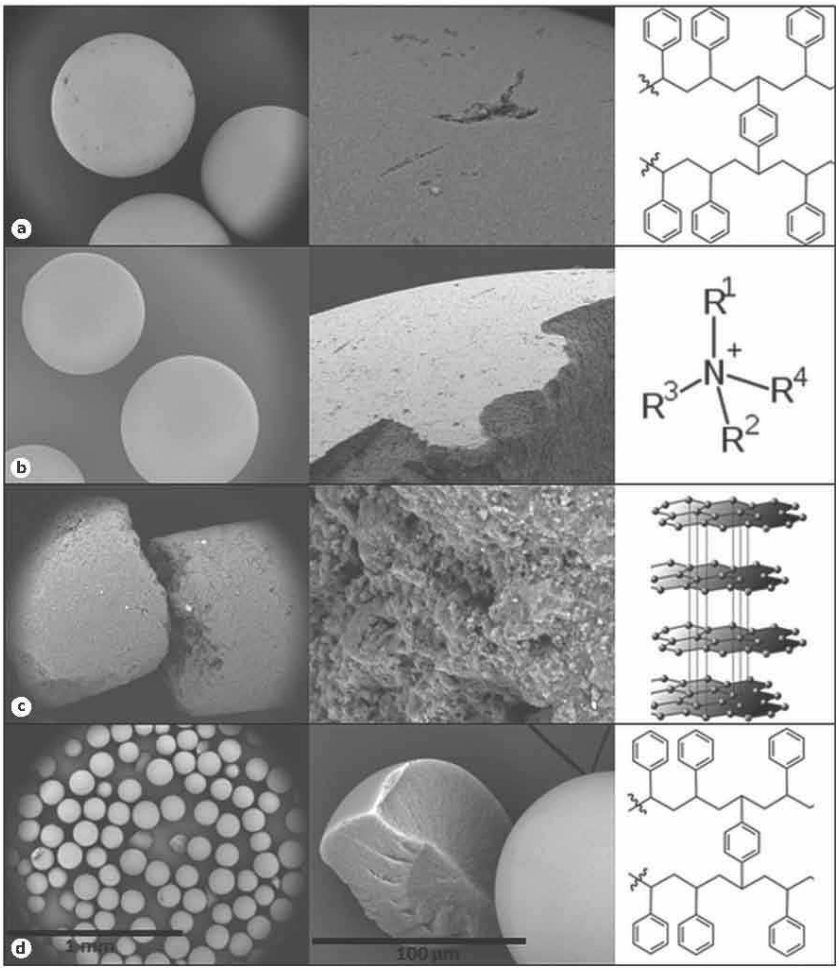
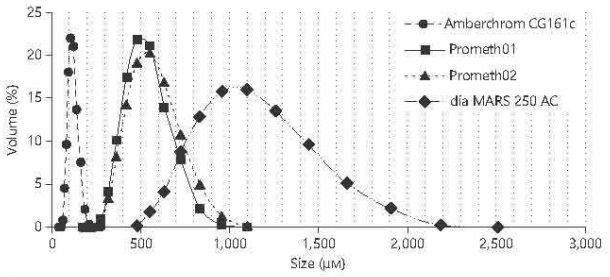


Fig. 2. SEM images. Electronic microscopy of the tested adsorbents at 200- and 5,000-fold magnifications was carried out with gold-coated adsorbent samples. The scale bare in D applies for the whole column. **a** Prometh01, a hydrophobic resin consists of polystyrene-divenylbenzene (PS-DVB), where polystyrene macromolecules are cross-linked by divenylbenzene; **b** Prometh02 an anion exchanger, which has quaternary ammonium cations on the surface; **c** dia MARS 250 AC is an activated charcoal; **d** Amberchrom CG161c is a neutral resin and consists of the same material like Prometh01 and differs only in particle and pore size.



Adsorbent	D(0.1) [µM]	D(0.5) [µM]	D(0.9) [µM]	Volume weighted mean D [µM]
CG161c	86	117	158	120
Prometh01	395	538	730	551
Prometh02	404	566	789	584
dia MARS 250 AC	481	929	1,383	910

Fig. 3. Particle diameters of adsorbents. Size distributions of the tested adsorbents were determined using a laser light scattering particle size analyser. D(0.5) is the median diameter of the volume of distribution, D(0.1) indicates that 10%, while D(0.9) indicates that 90% have a smaller diameter than the value.

drophobic toxic compounds, shows a high adsorption rate for all tested antibiotics. The results (fig. 4 and table 4) show that the adsorption kinetics are slower due to the larger particle size of 200–800 µm. There are long diffusion distances for the adsorbate to reach the inner surface of the adsorbent. Ciprofloxacin, Metronidazole, Ofloxacin and Polymyxin B were reduced to a level below 10% of the initial value after 60 min reaction time. Meropenem, Piperacillin and Tazobactam were removed below 20% and Vancomycin, Cilastatin and Imipenem were reduced to a value between 40 and 20% of the starting concentration in plasma. In general, substances that contain hydrophobic groups are adsorbed very fast and effective by neutral resins based on hydrophobic interactions. The anion exchanging resin Prometh02, which is used in the Prometheus® device for bilirubin removal, shows the weakest adsorption of all tested adsorbents. Only the antimicrobial agent Tazobactam, which contains an anionic carboxyl group, was reduced to a level of 30% after 60 min incubation time.

DiaMARS AC 250, a porous activated charcoal, is used in the commercially available MARS, which is a liver-support system in intensive care units. Similar to Prometh01, the adsorption kinetics of the tested antimicrobial agents is slow, due to its large particle size of about 900 µm. With the exception of Vancomycin and Piperacillin, which were reduced below 20%, all other tested agents were nearly totally removed from the plasma after 60 min incubation time. Activated charcoal, in general, is due to its effective, unspecific and universal characteristics for many drugs and chemicals used for decontamination treatment and for most poisonings. Substances are bound by weak electrostatic forces between the activated charcoal's carbon and the side chains of carbon-based molecules, known as van der Waals forces [20].

The Amberchrom CG161c is a hydrophobic resin like Prometh01, but it has smaller particle size (120 µm) and larger pores (15 nm according to manufacturer) on the surface. It is a promising adsorbent for removing of cytokines and protein-bound toxins for sepsis treatment [21]. The results for the adsorption of the antibiotics show that Amberchrom CG161c removes the antibiotics quickly and efficiently. In case of Tazobactam, Meropenem, Cilastatin, Piperacillin, Imipenem and Metronidazole, the kinetics show that the adsorption rate in the first 15 min is higher than after 60 min. This is at least partly related to their lower molecular weight (table 2). Small substances are adsorbed very fast at the beginning, even if their affinity to the adsorbent is low. Other substances diffuse slower through the small pores, but have higher binding constants to the adsorbent material. Because of the higher affinity, they are able to displace the antibiotics by competitive binding. This phenomenon is known as the Vroman effect, which describes the desorption of smaller substances over time caused by the competitive adsorption of larger plasma proteins with higher binding affinity [22, 23].

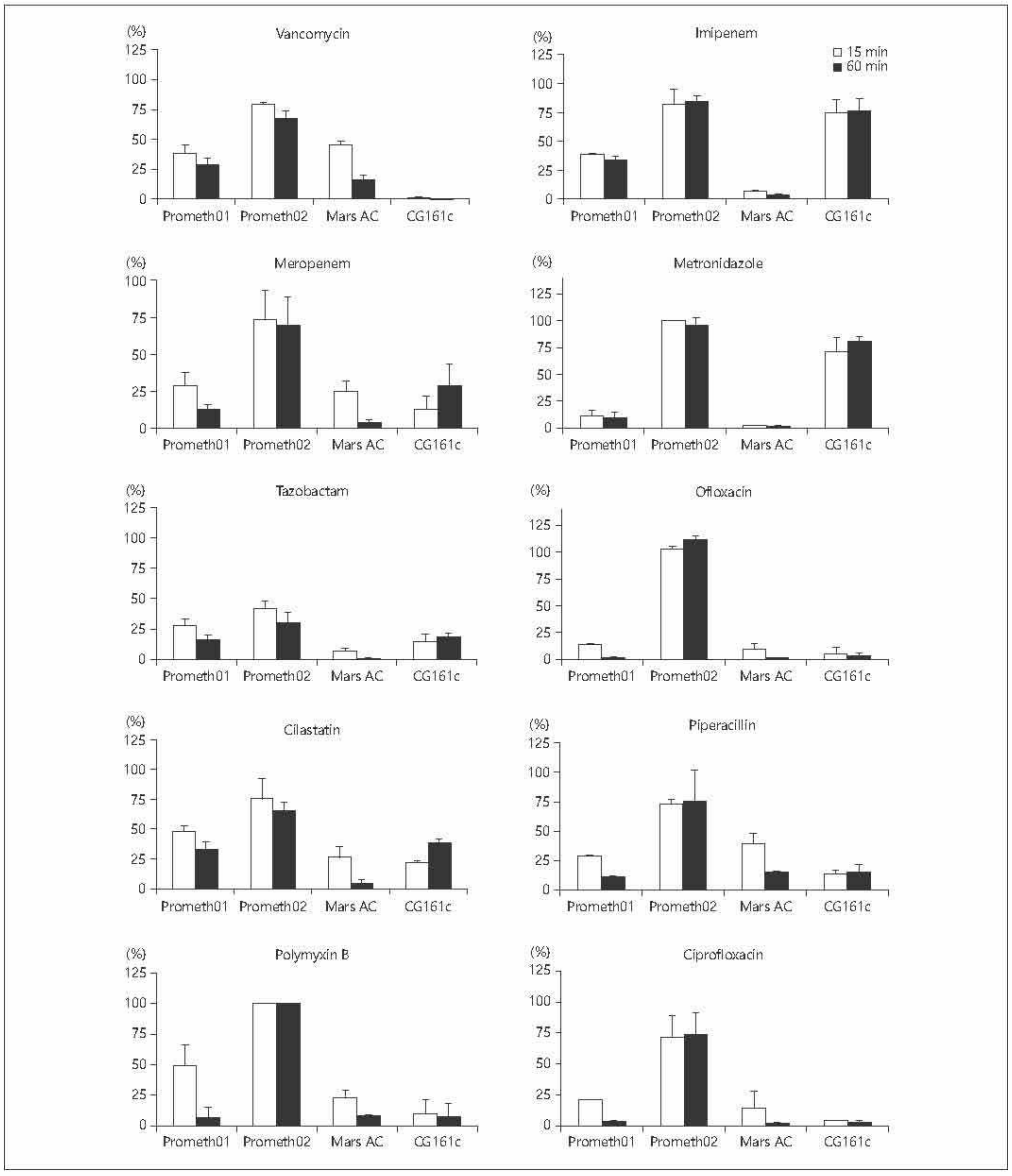


Fig. 4. Comparison of the retaining ratio (%) of various antimicrobial agents in plasma after incubation with different adsorbents. The white bares show the drug level at time point 15 min and the stripped bares show the retaining drug level in plasma after 60 min incubation with the adsorbents.

Table 4. Comparison of retaining ratio (%) of various antibiotics in plasma using different adsorbents in batch tests (mean ± SD, n = 4). The drug level was determined after 15 and 60 min of incubation with each adsorbent

	Prometh01		Prometh02		dia MARS AC 250		Amberchrom CG161c	
	15 min	60 min	15 min	60 min	15 min	60 min	15 min	60 min
Vancomycin	38±7	29±5	79±2	67±5	45±3	16±4	1.0±0.3	0.4±0.1
Meropenem	28±9	13±3	73±20	70±19	24±8	4.7±1.7	13±9	30±14
Piperacillin	29±1	12±1	73±4	75±26	40±8	15±1	14±4	16±6
Tazobactam	28±5	17±3	42±6	30±8	7±2	0.7±0.1	15±6	18±3
Cilastatin	48±5	33±5	75±16	67±7	26±9	4.9±3.2	22±2	39±3
Imipenem	39±1	34±4	82±13	85±5	7.0±0.6	3.7±0.1	74±12	77±11
Ciprofloxacin	21±0.5	3.6±0.5	71±17	74±17	15±13	2.3±1.0	4.1±0.4	2.7±1.9
Metronidazole	11±5	9.0±5.6	100±0	95±7	2.4±0.2	1.6±1.2	72±12	81±5
Ofloxacin	13±1	1.6±0.3	100±3	112±4	9.8±4.9	1.1±0.1	6.2±2.4	4.9±2.1
Polymyxin B	49±17	9.2±6.5	100±0	100±0	23±6	8.6±0.0	9.9±3.1	7.5±3.0

The adsorption is dependent on the chemical structure of the antibiotic and the used adsorbent. Concerning the hydrophobic resins Prometh01 and Amberchrom CG161c, especially antibiotics that possess hydrophobic parts in their structure are prone to be removed. Using activated charcoal in blood purification would completely remove these antibiotics. In case of patients who receive immunosuppressives, a parallel treatment with an extracorporeal blood purification system (EBS) such as MARS would remove the drug and consequently could lead to transplant rejection. The adsorption characteristic of anionic resins such as Prometh02 is well predictable if the chemical structure of the antibiotics is known.

The chemical characteristics of the adsorbents as well as the particle and pore size play a major role in the adsorption of antibiotics and other drugs. Large particles cause slow adsorption kinetics due to long diffusion distances. Furthermore, the pores of adsorbents act as molecular sieves and exclude molecules with a larger molecular weight than the molecular cut off of the pores [21]. In general, when EBP and antibiotic treatment are applied simultaneously or close to each other, the removal of the antibiotic has to be considered in order to optimize the treatment.

Conclusion

Adsorbent particles with different surface characteristics show different removal rates for various antibiotics and enzyme inhibitors in plasma. It can be concluded that if antibiotic therapy is combined with adsorption-based EBP simultaneously, the drug level has to be monitored. Since the removal of toxic substances in liver failure and sepsis has a high priority, the outcome of our study should not prevent the use of a combined therapy (EBS & antibiotics), but shows the necessity of an adaptation of the antibiotic dosage.

However, for treatment of patients with drug overdose or abuse, EBS such as the Prometheus® system has been applied successfully [24]. Especially protein-bound drugs that are difficult to remove with membrane-based blood purification systems [25] could be potential targets for the removal with these systems.

Further systematic investigations that measure the influence of EBP treatment onto plasma levels of drugs have to be conducted. Especially in vivo data would be helpful for antibiotic dosage adjustment because in vitro experiments don't consider the drug pharmacokinetics. An online antibiotic monitoring during treatment is clinically not applicable because routine laboratories do not offer these analytics. The practicable way would be to create clearance data for each EBP system, which enable the calculation of the adapted dosage for each drug during or after EBP treatment.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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2.1.5 Charakterisierung von Adsorbern zur Zytokinentfernung aus dem Blut an Hand eines *in vitro* Modells

Stephan Harm, Franz Gabor, Jens Hartmann: *Characterization of Adsorbents for Cytokine Removal from Blood in an In Vitro Model*. J Immunol Res. 2015; 2015:484736, DOI:10.1155/2015/484736

Kurzfassung:

Einleitung/Ziel: Extrakorporale Blutreinigungsverfahren werden in der Sepsis- und Lebertherapie eingesetzt, um unter anderen auch Zytokine aus dem Blut zu entfernen. Obwohl es verschiedene Adsorber (Cytosorb®, Bellco®) kommerziell erhältlich sind, ist der Erfolg in ihrer klinischen Anwendung begrenzt. In dieser Arbeit wurdedie Elimination von Zytokinen aus dem Plasma mit verschiedenen Adsorbern getestet. Weiteres wurde überprüft, in wie weit die Reduktion der Zytokinspiegel im Plasma die Endtothelzellen-Aktivierung beeinflusst.

Material & Methoden: Für die Zytokin-Adsorptionsversuche wurden drei Polystyrol-Divinylbenzol (PS-DVB) basierte Adsorber: Amberchrom® CG161c, CG300m und ein klinisch zugelassener Hämoperfusions-Adsorber (Cytosorb®) verwendet. Um die Zytokinfreisetzung aus Leukozyten zu induzieren, wurde frisches Blut vier Stunden lang mit 1 ng/ml LPS stimuliert. Danach wurde das zytokinhaltige Plasma abgetrennt und mit diesem die Adsorptionsexperimente in einem dynamischen Modell durchgeführt. Der Einfluss der Zytokinentfernung auf die Aktivierung von Endothelzellen wurde mit Hilfe eines Endothelzell-basierten Zellkulturmodells (Human umbilical vein endothelial cells, HUVEC) untersucht. Zusätzlich wurde die Mindestkonzentration für die HUVEC-Aktivierung durch TNF-α und IL-1β bestimmt.

Ergebnisse: Der CG161c Adsorber war für die Entfernung von Zytokinen aus dem Plasma am effektivsten. Zusätzlich konnte dieser im Vergleich zu den anderen untersuchten Adsorbern auch TNF-α großteils entfernen. Die CG161c-Behandlung reduzierte die Zytokinsekretion und Expression von Zelladhäsionsmolekülen durch HUVEC und unterstreicht die Bedeutung einer effektiven Entfernung von TNF-α bei entzündlichen Erkrankungen.

Schlussfolgerung: Diese Ergebnisse bestätigen die Hypothese, dass die Zytokinspiegel im Blut durch extrakorporale Adsorber auf physiologische Niveaus abgesenkt werden können, um die Aktivierungsrate der Endothelzellen zu reduzieren.

Mein Beitrag für diese Publikation:

- Planung der Versuche
- Verfassen der Publikation
- Durchführung der Tests
- Analytik: Größenausschlusschromatographie, Messung der Größenverteilung
- Datenauswertung

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Research Article

Characterization of Adsorbents for Cytokine Removal from Blood in an *In Vitro* Model

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Introduction. Cytokines are basic targets that have to be removed effectively in order to improve the patient's health status in treating severe inflammation, sepsis, and septic shock. Although there are different adsorbents commercially available, the success of their clinical use is limited. Here, we tested different adsorbents for their effective removal of cytokines from plasma and the resulting effect on endothelial cell activation. *Methods.* The three polystyrene divinylbenzene (PS-DVB) based adsorbents Amberchrom CG161c and CG300m and a clinically approved haemoperfusion adsorbent (HAC) were studied with regard to cytokine removal in human blood. To induce cytokine release from leucocytes, human blood cells were stimulated with 1 ng/ml LPS for 4 hours. Plasma was separated and adsorption experiments in a dynamic model were performed. The effect of cytokine removal on endothelial cell activation was evaluated using a HUVEC-based cell culture model. The beneficial outcome was assessed by measuring ICAM-1, E-selectin, and secreted cytokines IL-8 and IL-6. Additionally the threshold concentration for HUVEC activation by TNF-α and IL-1β was determined using this cell culture model. *Results.* CG161c showed promising results in removing the investigated cytokines. Due to its pore size the adsorbent efficiently removed the key factor TNF-α, outperforming the commercially available adsorbents. The CG161c treatment reduced cytokine secretion and expression of cell adhesion molecules by HUVEC which underlines the importance of effective removal of TNF-α in inflammatory diseases. *Conclusion.* These results confirm the hypothesis that cytokine removal from the blood should approach physiological levels in order to reduce endothelial cell activation.

1. Introduction

Sepsis is a systemic inflammatory response syndrome (SIRS) that results from the body's innate immune response triggered by any of the several infectious stimuli. Lipopolysaccharides (endotoxins), peptidoglycan, flagellin, lipoteichoic acid from bacteria, mannan from fungi, and other antigens from infectious agents stimulate monocytes and macrophages to release tumour necrosis factor alpha (TNF-α) as well as interleukins 1 and 6 (IL-1, IL-6) into the circulation [1–4]. These again activate additional proinflammatory pathways within endothelial cells and leukocytes. A very high and uncontrolled release of proinflammatory cytokines also stimulates leukocytes to release anti-inflammatory mediators and transforming growth factor-beta, which inhibit the synthesis of proinflammatory cytokines and exert direct anti-inflammatory effects on monocytes, macrophages, and endothelial cells [5]. In many cases progress (the further course) of the disease will lead either to an unbalanced coexistence of pro- and anti-inflammatory mediators (mixed antagonistic response syndrome) or to an excess of anti-inflammatory cytokines which end up in immunosuppression. This so-called sepsis-induced "immunoparalysis" is characterized by restricted innate and adaptive immune responses, including enhanced apoptosis and dysfunction of lymphocytes and impaired phagocyte functions [6]. A sensitive balance between proinflammatory and anti-inflammatory response is necessary for cytokine release to achieve homeostasis. Attempts were made to restore the cytokine imbalance by using anticytokine monoclonal antibodies. These attempts, where particular cytokines were blocked, yielded no clinically detectable benefits but

indicated that the modulation of several cytokines at the same time to reach rather physiological blood levels may help to achieve homeostasis [7]. Consequently, extracorporeal blood purification (EBP) techniques were applied to modulate pro- and anti-inflammatory cytokines of sepsis patients. Currently, there are four main techniques in clinical use for cytokine removal: high-flux hemofiltration, high cutoff membranes, adsorption techniques, and combined plasma filtration adsorption [7]. A hemoperfusion cartridge that is used for cytokine removal in intensive care medicine is the Cytosorb cartridge, which is filled with 300 mL hemadsorption beads [8]. Cytosorb hemadsorption beads are porous polystyrene-divinylbenzene (PS-DVB) particles coated with biocompatible polyvinylpyrrolidone exhibiting 450 μm average particle diameter and 0.8–5 nm pores [9, 10]. Another device for cytokine removal is the Coupled Plasma Filtration Adsorption (CPFA). CPFA is an extracorporeal therapy that was developed and patented by Bellco for the treatment of patients with multiorgan failure or sepsis. CPFA combines plasma sorption and hemofiltration for cytokine elimination in patients' blood. The unspecific removal of inflammatory mediators is achieved by an Amberchrom adsorbent [11]. This hydrophobic polystyrene resin with an average pore size of 30 nm has a high affinity and capacity for many cytokines and mediators [12]. Both adsorbents were clinically tested and capable of decreasing proinflammatory cytokines significantly, but a reduction of mortality in patients with septic shock was not observed [13, 14]. Probably the removal rate of cytokines was not sufficient to reach homeostasis. In a previous study conducted by our group, the optimal pore size for cytokine removal was investigated [15] and revealed that the Amberchrom CG161c, a neutral PS-DVB based adsorbent with 15 nm pores, shows promising results for cytokine removal from human plasma. The aim of this study was to compare, by *in vitro* experiments using human plasma, the capability of cytokine removal between the new CG161c adsorbent and the two PS-DVB based cytokine adsorbents available for clinical use. Furthermore, the consequence of the level of cytokine removal achieved by each adsorbent on endothelial cell activation was tested using human umbilical vein endothelial cells (HUVECs).

2. Materials and Methods

2.1. Materials. The clinically approved hemoperfusion adsorbent for cytokine removal (HAC) was obtained from Euromed (Euromed GmbH, Vienna, Austria) and the two Amberchrom adsorbents CG300m and CG161c were provided by Dow Chemical (Philadelphia, PA, USA). Tetrahydrofuran, toluene, and polystyrene standards for inverse size exclusion chromatography (iSEC) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and ethanol was obtained from VWR (Vienna, Austria). Blood bags were ordered from the Red Cross (Vienna, Austria) and the Bio-Plex cytokine array was purchased from Biorad (Bio-rad, Vienna, Austria). Recombinant TNF-α and IL-1β were obtained from R&D Systems (Minneapolis, MN). Hank's Balanced Salt Solution, cell culture medium M199, penicillin, streptomycin, fetal bovine serum (FBS), endothelial cell growth supplement (ECGS), and HEPES buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Adsorbent Characterization

2.2.1. SEM. The structural characteristics and accessible pore size of each adsorbent were determined by scanning electron microscopy (SEM) and inverse size exclusion chromatography. The adsorbent particles were washed with pure ethanol and dried at 100°C for 12 hours. The particles were then sputtered with gold (Q150R ES, QUORUM) and imaged by SEM (TM-1000, Table Microscope, Hitachi).

2.2.2. Particle Size. Particle size distributions of the microspheres were determined by laser-light scattering (Mastersizer 2000, Malvern Instruments, Malvern, UK). Approximately 500 μL of microspheres suspension was dispersed in 100 mL distilled water and sonicated to avoid agglomeration of particles during measurements. The particle size distribution results are volume based.

2.2.3. Pore Size. Inverse size exclusion chromatography was used to determine the accessible pore size and intraparticle porosity of each adsorbent based on the retention of toluene and polystyrene standards with molecular masses between 0.5 and 1,000 kDa. For this purpose, each adsorbent was flow packed in 0.46 × 15 cm HPLC columns from Grace Davison Discovery Sciences (GRACE). A Waters HPLC System (Milford, USA) with a Waters 2487 UV detector was used to determine the retention volume of individual standards after injection of 20 μL samples containing 10 mg/mL polystyrene at a flow rate of 0.5 mL/min. The retention volume of each polystyrene standard was experimentally determined and the SEC distribution coefficient has been calculated according to the following:

$$K_d = \frac{V_R - V_0}{V_T - V_0}, \tag{1}$$

where V_R is the retention volume, V_0 is the interparticle void volume, and V_T is the total mobile phase volume. The mobile phase was represented by Tetrahydrofuran. Toluene was used as a small molecule tracer and acetonitrile only for washing. K_d values range between 0, for a compound that is excluded completely corresponding to polystyrene with a molecular mass of 1,000 kDa, and 1, for compounds able to access and permeate the total pore volume represented by toluene with a molecular mass of 92 Da. Since $(V_T - V_0)$ represents the intraparticle mobile phase volume, K_d represents the extent of permeation for molecules into the pore volume of the stationary phase. The following correlation was used in order to interrelate the molecular mass M_W of a polystyrene sample and the size of the pores (Φ) from which it is excluded:

$$M_W = 2.25 \times \Phi^{1.7}, \tag{2}$$

where the pore size diameter is given in Å [16, 17].

The adsorbent porosity ε_P was calculated from the following [18, 19]:

$$\varepsilon_P = \frac{V_T - V_0}{V_B - V_0}, \quad (3)$$

where V_B is the column bed volume.

The pore volume (V_P) of the adsorbent materials was calculated according to

$$V_P = V_T - V_0. \quad (4)$$

2.3. An In Vitro Sepsis Model. The three PS-DVB based adsorbents Amberchrom CG161c, Amberchrom CG300m, and HAC were studied in a dynamic model with regard to cytokine removal in human plasma. Furthermore, the effect of cytokine removal on endothelial cell activation was evaluated using human umbilical vein endothelial cells (HUVECs). This model comprises three steps: whole blood stimulation, the adsorption study in a dynamic model, and the cell culture model (see Figure 1).

2.3.1. Whole Blood Stimulation. Blood bags containing between 400 and 500 mL fresh donated blood were ordered from the Red Cross (Vienna, Austria). The overproduction of cytokines by leucocytes was induced by stimulating human blood with 1 ng/mL LPS from *E. coli* (Sigma, St. Louis, MO, USA) at 37°C for 4 hours. The plasma, including the inflammatory mediators, was separated by centrifugation at 3000 ×g for 10 min and then stored at −80°C until adsorption experiments were performed in a dynamic model.

2.3.2. Adsorption Studies in a Dynamic Model. The dynamic model consists of a commercially available 5 mL Rezorian cartridge (Sigma, St. Louis, MO, USA) packed with 5 mL of adsorbent material. The bead volume of the cartridge was downscaled (approximately 60x) in comparison to the 300 mL cartridge which is normally used clinically for the HAC device. The recirculation reservoir volume, 60 mL, and flow rates, 1 mL/min (55 cm/h), used in the experiments were also scaled down from clinical hemadsorption, 100 to 300 mL/min (212–635 cm/h), and a total blood volume of 4 to 6 liters in the average adult, using this factor (see Figure 1). A circuit with an empty cartridge acted as a control. The experiment was carried out for 6 hours at 37°C, and samples were taken hourly and stored at −80°C until cytokine quantification using the Bio-Plex cytokine array and the cell culture model for endothelial cell activation were performed. In order to ensure the plasma stability during the experiment, albumin, total protein, antithrombin III, protein C, and fibrinogen were measured at the beginning and at the end of the experiment using a Hitachi/Roche 902 automated analyser with the according test kits (Roche, Penzberg, Germany).

2.3.3. Endothelial Cell Activation

(1) Cell Culture. The effect of cytokine removal on endothelial cell activation was evaluated using a cell culture model with HUVEC. The beneficial outcome was assessed by measuring the adhesion molecules ICAM-1 and E-selectin and an array of secreted cytokines after incubation of HUVEC with 10% of plasma from the adsorption experiments. Primary HUVECs were isolated from umbilical cord veins provided by the local hospital (LKH-Krems, Austria) after informed

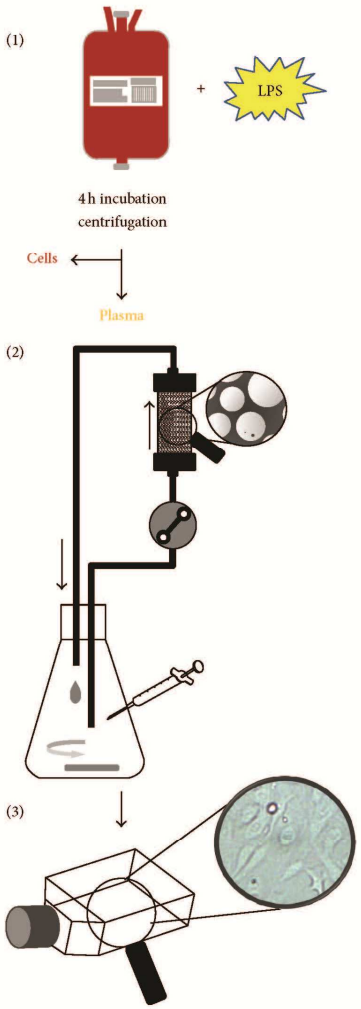

FIGURE 1: Schematic procedure of the experiments. The experiments were conducted in three parts: (1) blood stimulation and centrifugation, (2) adsorption experiments by a dynamic model, and (3) cell culture model with HUVEC.

consent of the donors and stored at 4°C in sterile HBSS. HUVECs were isolated according to Marin et al. with minor changes [20]. Cannulated umbilical veins were perfused with M199 containing 0.02 M HEPES and 100 mM penicillin-streptomycin (M199/HEPES/PS) at 37°C to remove the blood. The veins were filled with dispase (BD Biosciences Europe, Vienna, Austria) and incubated at 37°C for 15 min. After incubation, the dispase solution containing the HUVEC was collected by perfusion of the cord with basal medium (M199/HEPES/PS). The cells were collected by centrifugation at 500 ×g for 5 min and resuspended in growth medium (M199/HEPES/PS containing 20% FBS, 15 IU/mL heparin, and 10 mg/mL ECGS) and transferred to a 75 cm^2 cell culture flask. One day after isolation, cells were washed with basal medium and supplied with fresh growth medium. Isolated HUVECs were used between passages 4 and 7 for the assays.

(2) Stimulation of HUVEC. HUVECs (8.5×10^5) were seeded in 25 cm^2 cell culture flasks with 5 mL growth medium and incubated for 24 hours at 37°C and 5% CO_2. The cell activation tests were performed after cell vitality and near confluency were confirmed by microscopy, as follows: plasma samples from the adsorption experiments were thawed and diluted 1 : 10 with 5 mL of basal medium. The HUVEC monolayer was washed once with basal medium and the sample medium was added to the corresponding cell culture flask. The cells were incubated with the sample medium and control medium (basal medium including 10% native plasma) for 16 hours at 37°C and 5% CO_2 atmosphere. After incubation the supernatants were centrifuged for 10 min at 1000 g, aliquoted, and stored at −80°C until cytokine analysis by the Bio-Plex cytokine array. The cells were washed with 3 mL of ice-cold PBS and detached with 1.5 mL 0.02% EDTA per flask. After addition of 3 mL PBS, cells were pelleted at 500 ×g for 5 min and used for flow cytometry analysis.

(3) Flow Cytometry Analysis. The detached cells were counted and aliquots of 2.5×10^5 cells per sample were prepared in FACS tubes. The cells were washed with ice-cold PBS and stained by incubation with FITC-conjugated anti-CD31, PE-conjugated anti-ICAM-1, PE-Cy5-conjugated anti-E-selectin (BD, Franklin Lakes, NJ, USA), or the corresponding control antibodies for 30 min on ice in the dark. All antibodies were from the IgG isotype. After two further washing steps with PBS, cells were analysed on a flow cytometer (Cytomics FC 500 MPL, Beckman Coulter, CA, US), using the FlowJo 7.6.5 software (Tree Star Inc., Ashland, OR, USA).

2.4. TNF-α and IL-1β Dependent Activation of HUVEC in the Cell Culture Model. To evaluate the level, to which the cytokines have to be lowered by any extracorporeal treatment, for preventing or reducing the endothelial cell activation, a separate experiment was performed. Heparinized (5 IU/mL) human plasma with different recombinant TNF-α and IL-1β concentrations (0, 50, 100, 500, 1000, 5000, and 10000 pg/mL) was used in our cell culture model to determine their threshold level for endothelial cells activation. The HUVECs were cultivated with sample medium (as described above) including 10% of spiked plasma which leads to a tenfold dilution of the spiked recombinant cytokines. After 16 hours of incubation, the supernatants were aspirated, centrifuged for 10 min at 1000 ×g, aliquoted, and stored at −80°C until IL-8 and IL-6 were quantified by the Bio-Plex cytokine array. To verify the expression of the adhesion molecules ICAM-1 and E-selectin, the HUVECs were washed and analysed by flow cytometry as described above.

3. Results and Discussion

3.1. Adsorbent Characterization

3.1.1. SEM, Particle Size, and Inverse Size Exclusion. SEM of the manually cracked particles illustrates that the outer thin shell of the adsorbent particles acts as a molecular sieve for entering the inner surface, which is the adsorbent surface for the target molecules (see Figure 2 and Table 1). The K_d values obtained for toluol and polystyrene probes from the iSEC experiments are shown in Table 2. Complete molecular exclusion is achieved when the K_d value reaches zero at a certain molecular weight. For the largest pore size, an acceptable K_d was defined with a value of 0.1, which means that molecules with a K_d of 0.1 are allowed to pass through the outer pore shell and reach the inner adsorbent surface. As shown in Table 2, K_d approaches 0.1 at a pore size between 10.0 and 16.2 nm for CG161c, 20.6 and 26.0 nm for CG300m, and 7.6 and 10.0 nm for HAC. The porosities ε_P (see Table 2) of the three tested adsorbent particles were similar and always above 80%. HAC was found to have the highest porosity at 86.6% followed by CG161c, 86.2%, and CG300m, 82.3%.

3.2. Adsorption Studies in Dynamic Model. The ability of the adsorbents to remove cytokine was investigated in dynamic model experiments using inflammatory mediator rich human plasma obtained after whole blood stimulation. The concentrations of the following cytokines were measured hourly over a 6-hour period: TNF-α, IL-1β, IL-6, IL-8, and IL-10. These cytokines are considered to be key factors as well as markers in inflammation [21–23]. However, there are many other cytokines and mediators involved in this complex and dynamic process. The interleukins were efficiently removed by both CG161c and CG300m at comparable levels. HAC performed consistently worse than the other adsorbents for all tested cytokines. Sufficient TNF-α removal could only be observed in case of CG161c (Figure 3 and Table 3) with a removal rate of 94.3 ± 0.23%. The two commercially available cytokine adsorbents offered limited removal of TNF-α: 63.5± 0.5% for CG300m and 53.4 ± 6.8% for HAC. The molecular mass of TNF-α ranges from 17 to 51 kDa depending on oligomerization, that is, monomer, dimer, or trimer. The homotrimer is the most active form of TNF-α, which is the largest cytokine with respect to the crystal structure and viscosity radius [15]. Because of the large size of the trimer, the removal of TNF-α from the bloodstream represents a considerable significant challenge. The mechanism of adsorption of the three adsorbents under investigation is the same. The target molecules have to enter the pores of the outer surface to reach the inner surface composed of PS-DVB copolymer, where they will be adsorbed. The particle size, the pore size

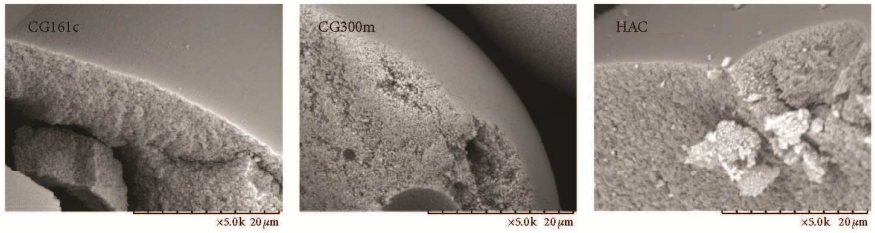

FIGURE 2: Imaging and particle size of the used adsorbents. SEM images at 5000x magnification and particle size distribution using laser-light scattering of the three tested adsorbents.

TABLE 1

Adsorbent	$D(0.1)$ [μm]	$D(0.5)$ [μm]	$D(0.9)$ [μm]	Vol. weighted mean D [μm]
CG300m	60	82	112	84
CG161c	86	117	158	120
HAC	363	492	656	504

(determined by iSEC), and the blood compatible outer PVP layer in case of HAC are the only differentiation factors suggesting that the reduced adsorption is primarily due to the different pore sizes of the adsorbents. When the pores are too small, TNF-α cannot enter the adsorbent beads to be immobilized at the inner surface. Contrariwise, if the pore size is too large, bound TNF-α may be replaced by high molecular weight plasma proteins and plasma lipids due to their high binding affinity via hydrophobic interactions according to the Vroman effect [24]. There was no significant change in the parameters (albumin, total protein, antithrombin III, protein C, and fibrinogen) which were observed in order to ensure plasma stability during the experiment (data not shown).

3.3. Cell Culture Model. The endothelium takes part in the regulation of numerous physiological functions and lies at the interface of circulating blood and the vessel wall. Under physiological conditions, it is responsible for anticoagulant and antiadhesive properties and it regulates vasomotor tone and vascular homeostasis. Endothelial dysfunction has been associated with many pathophysiological processes, such as inflammation and oxidative stresses. Endothelial cells are precociously exposed to circulating signalling molecules and physical stresses, like in sepsis and septic shock [25]. It is well known that sepsis in humans is associated with activation of the endothelium as evidenced by increased levels of expressed ICAM-1 and E-selectin and secreted cytokines/chemokines such as IL-6 and IL-8. To test whether cytokine removal has a positive effect on endothelial cell activation, the treated plasma derived from the adsorbent experiments was used to stimulate HUVEC. The results of the flow cytometry analysis indicate that the CG161c adsorbent is most effective at reducing the expression of cell adhesion molecules by HUVEC.

TABLE 2: Summary of inverse size exclusion chromatography. The pore size of the adsorbents was determined by SEC using polystyrene standards. R_S: stokes radius; K_d: SEC distribution coefficient.

M_r	R_S [nm]	K_d CG161c	K_d CG300m	K_d HAC
92	0.18	1.00	1.00	1.00
570	0.51	0.82	0.85	0.56
1920	1.05	0.67	0.76	0.29
3460	1.48	0.59	0.70	0.21
9630	2.71	0.41	0.60	0.11
17300	3.82	0.29	0.53	0.10
27500	5.01	0.15	0.44	0.08
62300	8.11	0.02	0.26	0.04
96000	10.46	0.00	0.13	0.03
139000	13.00	0.00	0.07	0.02
319000	21.19	0.00	0.01	0.01
524000	28.37	0.00	0.00	0.00
925000	39.63	0.00	0.00	0.00
		CG161c	CG300m	HAC
$V_{T\,(\text{Toluol})}$ [mL]		2.30	2.25	2.31
$V_{0\,(\text{PS1000 kDa})}$ [mL]		1.06	1.14	1.13
$V_{B\,(\text{column})}$ [mL]		1.792	1.792	1.792
V_P [mL]		1.23	1.11	1.18
ε_P		86.2	82.3	86.6
*iSEC pore radius r [nm]		$5.0 < r < 8.1$	$10.3 < r < 13.0$	$3.8 < r < 5.0$
+Pore radius [nm]		7.5	15	2.5

*$K_d > 0.1$.
+Manufacturer data.

The expression of the adhesion molecule E-selectin could effectively be suppressed by all three adsorbent treatments. A marked difference, however, was observed in case of ICAM-1

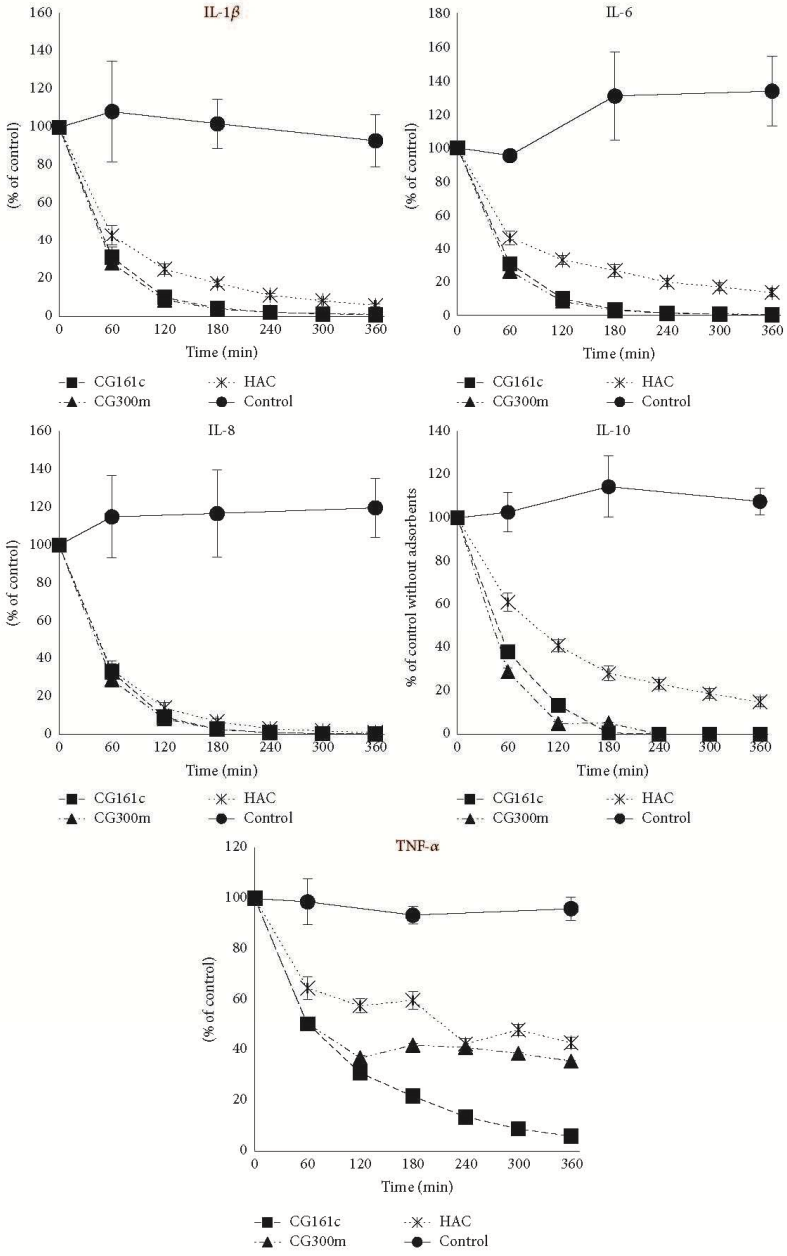

Figure 3: Cytokine removal in the dynamic model. Cytokine levels (TNF-α, IL-1β, IL-6, IL-8, and IL-10) in the plasma pool during 6 hours of treatment with the three tested adsorbents in the dynamic model. The results are shown in mean ± SD.

Table 3: Cytokine levels of treated plasma. Mean cytokine concentration ± SD (n = 3) in plasma after 6 hours of treatment with different adsorbents in the dynamic model. Plasma from LPS treated blood circulating through an empty cartridge acted as control.

	TNF-α [pg/mL]	IL-1β [pg/mL]	IL-6 [pg/mL]	IL-8 [pg/mL]	IL-10 [pg/mL]
Control	3102 ± 533	830 ± 190	24273 ± 13446	4837 ± 2300	51 ± 9
CG161c	177 ± 7	7 ± 1	59 ± 18	8 ± 7	<2
CG300m	1131 ± 16	10 ± 1	65 ± 3	10 ± 6	<2
HAC	1445 ± 212	45 ± 3	2587 ± 1254	60 ± 52	7 ± 4

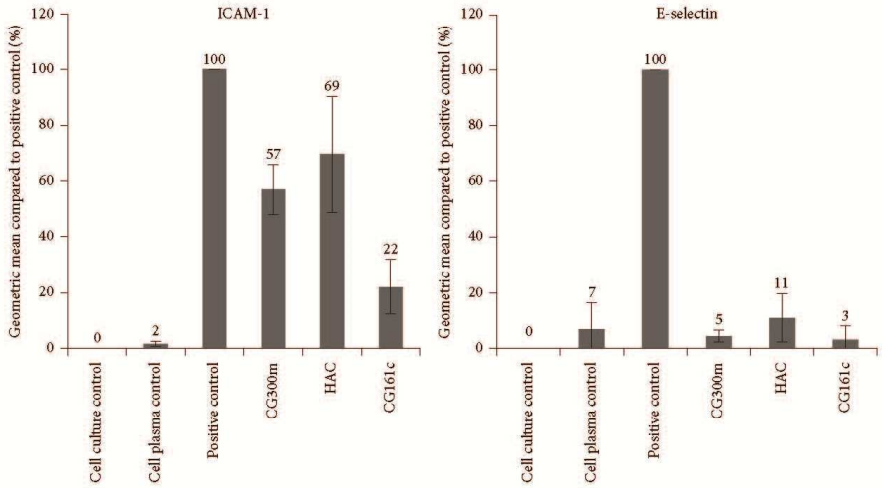

Figure 4: ICAM-1 and E-selectin expression by HUVEC. The effect of cytokine removal on endothelial cell activation was assessed by measuring the expressed adhesion molecules ICAM-1 and E-selectin after incubation of HUVECs with 10% of sample plasma from the adsorption experiments. The results are shown in mean ± SD.

with a highly reduced expression to 22 ± 10% for CG161c and a moderate beneficial effect for CG300m (57 ± 9%) as well as for HAC (69 ± 21%) compared to the untreated cytokine rich plasma. It is well documented that ICAM-1 expression in vascular endothelium can be induced by IL-1 and TNF-α [26]. This fact should be considered when the results of TNF-α removal (Figure 3) and the resulting ICAM-1 expression (Figure 4) are compared. It confirms that the lower the TNF-α content in plasma, the lower the ICAM-1 expression on HUVEC's surface. Thus, only an effective removal of cytokines to a physiological concentration in plasma, which are usually below 100 pg/mL [27], can significantly reduce endothelial cell activation.

A similar effect was observed when the secreted cytokines of HUVEC were determined. The plasma treated with the CG161c adsorbent elicits the lowest IL-6 and IL-8 levels in cell culture but also the other two tested adsorbents provoke a high reduction in cytokine release compared to untreated plasma (Figure 5 and Table 4). Makó et al. reported that the expression of E-selectin, IL-6, and IL-8 was induced most efficiently by IL-1β, while that of LPS and TNF-α was less efficient, and ICAM-1 expression was not different between stimuli [28]. Our findings are in agreement with those of Makó et al. (see Figure 6), because IL-1β was removed very efficiently by all tested adsorbents; also the ICAM-1 expression as well as IL-6 and IL-8 secretion from the HUVEC was reduced.

Table 4: IL-6 and IL-8 secretion by HUVECs. Mean IL-6 and IL-8 secretion ± standard deviation (n = 3) of HUVECs after a 16-hour treatment with cell media containing 10% of plasma from the different adsorption studies in the dynamic model. Untreated plasma from LPS stimulated blood acts as positive control and cell plasma control denotes plasma from blood without LPS stimulation.

	IL-8 [pg/mL]	IL-6 [pg/mL]
Cell plasma control	326 ± 15	231 ± 187
Positive control	6249 ± 858	4056 ± 2124
CG300m	657 ± 72	141 ± 23
HAC	600 ± 53	143 ± 14
CG161c	130 ± 15	66 ± 6

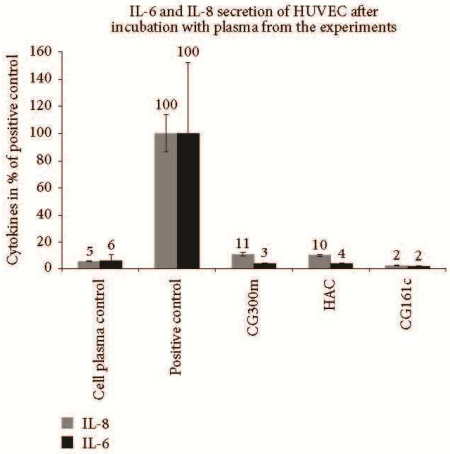

FIGURE 5: IL-6 and IL-8 secretion by HUVEC. The effect of cytokine removal on endothelial cell activation regarding IL-8 and IL-6 secretion after 16 h incubation of HUVEC with 10% of sample plasma from the adsorption experiments. Untreated plasma (empty cartridge without adsorbent) acts as positive control. The results are shown in mean ± SD.

3.4. TNF-α and IL-1β Dependent Activation of HUVEC in the Cell Culture Model. Endothelial cells are activated primarily by the two cytokines TNF-α and IL-1β [28, 29]. In our cell culture model the threshold concentrations of IL-1β and TNF-α for HUVEC activation were between 10 and 50 pg/mL (see Figure 6) regarding IL-8 and IL-6 secretion as well as ICAM-1 expression (Figures 6 and 7). E-selectin expression was induced by low IL-1β concentration (50 pg/mL) in contrast to TNF-α which activates the expression of E-selectin not below 500 pg/mL. Thus the three tested adsorbents which removed IL-1β very efficiently also were able to reduce the expression of E-selectin in contrast to ICAM-1 expression which was only suppressed by the CG161c treatment because of efficient TNF-α removal. These results confirm the assumption that it is not sufficient merely to reduce the cytokine levels, for example, by EBP. The cytokine levels have to be reduced to physiological levels in order to prevent endothelial cell activation. Based on their molecular size, especially trimeric TNF-α with 52 kDa, the cytokines are not able to rapidly cross the usually applied dialysis membranes. Consequently, an effective removal of a wide array of cytokines from the plasma cannot be achieved using only membrane based technologies like high-volume hemofiltration and high-cutoff hemodialysis or hemofiltration. This can only be realised by using adsorption techniques or by a combination of adsorption and membrane technologies.

4. Conclusion

Cytokines are considered to be targets that have to be modulated in order to improve the patient's health in case of severe inflammation, sepsis, and septic shock. Although there are different adsorbents commercially available, their clinical utility is limited [30]. In order to suppress systemic effects in these disease patterns, effective removal of cytokines below a critical threshold is necessary. The three PS-DVB based adsorbents Amberchrom CG161c, Amberchrom CG300m, and HAC were studied with regard to cytokine removal capacity from human plasma. The new PS-DVB based cytokine adsorbent CG161c exhibited promising results in terms of all tested cytokines. Especially in case of removing the key factor TNF-α, it outperforms commercially available adsorbents such as HAC or CG300m due to its optimized pore size. With respect to endothelial cell activation, the CG161c treatment highly reduced cytokine secretion and expression of cell adhesion molecules in HUVEC, which emphasizes the importance of the effective removal of TNF-α in inflammatory diseases when using a cytokine adsorber. A successful sepsis treatment strategy regarding effective cytokine modulation may use a combination of membrane and adsorption based technique. A promising adsorbent for such a blood purification device could be the CG161c adsorbent. However, the findings here are based on *in vitro* studies and are not yet confirmed by clinical data.

Key Messages

(i) The Amberchrom adsorbent CG161c is promising for cytokine removal from human plasma compared to other tested cytokine adsorbents.

(ii) The threshold concentrations of TNF-α and IL-1β for HUVEC stimulation are below 50 pg/mL.

(iii) Cytokines circulating in the blood should be modulated to physiological levels during treatment of sepsis in order to reduce endothelial cell activation.

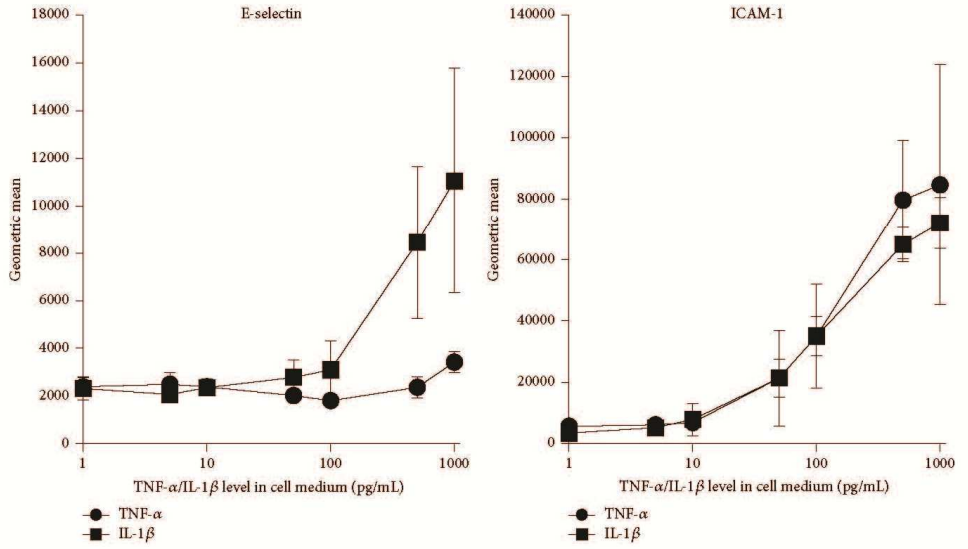

FIGURE 6: E-selectin and ICAM-1 expression of HUVEC as a function of TNF-α or IL-1β level. ICAM-1 and E-selectin expression by HUVEC after 16-hour incubation in cell media with 10% of plasma spiked with increasing amounts of TNF-α or IL-1β (mean ± SD, n = 3).

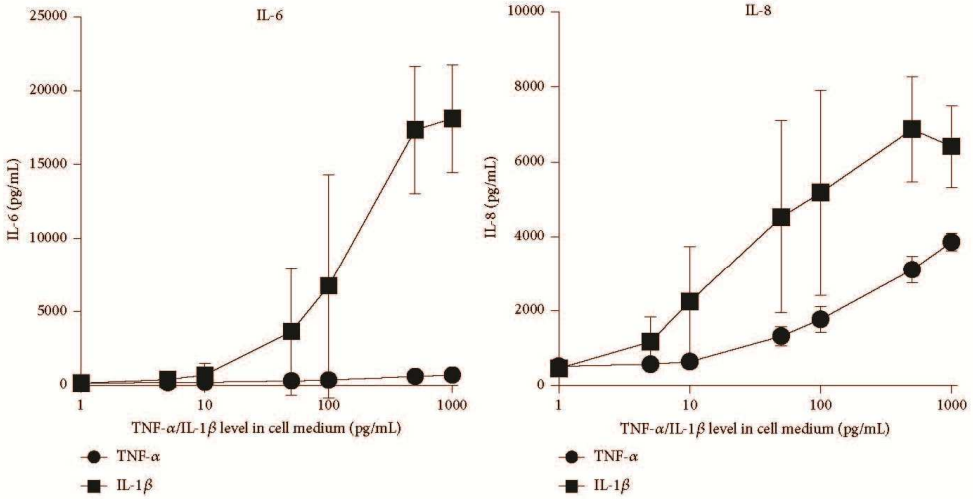

FIGURE 7: TNF-α or IL-1β dose-dependent production of IL-6 and IL-8 by HUVEC. IL-6 and IL-8 secretion by HUVEC after 16-hour incubation in cell media with 10% of plasma spiked with increasing amounts of TNF-α or IL-1β (mean ± SD, n = 3).

Conflict of Interests

This study has received research funding from Fresenius Medical Care (Bad Homburg, Germany).

Authors' Contribution

Stephan Harm, Franz Gabor, and Jens Hartmann participated in research design, writing of the paper, performance of the research, and data analysis. The authors contributed to the critical review and revision of the paper and approved it for publication.

Acknowledgments

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2.1.6 Verabreichung von geringen Polymyxin Mengen: Eine mögliche Option zur Behandlung von Gram-negativer Sepsis

Stephan Harm, Franz Gabor, Jens Hartmann: *Low-dose polymyxin: An option for therapy of Gram-negative sepsis*. Innate Immun. 2016; 22(4):274-83, DOI:10.1177/1753425916639120

Kurzfassung:

Einleitung/Ziele: Endotoxine (Lipopolysaccharide, LPS) sind die Hauptbestandteile der äußeren Membran gramnegativer Bakterien und oftmals Auslöser von entzündlichen Erkrankungen. Das Auftreten von Endotoxinen im Blut (Endotoxämie) kann bei ausgeprägter Immunantwort einen septischen Schock auslösen. Es ist unumstritten, das die Entfernung von Endotoxinen aus dem Blut bei Leberversagen und Sepsis einen Vorteil für den Patienten bieten kann. Die Effizienz der momentan im Handel erhältlichen Endotoxinadsorber in der Klinik ist umstritten. In dieser Arbeit zeigen wir, dass es neben der adsorptiven Entfernung von Endotoxinen auch die Möglichkeit gibt, diese mit Hilfe von Polymyxin B (PMB) zu neutralisieren.

Material & Methoden: Die stimulierenden Eigenschaften des LPS-PMB Komplex auf Leukozyten wurden in einem *in vitro*-Modell mit frisch entnommenem Blut untersucht. Analysiert wurde die biologische Aktivität der Endotoxine mittels LAL-Test sowie die Aktivierung der Leukozyten durch Quantifizierung von Zytokinen. Weiteres wurde die Proteinbindung von PMB und die PMB-Clearance während der Dialyse mit dem AV1000 S-Filter ermittelt.

Ergebnisse: Liegen Endotoxine als i LPS-PMB-Komplexe vor, so besitzen sie eine geringere biologische Aktivität im Blut. Dies führt zu einer stark reduzierten Zytokinsekretion der Leukozyten. Auf Grund der *in vitro*-Ergebnisse liegt der ideale PMB-Serumspiegel für die LPS-Inaktivierung zwischen 100 und 200 ng/ml. Hier sind etwa 90 % des PMB ist an Plasmaproteine gebunden und die Clearance-Rate von PMB liegt zwischen 12 und 17 %, sofern ein kommerziell erhältlicher Dialysator mit einem Dialysatfluss von 2000 - 4800 ml/h verwendet wird. Darüber hinaus konnte in einem *in vitro* Vollblut-Modell gezeigt werden, dass die Kombination der adsorptiven Zytokinentfernung und der LPS-Neutralisierung durch PMB zu einer starken Unterdrückung der proinflammatorischen Immunantwort führt.

Schlussfolgerung: Die immunstimulierende Eigenschaft von Endotoxinen kann durch niedrig dosiertes, intravenös verabreichtes PMB zum größten Teil neutralisiert werden. Es besteht auch die Möglichkeit, PMB in kontrolliert niedrigen Dosen während der extrakorporalen Blutreinigungstherapie zu verabreichen. Diese einfache, neuartige Therapie könnte zur dringend notwendigen Endotoxineliminierung bei der Sepsis- und Leberbehandlung eingesetzt werden.

Mein Beitrag für diese Publikation:

- Planung der Versuche
- Verfassen der Publikation
- Durchführung der Tests
- Datenauswertung

Original Article

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Low-dose polymyxin: an option for therapy of Gram-negative sepsis

Stephan Harm[1,2], Franz Gabor[2] and Jens Hartmann[1]

Abstract

Endotoxins are the major components of the outer membrane of most Gram-negative bacteria and are one of the main targets in inflammatory diseases. The presence of endotoxins in blood can provoke septic shock in case of pronounced immune response. Here we show *in vitro* inactivation of endotoxins by polymyxin B (PMB). The inflammatory activity of the LPS–PMB complex in blood was examined *in vitro* in freshly drawn blood samples. Plasma protein binding of PMB was determined by ultracentrifugation using membranes with different molecular cut-offs, and PMB clearance during dialysis was calculated after *in vitro* experiments using the AV1000S filter. The formed LPS–PMB complex has lower inflammatory activity in blood, which results in highly reduced cytokine secretion. According to *in vitro* measurements, the appropriate plasma level of PMB for LPS inactivation is between 100 and 200 ng/ml. Furthermore, the combination of cytokine removal by adsorbent treatment with LPS inactivation by PMB dosage leads to strong suppression of inflammatory effects in blood in an *in vitro* model. Inactivation of endotoxins by low-dose intravenous PMB infusion or infusion into the extracorporeal circuit during blood purification can be applied to overcome the urgent need for endotoxin elimination not only in treatment of sepsis, but also in liver failure.

Keywords

Polymyxin, lipopolysaccharide, sepsis, Inflammation, cytokines, endotoxin

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Introduction

In recent years, endotoxins (LPS) have increasingly become the focus of interest for therapy of diseases that are treated with extracorporeal blood purification. LPS is a major constituent of the outer cell envelope of most Gram-negative bacteria and may strongly trigger inflammatory responses in humans, even at doses as low as 1 ng/kg body mass/h.[1] LPS is released from the cell envelope of growing bacteria, as well as by lysis via antibiotics or complement.[2,3] Endotoxins that enter the circulatory system bind to the soluble lipopolysaccharide binding protein (LBP). This complex initiates the inflammatory response by binding to the CD14 membrane protein of monocytes and macrophages, subsequently triggering the production of cytokines via TLR. LPS activation of TLR4 triggers the biosynthesis of diverse mediators of inflammation, such as TNF-α and IL-1β,[4] and activates the production of co-stimulatory molecules required for the adaptive immune response.[5] In mononuclear and endothelial cells, LPS also stimulates tissue factor production,[6] and can therefore trigger the extrinsic coagulation pathway. As long as this process is limited to a local increase of pro- or anti-inflammatory cytokines, this is a normal response of the patient's immune reaction against pathogens. However, in severe cases, the production of cytokines gets out of control leading to the more severe medical conditions such as systemic inflammatory response syndrome (SIRS) or sepsis. Thus, the effective removal of endotoxins is essential in order to reduce cytokine production in the case of Gram-negative sepsis.

Furthermore, endotoxins play an important role in liver failure. Endotoxins from patients' intestines can pass the liver owing to reduced endotoxin removal via the reticuloendothelial system, which can lead to

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endotoxemia and, finally, to the symptoms described above.[7,8]

Different approaches have been followed to remove or inactivate endotoxins upon inflammation and sepsis. Antimicrobial peptides (AMPs) can block the endotoxin-initiated inflammatory cascade, which leads to a reduction of cytokine production. These AMPs are currently intensively investigated and provide, to some extent, promising results.[9–11] While polymyxins are well known to inactivate the biological activity of LPS by shutting down the NF-κB pathway, owing to direct binding of LPS,[12] the mechanisms of action for some other AMPs are not yet fully understood. There is some evidence that they enter another mode of action by inserting into CD14-positive cells and reducing the endotoxin activity by competitive inhibition due to their high affinity to LPS.[13]

Polymyxin B (PMB) is an antibiotic preferably applied to treat infections provoked by multidrug resistant Gram-negative bacteria. It is a cyclic, highly cationic decapeptide derived from *Bacillus polymyxa*.[14] PMB has been applied for *in vitro* investigations, as well as intravenously in animal models, to demonstrate its capacity to inactivate endotoxins and to break down endotoxin aggregates in Gram-negative septicemia models.[15–20] The bactericidal activity of the antibiotic PMB against Gram-negative bacteria relies on the ability to destabilize the outer bacterial wall by direct interaction with the lipid A moiety of LPS molecule. The model for action of PMB involves interaction of the positively charged diaminobutyric acid residues and the negatively charged phosphate groups of lipid A. This initial electrostatic interaction temporarily stabilizes the LPS–PMB complex and facilitates the interaction of the N-terminal fatty acyl chain of the PMB molecule into proximity with the lipid A fatty acyl chains.[21] The LPS–PMB complex is very stable and has an association constant (Ka) according to the LPS type between 1.8×10^{-6} and 2.3×10^{-6} M.[22] However, although these studies were rather focused on endotoxins than on bacteria, the applied PMB amounts mostly exceeded the minimal inhibitory concentration (MIC) of this antibiotic.

Currently, knowledge about pharmacokinetics and pharmacodynamics of polymyxins is very limited as intravenous administration was avoided within the last 50 yrs. Furthermore, nephro- and neurotoxicity restricted clinical application. Because of increasing numbers of multidrug-resistant Gram-negative pathogens and limited development of new antimicrobials, PMB experiences a revival as a therapeutic option for Gram-negative infections.[23] In particular, the use of affinity chromatographic sorbents based on PMB ligands is reported as an appropriate method to remove endotoxins from protein solutions without denaturation and loss of products.[24] Here we show that endotoxin inactivation by PMB or polymyxin E (PME; colistin) in patients with Gram-negative sepsis or endotoxemia could be an additional therapeutic option.

Materials and methods

Materials

PMB and endotoxins (LPS) from *Pseudomonas aeruginosa* and *Escherichia coli* were purchased from Sigma-Aldrich (Vienna, Austria). The PMB analyses were conducted using the Colistin & Polymyxin ELISA kit from Kwinbon Biotechnology Co. LTD (Beijing, China). The analyses of endotoxins were performed in pyrogen-free tubes and the kinetic chromogenic *Limulus* amebocyte lysate (LAL) test from Charles River Laboratories (Wilmington, MA, USA). Tubes (Vacuettes) for blood donation were obtained from Greiner (Kremsmünster, Austria). The Amicon Ultra-2 centrifugal filter devices with molecular cut-offs of 10, 30, 50 and 100 kDa were from Merck (Darmstadt, Germany). The ALBplus reagent set for albumin quantification and the TP set for measurement of total protein level was purchased from Roche (Mannheim, Germany).

LPS inactivation as a function of PMB concentration

To elucidate the dependency of LPS inactivation on the PMB concentration, fresh human heparinized plasma containing 5 ng/ml or 0.5 ng/ml LPS from either *P. aeruginosa* or *E. coli* were incubated with increasing amounts of PMB (0, 10, 100, 250, 500 and 1000 ng/ml) for 60 min at 37°C. The tests were performed in pyrogen-free 3-ml glass vials and LPS activity was determined using the LAL test. In order to compare the endotoxin neutralizing capability of PMB, the endotoxin neutralizing concentration (ENC_{50}) was calculated. This is the PMB concentration that is capable of reducing the endotoxin activity compared with the LPS-spiked plasma control without PMB, by 50%.

Influence of PMB–LPS complex on cytokine induction

The PMB-dependent reduction of the LPS activity, as indicated by the LAL test, is not necessarily associated with a reduced inflammatory effect of LPS, namely the induction of the cytokine production. Therefore, further experiments were conducted in order to check if cytokine induction can be modulated by PMB-dependent inactivation of LPS. For these experiments, freshly drawn human heparinized blood was spiked with PMB to yield 0, 250, 500 and 1000 ng/ml PMB. Then, 0.5 ng/ml LPS from *E. coli* was added. The negative control was blood without LPS, spiked with 1000 ng/ml PMB to assess the influence of PMB alone on cytokine release. The samples were incubated in sterile

polypropylene tubes (Greiner) for 4 h at 37°C on a roller mixer and stored at −80°C until the cytokines were quantified by ELISA. All assays were conducted at least in triplicates.

Combined use of PMB and cytokine adsorbent

To verify whether the combination of adsorptive cytokine removal and PMB infusion is more effective than one of these treatments, an experiment as schematically displayed in Figure 1 was conducted. This experiment was carried out with blood from three different volunteers.

Binding of PMB to plasma proteins

Depending on the affinity to plasma proteins, which is a specific characteristic of a drug, one part of the drug is transported by plasma proteins and another part is freely distributed throughout the circulation. If protein binding is reversible, an equilibrium will exist between the bound and unbound proportion. To evaluate plasma protein binding, fresh citrate anticoagulated plasma from three donors was spiked with 1 µg/ml PMB. Five hundred µl PMB containing plasma was centrifuged (10,000 *g*, 20 min) through different ultrafiltration membranes with a molecular mass cut-off (MMCO) of 10, 30, 50 or 100 kDa. The filtrates were stored at −20°C until quantification of PMB by ELISA and total protein content by an automated analyser (Hitachi 902; Hitachi, Tokyo, Japan). The positive control was unfiltered plasma spiked with 1 µg/ml PMB and the negative control was plasma without PMB. To detect any PMB-binding to the filter material, the same experiment was performed in 0.9% sodium chloride solution.

Clearance measurement of PMB

Since sepsis patients with acute kidney injury are treated with renal replacement therapy, a dialysis experiment was performed to estimate the clearance of PMB during a treatment. As intensive care treatment sessions usually take several hours, it can be assumed that most of the non-protein-bound fraction of PMB is removed during treatment. In order to avoid that the inflammatory activity of LPS is restored by PMB removal, PMB monitoring would be useful. To check the PMB clearance by a conventional dialysis filter, 1500 ml plasma spiked with 5 µg/ml PMB was circulated through a dialyzer (AV1000S, FMC, Bad Homburg, Germany) using the multiFiltrate device from Fresenius Medical Care (Bad Homburg, Germany). The schematic setup of this experiment is shown in Figure 2. The plasma flow rate (Q_b) was 100 ml/min and dialysate (Q_D) flow rates of 2000 ml/h and 4800 ml/h were chosen. Samples for PMB quantification were collected pre- and postfilter after 5, 10, 15, 20, 25 and 30 min. The experiment was performed three times with different plasma and dialyzers and the PMB clearance was calculated according to the following formula:

$$Cl_{(PMB)} = \frac{c_{in(PMB)} - c_{out(PMB)}}{c_{in(PMB)}} \times Q_b$$

PMB quantification

The blood and plasma levels of PMB were determined by use of a competitive enzyme immunoassay kit for analysis of PMB and PME with a detection limit of 1 ng/ml. If necessary, the samples were diluted with dilution reagent provided with the ELISA kit to reach the measuring range between 0 and 100 ng/ml PMB.

Cytokine quantification

The analysis of cytokines was conducted by ELISA with a Bio-Plex cytokine array system (Biorad, Vienna, Austria).

Statistics

Mean and SD were calculated with Excel 2010 (Microsoft, Redmond, WA, USA). The tests for normal distribution and the *t*-tests were conducted with SigmaStat for Windows 2.03.

Results

LPS inactivation as a function of PMB concentration and ENC_{50}

To estimate the plasma level of PMB required to decrease the LPS activity to a certain level, the LPS inactivation in plasma was measured as a function of PMB concentration. At 0.5 ng/ml LPS, the ENC_{50} is 84 and 63 ng/ml for LPS from *P. aeruginosa* and *E. coli*, respectively. For 5 ng/ml LPS, the ENC_{50} is 219 and 381 ng/ml for LPS from *P. aeruginosa* and *E. coli*, respectively (Figures 3 and 4). The differences in the ENC_{50} for LPS from *E. coli* and *P. aeruginosa*, however, are not significant (*t*-test, $P = 0.88$ and $P = 0.26$, respectively) at both concentration levels.

Influence of PMB–LPS complex on cytokine induction

Although the LAL test revealed inactivation of LPS by PMB in a concentration-dependent manner, the influence on inflammation was tested by stimulating leukocytes from human blood with increasing amounts of PMB. The results of cytokine release show clearly that the formed LPS–PMB complex exerts a by far

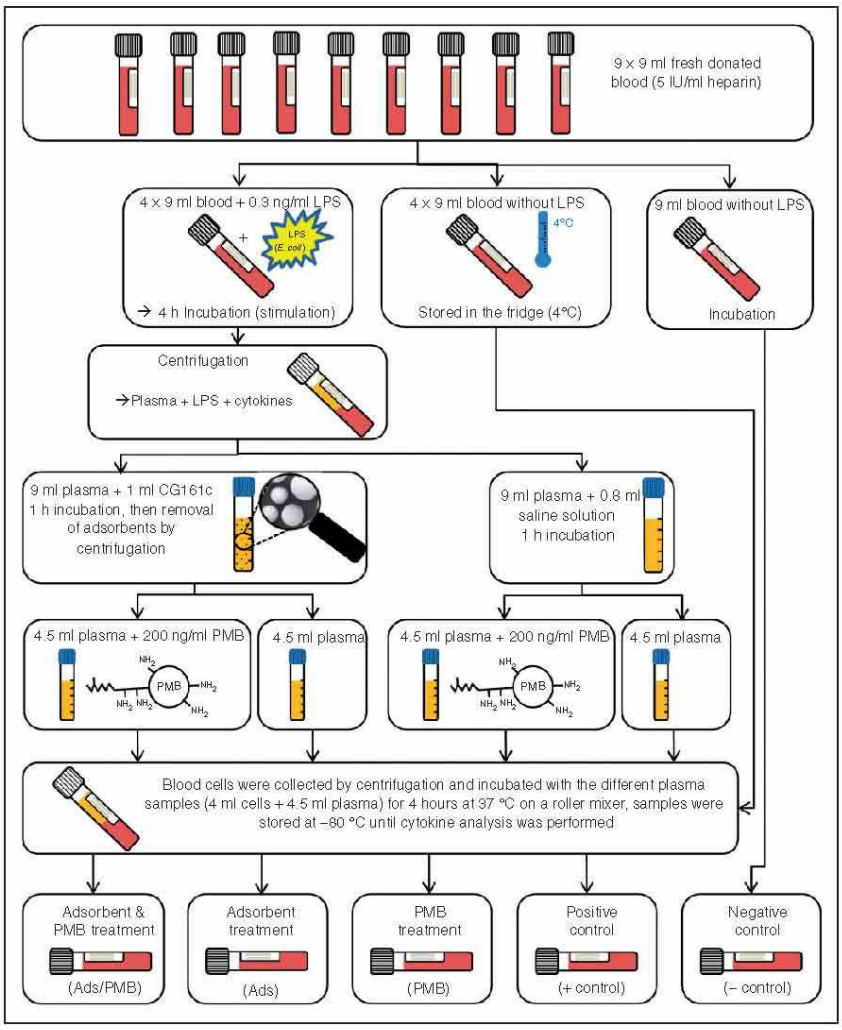

Figure 1. Scheme of the experiment where three different treatment options: (i) adsorbent combined with PMB; (ii) adsorbent treatment only; (iii) PMB treatment only. The influence on inflammation was tested by determination of the cytokine levels after incubation of differently pre-treated plasma samples with native blood cells.

lower stimulating effect on blood cells than the native LPS molecule (Table 1; Figure 5). Among all cytokines under investigation, the highest impact was observed on TNF-α release. Only 50 ng PMB per ml blood reduced TNF-α secretion from leukocytes by 87.4 ± 5.9%. Increasing the PMB 2.5-fold to 125 ng/ml decreased the TNF-α level by about 94.8 ± 2.3%. A further increase of PMB caused no considerable decrease of cytokine concentrations (Table 1). The lowest impact of PMB was observed on IL-8 secretion. However, even the negative control without LPS showed high levels of IL-8. In general, the IL-10 level was very low (23 ± 8 pg/ml) because IL-10 is a late-related cytokine and a high level can only be reached if stimulation of the blood cells is prolonged to 12 h. The secretion of IL-1β was reduced by 75.1 ± 10.8%

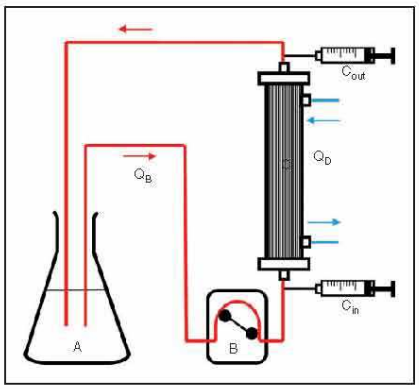

Figure 2. Setup for determination of PMB clearance during hemodialysis using the multiFiltrate. The experiment was performed with (A) 1500 ml plasma, (B) a blood pump with (Q_B) flow rates between 50 and 200 ml and (C) the dialyzer AV1000 S (Fresenius Medical Care). Samples were taken pre- (C_{in}) and post- (C_{out}) filter after 5, 10, 15, 20, 25 and 30 min.

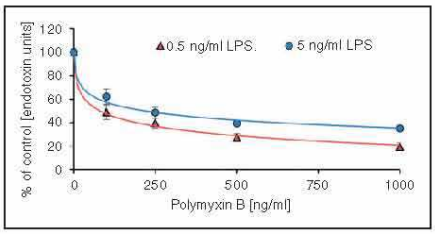

Figure 3. PMB-dependent inactivation of endotoxins from *P. aeruginosa* in human plasma.

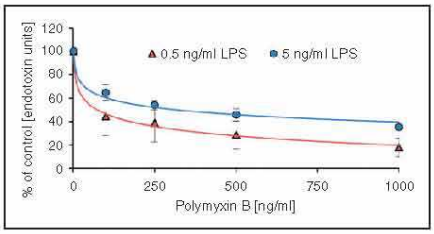

Figure 4. PMB-dependent inactivation of endotoxins from *E. coli* in human plasma.

and that of IL-6 by 78.0 ± 12.0% in the presence of 125 ng PMB per ml blood.

Combined use of PMB and cytokine adsorbent

As pre-existing cytokines cannot be reduced by PMB infusion and the inflammatory acting LPS cannot be removed from blood by adsorption techniques sufficiently, these two types of treatments were simulated in an *in vitro* model (see Figure 1). Plasma that contained inflammatory mediators like LPS and secreted cytokines was pre-treated with adsorbent or PMB or both. Then, the treated plasma samples were incubated with blood cells from the same donor for 4 h and the cytokine levels were determined. Untreated plasma containing the inflammatory mediators served as a positive control and native blood as a negative control. According to the results, the addition of PMB after LPS stimulation scarcely reduced the cytokine secretion compared with the untreated plasma. The cytokine levels, however, were still high because of the presence of cytokines like TNF-α that still act as stimulating agents. Interestingly, the adsorbent CG161c decreased the cytokine level in plasma considerably to 30 ± 9% (TNF-α), 6 ± 5% (IL-1β), 2 ± 2% (IL-6), 1 ± 1% (IL-8) and 9 ± 13% (IL-10) compared with the untreated positive control. The styrene-divinylbenzene based CG161c adsorbent has a particle size of 120 μm and pores with an average diameter of 15 nm. In earlier studies, we have shown that CG161c is very effective in removing cytokines from plasma.[25,26] After incubation of the different plasma samples with blood cells only the combination of adsorbent with PMB exhibited cytokine levels similar to the negative control where no LPS was added (see Table 2 and Figure 6). Obviously, PMB inactivates LPS and reduces leukocyte stimulation. The results show high SDs of the cytokine level due to the fact that blood from different donors was used.

Binding of PMB to plasma proteins

Most drugs bind to plasma proteins such as albumin, α1-acid glycoprotein, lipoproteins, α-, β- and γ-globulins, and to erythrocytes. Ultracentrifugation of PMB-spiked plasma through membranes with different MMCOs for separation of protein-bound PMB from free PMB revealed that this drug with a molecular weight of 1.3 kDa is mostly bound to plasma contents. Only the membrane with 100 kDa MMCO was permeable for higher amounts of PMB (25 ± 13%). The PMB-content of filtrates obtained from centrifugation through membranes with smaller pores was <10% compared with a PMB-solution without plasma (Figure 7). Thus, >90% of PMB is bound to plasma proteins and not even one-tenth exists in its free form in circulation.

Clearance measurement of PMB

The PMB clearance (Cl_{PMB}) for the AV1000S filter was determined using the multiFiltrate device (FMC) at a blood flow rate of 100 ml/min. The PMB clearance was 12.9 ± 5.1 ml/min ($n = 18$) at a dialysate flow rate of

Table 1. The cytokine release in LPS-stimulated (*E. coli*) blood in presence of increasing amounts of PMB.

	TNF-α	IL-1β	IL-6	IL-8	IL-10
− Control	0.3 ± 0.2	1.6 ± 1.8	0.2 ± 0.1	20.1 ± 13.2	18.9 ± 10.9
+ Control	100 ± 17.6	100 ± 73.3	100 ± 19.1	100 ± 54.3	100 ± 32.8
50 ng/ml PMB	12.6 ± 5.9	31.1 ± 9.6	40.0 ± 18.6	59.1 ± 19.4	53.1 ± 16.8
125 ng/ml PMB	5.2 ± 2.3	24.9 ± 10.8	22.0 ± 12.0	53.6 ± 14.5	38.5 ± 16.2
250 ng/ml PMB	4.2 ± 2.3	18.4 ± 11.2	19.0 ± 13.8	49.1 ± 11.0	34.9 ± 16.7
500 ng/ml PMB	2.1 ± 0.7	10.2 ± 5.5	8.4 ± 4.5	40.6 ± 10.5	32.5 ± 15.8

The results are % SD of the positive control without PMB.

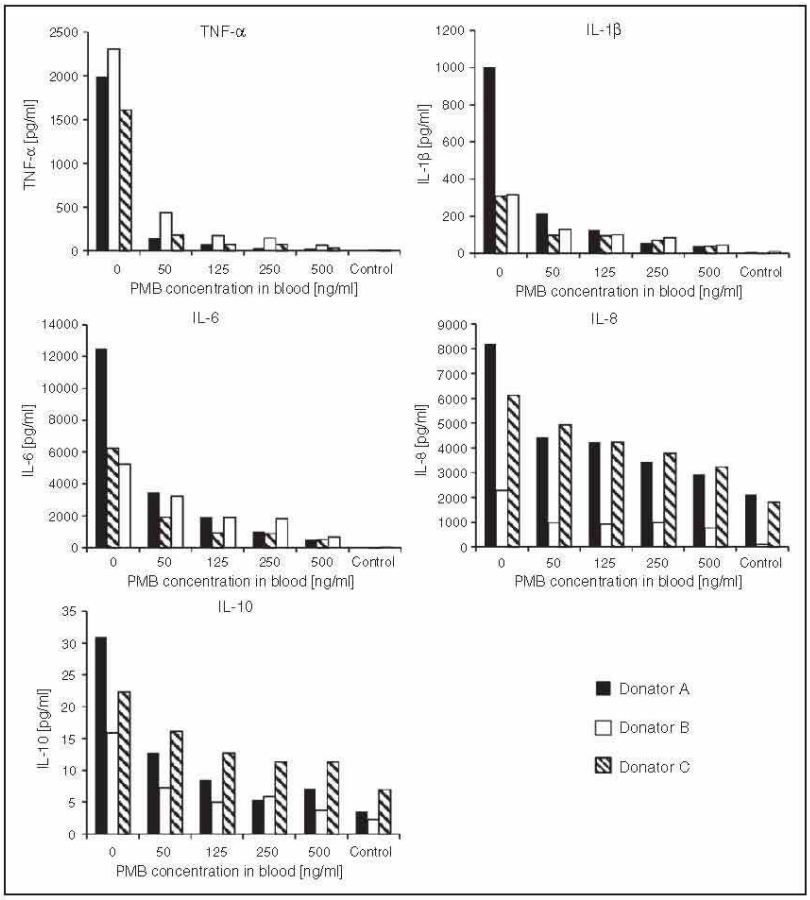

Figure 5. The effect of LPS inactivation by PMB on cytokine release in whole blood from three different donors (mean ± SD; $n = 3$).

Table 2. Cytokine release from blood cells after incubation with differently treated inflammatory plasma. The plasma treatments were (i) addition of PMB, (ii) adsorbent to remove cytokines or (iii) the combination of both (see also Figure 1).

	TNF-α	IL-1β	IL-6	IL-8	IL-10
Donators	A/B/C	A/B/C	A/B/C	A/B/C	A/B/C
Positive control	100	100	100	100	100
Negative control	73/2/0	69/58/1	20/3/0	11/13/13	54/56/14
PMB	92/38/95	98/80/82	101/80/88	91/91/92	91/41/67
Adsorbent	62/11/19	7/23/7	2/2/3	8/8/29	0/0/22
PMB/adsorbent	36/10/11	7/22/2	2/2/0	8/8/16	0/0/6

The results of each donator (A, B and C) are given in % as referred to the positive control without any treatment set at 100%.

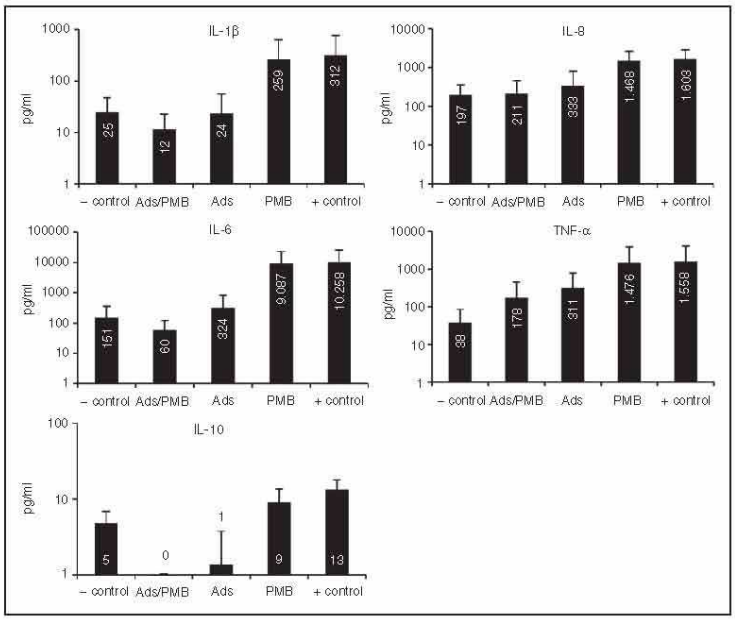

Figure 6. Comparison of the cytokine release pattern from blood cells after different ways of plasma pre-treatments: adsorbent combined with PMB (Ads/PMB); only adsorbent (Ads); only PMB (PMB); untreated plasma (+ control); native blood (– control).

2000 ml/h, and at a dialysate flow rate of 4800 ml/h the PMB clearance increased to 17.2 ± 8.5 ml/min ($n = 13$).

Discussion

In sepsis therapy, adsorption-based removal of LPS is still a challenge. The LPS molecule consists of a conserved hydrophobic domain known as lipid A, a non-repeating 'core' oligosaccharide and a highly variable distal polysaccharide. Until now, specific adsorption based on immobilized Abs did not offer promising results.[27–30] In the case of Abs against the lipid A region, this was attributed to the low affinity of anti-lipid A Abs to lipid A and to cross-reactivity to plasma proteins.[31,32] Adsorption based on Abs against the polysaccharide chain (O-antigen) is not feasible as the polysaccharide domain is highly variable. Although anion-exchanger resins efficiently remove endotoxins from aqueous solutions, as well as from protein solutions, they cannot be used in blood purification because of insufficient biocompatibility.[33] Since 1994, a PMB-based extracorporeal hemoperfusion device, called Toraymyxin, has been commercially available and is now approved as a therapeutic device by the health insurance system in Japan.[34] It is recommended for selective blood purification from endotoxins via direct

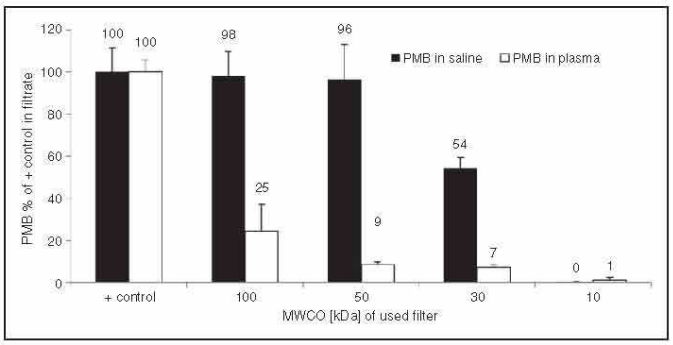

Figure 7. Binding of PMB to plasma proteins determined by ultrafiltration through membranes with different MMCO. The filtration rate of PMB in plasma was compared with that in 0.9% NaCl solution. The data represent the percentage of PMB in the filtrate as referred to the positive control (mean ± SD; $n = 3$).

hemoperfusion. Toraymyxin consists of polystyrene-derivative fibres with covalently immobilized PMB at a mass ratio of 0.5%.[35] Direct hemoperfusion using such a PMB-immobilized fiber column (PMX-F) has been used for about 15 yrs for the treatment of septic shock.[36] Direct hemoperfusion with PMX-F can be applied in patients with endotoxemia or suspected Gram-negative infection, who fulfill the conditions of SIRS and suffer from a septic shock requiring administration of vasoactive agents. Several studies claim that endotoxins are efficiently removed by PMX-F,[35,37–39] and, concurrently, *Staphylococcus aureus* lipoteichoic acid-induced TNF-α production is suppressed.[40] Even in clinical practice, adsorption techniques using Toraymyxin cartridges have been successfully applied in Japan since 1994 in more than 60,000 patients with severe sepsis.[35] The fact that polymyxins bind endotoxins in blood very effectively and that PMB or PME in the form of colistin is a clinically approved drug for intravenous application reveals the idea of using them not as an antibiotic, but as an LPS neutralizing agent. We showed that PMB concentrations <100 ng/ml in plasma for 0.5 ng/ml LPS and <400 ng/ml for 5 ng/ml LPS from *P. aeruginosa* and *E. coli* are required to reduce the LAL activity to 50% of the control serum without PMB. These ENC_{50} are in the range of AMPs with the highest endotoxin inactivation capabilities that are currently known, namely peptides derived from the recombinant factor c of the LAL cascade.[41] While LPS from *P. aeruginosa* is usually an order of magnitude less active than LPS from enterobacterial strains such as *Escherichia*, the inactivation ratio is similar. A potential explanation for this finding is that PMB directly interacts with the conserved lipid A region of the LPS molecule. *In vitro*, the formed LPS–PMB complex in blood exhibits lower inflammatory activity than the free LPS. Moreover, in presence of LPS the cytokine secretion of blood cells is clearly reduced when the PMB level in blood is ≥50 ng/ml (Figure 5). Although polymyxins are shown to reduce significantly the cytokine storm in endotoxemia, it has to be considered that patients developing or even suffering from sepsis already show high cytokine levels before starting any therapy. As polymyxins cannot reduce pre-existing cytokine levels, the administration of polymyxins should be supported by effective approaches for cytokine removal, such as adsorption or hemofiltration.[42,43] Combining adsorptive cytokine removal with PMB administration (Figure 1) is proposed to be an effective approach to suppress inflammatory effects of Gram-negative infections (Figures 1 and 6; Table 2). The concentration of PMB necessary for effective inactivation of LPS is in a very low range but still close to the MIC.[44] To minimize the risk of generating resistant germs, low-dose PMB for LPS inactivation should be applied only together with conventional antibiotic treatment. This procedure offers the advantage that endotoxins, which are released by antibiotic-induced bacterial lysis, are effectively inactivated by PMB.[13,45] Furthermore, the half-lives of the different polymyxins have to be considered upon application for inactivation of LPS. However, the data available from the literature are inconsistent, and depend on the type of polymyxin and on the renal function of the patients, especially for the PME prodrug colistin methanonsulfonate.[46–49] As polymyxins are removed by hemodialysis and adsorption,[50,51] the clearance of the dialyzer and/or the adsorption unit has to be taken into account when PMB is applied concomitantly with extracorporeal blood purification. An *in vitro* simulation with a commercially available dialyser at dialysate flow rates between 2000 and 4800 ml/h yielded a PMB plasma clearance between 12% and 17%. This clearance rate agrees with data from the literature.[52,53] Considering the small molecular mass of the PMB molecule (1.3 kDa), the calculated clearance

rate is relatively low, which can be explained by binding of PMB molecules to plasma proteins. Plasma protein binding of PMB was assumed to be up to 90%,[54] which is confirmed by our experiments (Figure 7). Titration calorimetry experiments show that in aqueous solution with 1–3 molecules of PMB are necessary to inactivate one LPS molecule.[22] The association constant (K_a) of this interaction is between 1.8×10^{-6} and 2.3×10^{-6} M, which explains why very low amounts of free PMB inactivate LPS.

Conclusions

As endotoxins induce a strong host immune response, there is an urgent therapeutic need to reduce their activity. As a promising alternative or add-on to endotoxin adsorbents, endotoxin inactivation by low-dose PMB intravenous infusion or infusion into the extracorporeal circuit during blood purification is proposed to reduce considerably endotoxin activity not only in treatment of sepsis, but also in liver failure. However, the findings presented here are based on *in vitro* experiments. For an optimal and safe endotoxin inactivation therapy by polymyxin administration, further systematic investigations regarding drug monitoring and pharmacokinetic studies, especially *in vivo* studies, should be conducted.

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Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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2.1.7 Polymyxin-immobilisierte, nanostrukturierte Materialien – Eine neue Möglichkeit zur Sepsis Behandlung

Stephan Harm, Jens Hartmann: *Polymyxin-Coated Nanostructured Materials: An Option for Sepsis Treatment*. J Nanomater Mol Nanotechnol. 2016; S4:003, DOI:10.4172/2324-8777.S4-003

Kurzfassung:

Einleitung/Ziel: Endotoxine (Lipopolysaccharide, LPS) sind die Hauptbestandteile der äußeren Membran gramnegativer Bakterien und oftmals Auslöser entzündlicher Erkrankungen. Das Vorhandensein von Endotoxinen im Blut (Endotoxämie) in einer Konzentration < 1 ng/ml kann bereits einen septischen Schock auslösen. Es ist unumstritten, dass die Entfernung von Endotoxinen aus dem Blut bei Leberversagen und Sepsis für den Patienten vorteilhaft ist. Neben der adsorptiven Entfernung von Endotoxinen gibt es die Möglichkeit, diese mit Hilfe von Polymyxin zu binden, um ihre immunstimulierende Aktivität zu verringern. Ziel dieser Arbeit ist es, für die extrakorporale Blutreinigung einen Adsorber zu entwickeln, welcher während der Behandlung einen konstanten Polymyxin-Serumspiegel aufrechterhält.

Material & Methoden: Ein auf Polystyrol-Divinylbenzol-Copolymer basierender Adsorber (CG161c) mit nanostrukturierten Poren wurde mit definierten Mengen an Polymyxin (PMB) über hydrophobe Wechselwirkungen beladen. Die PMB-Freisetzung des Adsorbers wurde sowohl in Plasma als auch in fraktioniertem Plasma ermittelt.

Ergebnisse: In But und Plasma stellt sich sehr rasch ein Gleichgewicht zwischen gebundenem und freiem PMB ein. Die PMB-Freisetzung wird durch die innere Oberfläche des Adsorbers und die Proteinkonzentration im Plasma beeinflusst. In fraktioniertem Plasma, welches eine geringere Proteinkonzentration aufweist, erfolgt eine geringere Freisetzung. Zusätzlich konnte gezeigt werden, dass die PMB-Immobilisierung am CG161c Adsorber dessen Zytokinadsorption nicht beeinflusst.

Schlussfolgerung: Unser *in vitro*-Modell zeigt, dass die Kombination von Zytokinadsorption und kontrollierter PMB-Freisetzung durch ein und denselben Adsorber erfolgen kann. Dies könnte eine neue extrakorporale Therapieoption für die Gram-negative Sepsis darstellen.

Mein Beitrag für diese Publikation:

- Planung der Versuche
- Verfassen der Publikation
- Durchführung der Tests
- Analytik: Rasterelektronenmikroskop, HPLC-Analytik
- Datenauswertung

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Polymyxin-Coated Nanostructured Materials: An Option for Sepsis Treatment

Stephan Harm[1,2*] and Jens Hartmann[1]

Abstract

Objective: Endotoxins (lipopolysaccharides, LPS) are among the main targets in extracorporeal therapies. LPS, which is the major component of the outer cell wall of Gram-negative bacteria, strongly induces inflammatory responses in humans at concentrations lower than 1 ng/kg body weight. Although the elimination of LPS is promising for the supportive therapy of sepsis and liver failure, endotoxin neutralization using endotoxin adsorbents is controversial. Earlier studies show that Polymyxin B (PMB) could be applied for endotoxin inactivation in blood. Aim of this study was to establish an adsorbent-based PMB release system which ensures a constant PMB level in plasma during extracorporeal therapies.

Methods: A polystyrene-divinylbenzene based cytokine adsorbent (CG161c) with nanostructured pores was coated with a defined amount of PMB by hydrophobic interactions. The PMB release by the PMB coated adsorbent was studied in plasma and fractionated plasma.

Results: In plasma or blood, an equilibration between the free and bound form of PMB leads to a constant PMB level in plasma. The PMB release was influenced by the adsorbent inner surface area and the protein concentration of the plasma. In fractionated plasma where the protein concentration is lower, the PMB release was much less than in whole plasma. Additionally we could show that the PMB coating doesn´t influence the cytokine removal of the CG161c adsorbent.

Conclusion: Our *in vitro* model shows that the combination of cytokine removal and controlled PMB release by the same adsorbent could be an option for Gram-negative sepsis treatment.

Keywords

Endotoxin; Nanostructured Materials; Polymyxin; Multidrug resistant

Introduction

Endotoxins are lipopolysaccharides (LPS) which form the outer cell wall of Gram-negative bacteria and are released by cell lysis and during proliferation. Lipopolysaccharides are pyrogenic substances and can cause strong inflammatory response in human. Endotoxin accumulation in the blood circulation can result in uncontrolled activation of leucocytes and can lead to a disorder of the coagulation system [1,2]. In further consequence this can cause sepsis, severe sepsis or septic shock where the mortality rate, as a function of the severity of the disease, is between 30-60%. Endotoxemia can also occur in patients with impaired immune defense which can be a consequent of chemotherapy or liver failure [3,4]. For sepsis treatment, antibiotics against infection and corticosteroids to restore cardiovascular homeostasis and terminate systemic inflammation are used beside conventionally applied intensive care. Polymyxin B (PMB) is a peptide based antibiotic especially applied to treat infections caused by multidrug resistant Gram-negative bacteria. According to the LPS type, PMB build a very stable complex, which has much lower endotoxin activity compared to free LPS. The association constant (K_a) of the PMB-LPS complex depends of the LPS type and has a value between 1.8×10^{-6} and 2.3×10^{-6} M [5]. By establishing this stable complex, PMB is able to inactivate endotoxins very effectively. The dose for LPS-inactivation in human blood is much lower than the Minimal Inhibitory Concentration (MIC) of this antibiotic which is in the range of 2 to 8 mg/L [6]. Because high PMB serum levels can cause nephrotoxic effects, intravenous administration was avoided the last 50 years and consequently knowledge about pharmacokinetics and pharmacodynamics of Polymyxins is very limited. Although some clinical studies were focused on endotoxin inactivation, the applied PMB concentration was as high as normally used in order to kill bacteria [7,8]. Recently we could show that the optimal PMB concentration in human plasma is between 50 and 200 ng/ml [9]. However, a big challenge is not only to reach and hold the desired PMB level by intravenous administration, but also the lack of a quick and solid clinical test for PMB quantification in blood.

Endotoxins are among the main targets in extracorporeal blood purification therapies. There are two endotoxin adsorbents for clinical use available, the Toraymyxin and the Alteco endotoxin adsorbent cartridge. In recently published *in vitro* experiments we could show that the efficiency for LPS removal for these adsorbents is limited [10]. Since endotoxins are negatively charged molecules, anion exchange resins (e.g. DEAE or PEI groups bound to cellulose) are able to bind LPS by ionic interactions to the phosphate groups of the Lipid A domain of the LPS [11]. The reason why anion exchangers are not established in blood purification systems is due the unwanted side effects. By removal of anionic charged proteins like some coagulation factors (protein C, protein S and fibrinogen) they can cause coagulation, consequently, anion exchangers lack the needed biocompatibility for blood purification systems.

Aim of this study was to establish an adsorbent-based PMB release system which ensures a constant PMB level in plasma during extracorporeal therapies. A polystyrene-divinylbenzene based cytokine adsorbent with nanostructured pores [12] was coated with a defined amount of PMB by hydrophobic interactions. In plasma or blood, an equilibration between the free and bound form of PMB will lead to a constant PMB level in plasma.

Materials and Methods

Materials

The polystyrene-divenylbenzene based adsorbents used in this study were obtained from Dow Chemical (former Rohm and Haas, Philadelphia, Pa, USA). Polymyxin B and lipopolysaccharide (LPS)

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from *Escherichia coli* O55:B5 was purchased from Sigma-Aldrich (Vienna, Austria). Human plasma we got from a local plasma donation centre (fresh-frozen plasma) and Cytokines (TNF-α, IL-1β, IL-6, IL-8 and IL-10) were purchased from R&D Systems, (Minneapolis, MN).

Adsorbent characterization

The size distributions of the used adsorbents were measured by using a laser light scattering particle size analyser (Mastersizer 2000, Malvern Instruments, UK). Therefore 500 µl of adsorbents were suspended in 100 ml distilled water and stirred during sonication to avoid agglomeration. The particle size distribution results are volume based and expressed in terms of equivalent spheres. The structural features of each adsorbent were determined by scanning electron microscopy (SEM). SEM images were obtained by washing the adsorbent particles with pure ethanol and dry these in a heating cabinet at 100°C for 12 hours. The particles were then sputtered with gold (Q150R ES, QUORUM) and imaged by SEM (TM-1000, Tabletop Microscope, Hitachi).

PMB coating of the adsorbent material

Before the experiments were carried out, the adsorbents were washed with ethanol, water and 0.9% saline solution for 60 min using 4 ml washing volume per ml adsorbent for each washing step. PMB coating of the adsorbent was done by incubation of 3 ml of adsorbent with different amounts of PMB solution containing 10 mg PMB/ml (Table 1). The coating was performed for 12 hours on a roller mixer at room temperature. Thereafter, the coated adsorbents were washed twice with physiological saline solution and were finally stored in a 50% (w/v) suspension at 4°C before they were used for adsorption experiments. To determine if the whole PMB is hydrophobically bound on the adsorbent, PMB quantification of the coating and washing solutions was conducted by an HPLC based method.

PMB-quantification

The concentration of PMB in aqueous solutions was determined by a high performance liquid chromatographic method [13] with minor changes as described earlier [10]: Two millilitre samples (washing solution) were transferred to a solid-phase extraction C18 cartridge (Sep-Pak, Waters). After the cartridge was washed with 500 µl of carbonate buffer (1%, w/w, pH 10), 110 µl of 9-fluorenylmethyl chloroformate (FMOC-Cl, Sigma) solution (containing 30 µl of 100 mM FMOC-Cl in acetonitrile and 80 µl of methanol) was added. Following 10 min of reaction in the dark, the PMB derivatives were eluted with 900 µl of acetone. The eluate was mixed with 600 µl of boric acid (0.20 M) and 500 µl of acetonitrile. After vortexing, 20 µl of the eluate was injected onto the HPLC column (50 × 4.6 mm Onyx Monolithic C18 column coupled with a 4 × 3.0 mm C18 guard column, Phenomenex). The mobile phase with a flow rate of 1 ml/min was acetonitrile-tetrahydrofuran-water (50:25:25) and the run time was 10 min. Fluorescence detection was performed at an excitation wavelength of 260 nm and an emission wavelength of 315 nm. The concentrations of PMB were calculated on the basis of the sum of the chromatographic peak areas of Polymyxin B1 and B2 in the HPLC assay. The limit of quantification was 0.025 mg/l. The quantification of PMB in blood, plasma and serum was measured using a competitive enzyme immunoassay kit for the analysis of Polymyxin B and E (Kwinbon Biotech, Beijing, China) with a detection limit of 1 ng/ml. To obtain a signal in the standard range between 0 and 81 mg/ml, the samples were diluted with the dilution reagent which was provided with the ELISA kit.

Table 1: PMB coating procedure of the PS-DVB based adsorbents.

Adsorbent [ml]	PMB solution 10 mg/ml [ml]	0.9 % NaCl solution [ml]	PMB/g adsorbent [mg]
3	0.00	10.00	0.0
3	0.30	9.70	1.0
3	0.75	9.25	2.5
3	1.50	8.50	5.0
3	4.5	5.5	15
3	7.5	2.5	25

PMB desorption experiments in plasma vs fractionated plasma

To illustrate that the amount of PMB desorption depends on the protein composition in the medium, desorption experiments of the PMB coated CG161c were carried out in plasma and fractionated plasma. Fractionated plasma was produced using the Alfuflow® filter (Fresenius Medical Care, Germany) which is a plasma filter with a sieving coefficient for albumin of ≥ 60% and a molecular weight cut-off of about 250 kDa [14]. This specially designed albumin-permeable filter is used in the Prometheus® system which is an extracorporeal liver support system where the detoxification of the blood takes place in a secondary circuit (fractionated plasma) by two adsorbent cartridges. For these PMB desorption experiments, one ml adsorbent coated with different amounts of PMB was incubated in 9 ml plasma or fractionated plasma for 60 min at 37°C on a roller mixer. Thereafter, the samples were centrifuged and the supernatants were stored at -80°C until PMB quantification was conducted using the HPLC method and the ELISA kit.

PMB desorption experiments as a function of the adsorbent surface area

To demonstrate that the desorption of PMB is not only effected by the adsorbent material but also by the available surface of the adsorbent, adsorbents with different porosity and surface areas were used for the desorption experiments. The CG161c with 900 m^2/g and the HPR10 with 600 m^2/g inner surface were coated with different amounts of PMB (0, 5, 10, 15, 20 and 25 mg PMB/ml adsorbent) using the coating protocol described above. Desorption experiments in plasma were conducted by incubation of 1 ml PMB coated adsorbent in 9 ml plasma for 60 min at 37°C. Thereafter, PMB in the supernatant was quantified.

Endotoxin inactivation experiments

To determine the PMB level which is needed for endotoxin inactivation, fresh human heparinized plasma spiked with 5 ng/ml LPS from *E. coli* was incubated with different PMB concentrations (0, 10, 50, 100, 500, 1000 and 1000 ng/ml) for 60 min at 37°C. The experiments were performed in pyrogen-free 3 ml glass vials and the endotoxin activity was measured using the Limulus Amoebocyte Lysate (LAL) test (Charles River, Oxford, UK). To determine the LPS inactivation by PMB which is released from the PMB coated adsorbent, batch tests using PMB coated CG161c adsorbent were performed. Therefor, 0.5 ml of different PMB coated adsorbent (0, 2.5, 5, 10, 15, 20 and 25 mg PMB/ml adsorbent) were incubated in 4.5 ml plasma spiked with 5 ng/ml LPS. The incubation was carried out on a roller mixer for 60 min and thereafter the LPS activity was measured in the supernatant using the LAL test.

Cytokine removal experiments

To check if the PMB coating of the CG161c adsorbent

influences the cytokine adsorption, cytokine removal batch tests were performed. Plasma was spiked with selected cytokines to achieve a concentration between 250 and 500 pg/ml for each (Table 2). One ml PMB coated adsorbent was incubated in cytokine spiked plasma for 60 min at 37°C on a roller mixer. Samples were centrifuged and the supernatants were collected and stored at -80°C until cytokine quantification using the Bio-Plex cytokine array (Biorad, Vienna, Austria).

Results and Discussion

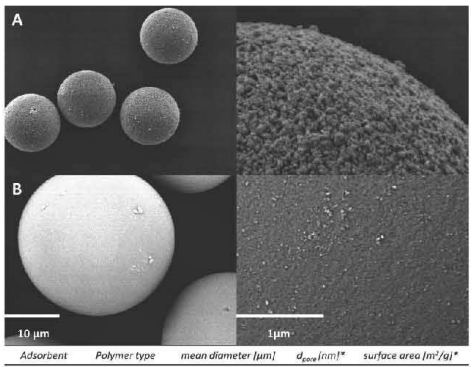
The SEM images (Figure 1) show the pore structures of the adsorbents surfaces. The pictures clearly show that the HPR10 adsorbent has a rougher pore structure than the CG161c adsorbent. These findings confirm the manufacturers which quote 30 nm pores and an inner surface of 600 m^2/g for the HPR10 adsorbent and 15 nm pores and an inner surface of 900 m^2/g for the CG161c adsorbent. Adsorbents with very high inner surface have to be loaded with higher amounts of PMB to obtain the desired PMB concentration in plasma. Consequently, the CG161c adsorbent can maintain the desired PMB concentration much longer.

The adsorption by adsorbent based extracorporeal blood purification systems can take place in blood, plasma or fractionated plasma. Fractionated plasma can be produced by specially designed plasma filters (e.g. Albuflow®) which have membranes with lower molecular weight cut-off and are for example used in the Prometheus® system. Due to the fact that this filter excludes the high molecular weight plasma fraction, the plasma composition and also the hydrophobicity of fractionated plasma is totally different to whole plasma. In our experiments we compared the PMB release of the CG161c adsorbents in plasma to fractionated plasma. The results clearly show that in fractionated plasma, where the hydrophobicity is lower, the PMB release is strongly reduced (Figure 2). To give an example: After incubation of PMB coated CG161c (25 mg PMB/ml CG161c) the PMB level in plasma was 7594 ng/ml whereas in fractionated plasma only 165 ng PMB/ml was measured. The results of these experiments suggest that the PMB release into plasma depends on the protein concentration. It makes a big difference whether the PMB coated adsorbent is used in plasma or in fractionated plasma where the hydrophobicity is much lower.

Because the equilibrium between adsorption and desorption of PMB depends not only on the material composition but also on the material property and especially on the available surface for the adsorbate we compared the PMB release between the CG161c with 900 m^2/g surface and the HPR10 with 600 m^2/g. Although both adsorbents are polystyrene-divinylbenzene based, the CG161c shows strongly reduced PMB release into plasma after coating compared to the HPR10 (Figure 3). The results clearly show that the adsorption and desorption is a function of the ratio of PMB concentration to adsorbent surface. The PMB coating will basically work with all hydrophobic based adsorbent material, but to get a desired PMB concentration in plasma, only the PMB loading per gram adsorbent material differs as a function of the material and the available inner surface of the adsorbent.

Table 2: Plasma concentrations of selected cytokines for the cytokine removal experiment.

Cytokine	Plasma concentration [pg/ml]
TNF-α	500
IL-1β	250
IL-6	200
IL-8	200
IL-10	300



Adsorbent	Polymer type	mean diameter [µm]	d_{pore} [nm]*	surface area [m^2/g]*
A HPR10	PS-DVB	10	30	600
B (CG161c)	PS-DVB	120	15	900

* Manufacturer data

Figure 1: SEM images at 200 and 5000 x magnification and particle size distribution using laser-light scattering of the HPR10 (A) and CG161c (B) adsorbents.

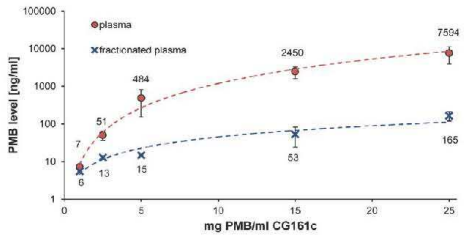


Figure 2: Comparison of PMB desorption from PMB coated CG161c adsorbent into plasma and fractionated plasma. The results are shown in mean ± SD (n=3).

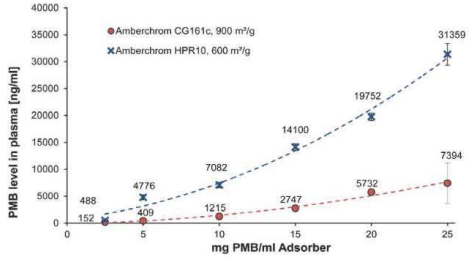


Figure 3: Comparison of PMB release into plasma between the CG161c adsorbent with an inner surface of 900 m^2/g and the HPR10 adsorbent with 600 m^2/g. Both adsorbent were coated with different PMB amounts and incubated in plasma. After incubation PMB quantification was done. The results are shown in mean ± SD (n=3).

Because, when using a PMB coated adsorbent, the constant PMB level in plasma is maintained by a permanent balancing act between adsorption and desorption of PMB on and from the adsorbent surface, a question arises: Does the permanent ad- and desorption of PMB influence the LPS inactivation? To answer this open question we compared the LPS inactivation between released and infused PMB. The results show that the presence of adsorbents does not influence the LPS inactivation by the released PMB (Figure 4).

To verify if the PMB coating influences the cytokine removal of the CG161c adsorbent, we compared PMB-coated and uncoated adsorbent regarding cytokine removal in plasma. The experiments suggest that the PMB coating of the CG161c adsorbent doesn't influence the cytokine removal which can take place in parallel (Figure 5).

In general, for safe and optimal endotoxin inactivation therapy by intravenous PMB administration, further systematic investigations concerning pharmacokinetic studies and drug monitoring should be performed. Because of the fact that Polymyxins are small molecules, they are removed by hemodialysis [7] and even by adsorption. Consequently the clearance of the dialyzer and/or the adsorption unit has to be taken into account when PMB is applied simultaneously with extracorporeal blood purification. Unlike to intravenous PMB treatment, a PMB desorption model for endotoxin inactivation during extracorporeal blood purification can be realized in different systems and guarantee the maintenance of the desired PMB level during treatment. One example is shown in Figure 6 where a PMB coated adsorbent is used in a closed plasma circuit system. This PMB release system is not limited for adsorbents used in plasma. For example the clinically proofed Cytosorb®, which is a polystyrene-divinylbenzene based cytokine adsorbent and used in hemoperfusion, can also be coated with PMB. *In vitro* tests will be necessary to validate how much PMB should be bound per ml adsorbent to get the desired PMB level in blood for endotoxin inactivation. Other possibilities to maintain equilibrium of PMB during extracorporeal sepsis treatment could be the use of PMB containing dialysate. Because of the nephrotoxicity of PMB, other LPS inactivating antimicrobial peptides (AMP) can be used for such a release system. Basic requirements for AMP are the binding on the used adsorbent by hydrophobic interactions. Promising AMP for future applications could be alkylated lactoferrin derived peptides [15,16]. Our *in vitro* model shows that the combination of cytokine removal and controlled PMB release for endotoxin inactivation by the same adsorbent is possible. This combination may be an option for Gram-negative sepsis treatment.

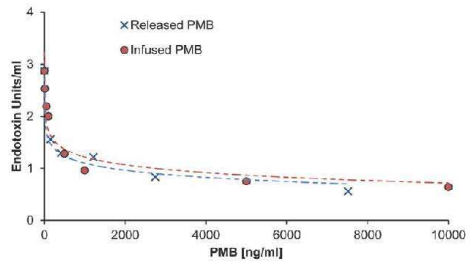


Figure 4: Endotoxin inactivation in plasma was compared between added PMB and the PMB coated adsorbent. The results are shown in mean (n=3).

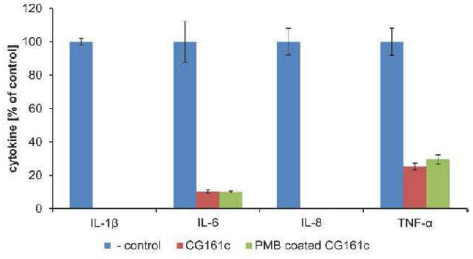


Figure 5: To test if PMB coating influences the cytokine adsorption, the CG161c and the PMB coated CG161c where compared regarding cytokine removal from plasma. The results are shown in mean ± SD (n=3).

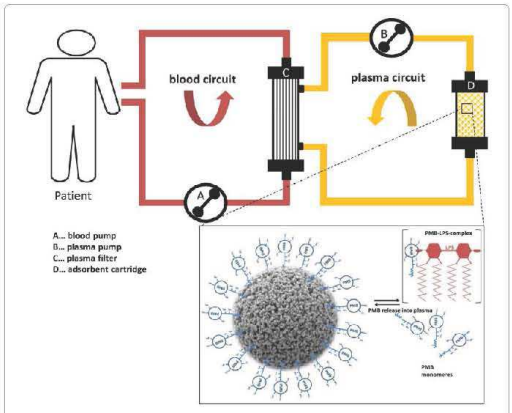


Figure 6: Setup of a blood purification system combining cytokine removal by adsorption and inactivation of lipopolysaccharides by released Polymyxin B. The system consists of a blood pump (A), a plasma pump (B), a plasma filter (C) and an adsorbent cartridge (D) containing the PMB-coated adsorbent.

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2.1.8 Ein neues Verfahren zur unterstützenden Sepsisbehandlung

Jens Hartmann, Stephan Harm: *A new integrated technique for the supportive treatment of sepsis*. Int J Artif Organs. 2017; 40(1):4-8, DOI:10.5301/ijao.5000550

Kurzfassung:

Einleitung/Ziel: Obwohl auf dem Gebiet der Sepsis-Behandlung seit vielen Jahren intensiv Forschung betrieben wird, sind die derzeit verfügbaren Therapien wenig erfolgreich und es mangelt noch an Verfahren, welche Endotoxine und Entzündungsmediatoren aus dem Blut effizient entfernen können. In dieser Arbeit stellen wir ein neues Blutreinigungsverfahren vor, welches in der Leber- und Sepsistherapie Anwendung finden kann.

Material & Methoden: Zur Entfernung von Zytokinen und anderen Entzündungsmediatoren soll in diesem Verfahren ein Polystyrol-Divinylbenzol-Copolymer Adsorber mit 15 nm Poren (CG161c) verwendet werden. Anstatt die Endotoxine zu entfernen, sollen durch eine niedrig dosierte Polymyxin B (PMB) Infusion die Endotoxine inaktiviert werden. Um die Blutverträglichkeit des Systems zu verbessern und die Filterstandzeit zu verlängern, soll mit Zitrat antikoaguliert werden. Zusätzlich ist im System ein High-Cut off Filter integriert, welcher zum einen die urämischen Toxine entfernt und zum anderen den Adsorber unterstützt, klein bis mittelmolekulare Entzündungsmediatoren aus dem Blut zu entfernen.

Ergebnisse: Es konnte gezeigt werden, dass mit einem PMB-Serumspiegel von 100-200 ng/ml die immunstimulierenden Eigenschaften der Endotoxine weitgehend inaktiviert sind. Der Zytokinadsorber CG161c zeichnete sich im Vergleich zu anderen konventionell erhältlichen Zytokinadsorbern durch eine gute Entfernung von TNF-α aus. Die Zitratantikoagulation zeigte in unseren *in vitro* Versuchen eine deutlich verbesserte Blutverträglichkeit der Filtermaterialien im Vergleich zu Heparin.

Schlussfolgerung: Eine Kombination von Endotoxin-Inaktivierung durch PMB-Infusion, Zytokinadsorption mit Hilfe von CG161c Adsorber, Zitratantikoagulation und der Verwendung eines High-Cut off Filters in einem extrakorporalen Blutreinigungssystem zeigte in *in vitro* Versuchen vielversprechende Ergebnisse. Weitere Versuche müssen noch folgen, um eine sichere Anwendung zu gewährleisten.

Mein Beitrag für diese Publikation:

- Planung der Versuche
- Durchführung der Tests
- Analytik: Rasterelektronenmikroskop,
- Datenauswertung

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ORIGINAL RESEARCH ARTICLE

A new integrated technique for the supportive treatment of sepsis

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ABSTRACT

Although there has been continuous, intensive research for many years in the field of sepsis treatment, currently available treatment options are limited, and there is still a lack of systems that efficiently remove endotoxins as well as mediators. Here, we discuss a newly developed, integrated technique that combines different aspects for their use in extracorporeal blood purification for the supportive treatment of liver failure and sepsis.

Keywords: Adsorption, Blood purification, Citrate anticoagulation, Liver failure, Polymyxin, Sepsis

Introduction

Although sepsis is a frequent cause of death in the intensive care unit and many research groups focus on sepsis, the treatment outcome is still very limited. Extracorporeal blood purification is an option to support the treatment of severe sepsis and septic shock, in addition to treatment with antibiotics, plasma exchange, and measures for stabilizing the patients' cardiovascular status. While some extracorporeal systems concentrate on the removal of only 1 group of potential target substances, such as the Toraymyxin™ or the Alteco™ adsorbent for the removal of endotoxins, or the Bellco and the Cytosorb™ adsorbents for the removal of cytokines, others target a group of factors. The clinical outcome of the former is very limited and/or an efficient removal of the crucial factor TNF-α could not be shown (1-4). Among the latter is the Modular Immune Adsorber System (MIAS) system which showed promising results in the recovery of the function of monocytes in immune paralysis (5) but still lacks clinical data. Although there are some promising approaches with adsorbents based on anion exchangers (6), their clinical benefit has not been shown yet in human trials.

Endotoxins are well known for their ability to trigger the inflammatory cascade, which can subsequently lead to Gram-negative sepsis and septic shock. In order to reduce or even block the production of cytokines via the TLR4-NFκB pathway, they have to be removed or inactivated efficiently. However, since in most cases patients who are considered to develop sepsis or even are diagnosed as septic patients have high levels of cytokines, inactivation or removal of endotoxins alone is not sufficient and, in most cases, too late. Therefore, in addition to the removal or inactivation of endotoxins, the efficient removal of cytokines seems mandatory.

The results from a clinical trial indicate that citrate anticoagulation can improve the mortality in critically ill patients (7). The reason for these findings could be a reduced activation of the complement system as well as the lower cytokine induction when citrate anticoagulation is applied. Although other studies could not confirm these findings and did not show any advantage of citrate over heparin regarding inflammation (8, 9), citrate offers other advantages such as longer filter lifetime (10) and prevention of Heparin-induced thrombocytopenia (HIT).

These approaches may offer new technologies in the long lasting battle against sepsis that has yet to be won. Here we discuss approaches to overcoming most issues as well as an integrated technique that combines these ideas into a multicomponent system for the potential treatment of sepsis.

Methods

Endotoxin inactivation

As has been shown recently, commercially available endotoxin adsorbents do not provide adsorptive removal of endotoxins (11). Polymyxin-B (PMB) is well known for its high binding to endotoxins (lipopolysaccharides, LPS) in solution. To prove endotoxin inactivation by PMB in vitro, whole blood from 3 healthy donors was spiked with endotoxins from *E. coli*, and different PMB concentrations were added. After 4 hours of incubation, cytokine analysis was conducted by ELISA.

Clearance tests with commercially available high-flux dialyzers were carried out to assess the removal of PMB in the extracorporeal circuit. Based on the results, our group has developed an infusion protocol for a controlled and safe

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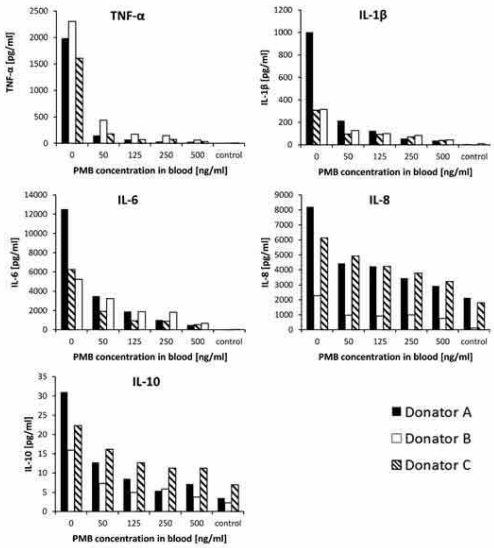

Fig. 1 - PMB-dependent reduction of cytokine induction after stimulation with endotoxins from *E. coli*, n = 3, (18).

application of low-dose PMB infusion that can maintain a defined PMB concentration in the patient's blood even during extracorporeal treatment. The protocol takes into account the clearance rate of the dialyzer as well as the patient's clearance (12). However, the protocol is only valid for patients with normal kidney function and does not cover special patient populations.

Cytokine adsorption

Commercially available adsorbents offer only limited removal of cytokines, especially of TNF-α, which has a molar mass of 51 kDa. The reason for the poor adsorptive removal is the pore size of these adsorbents. Small pores reduce the accessible surface area, since substances of higher molecular weight cannot come in contact with the inner surface of the adsorbents. In the case of pores larger than the optimal size, the inner surface is blocked by higher molecular-weight proteins and lipid fractions of the plasma/blood. The latter effect can be avoided when an albumin filter is used, providing a lower molecular-weight cut-off than conventional plasma filters. Therefore, a cartridge containing 350 mL of polystyrene-divinylbenzene (PS-DVB)-based adsorbent CG161c with optimal pore-size distribution in the range of 15 nm was introduced. It outperforms all tested commercially available adsorbents in our in vitro experiments (13).

The adsorption efficiency is further improved by using the Fresenius Albuflow® filter, which avoids the contact of high molecular-weight proteins with the adsorbents that would block their pores. The new adsorbent not only adsorbs cytokines efficiently, but also toxins that accumulate in liver failure, such as bilirubin and bile acids.

Citrate anticoagulation

Citrate anticoagulation is an upcoming method of anticoagulation in extracorporeal blood purification technologies. Compared to conventional anticoagulation with heparin, citrate offers several advantages such as longer filter lifetime, limitation of the anticoagulation to the extracorporeal circuit (regional anticoagulation), and the avoidance of HIT. Furthermore, citrate significantly improves the blood compatibility of many polymers. In order to visualize anticoagulant-dependent cell activation, we compared the cell adhesion to adsorbents and membrane materials using in vitro experiments with heparin- and citrate-anticoagulated blood by scanning electron microscopy (SEM). In earlier publications we were able to show that citrate suppresses the activation of the complement system (14) and reduces cytokine induction in the presence of endotoxins (15). A new device for citrate anticoagulation that enables a target-Ca^{2+} oriented infusion was developed (16), which can downregulate the Ca^{2+} concentration to ≤0.2 mmol/L in order to minimize complement activation in the extracorporeal circuit. A unique feature of this algorithm is that it takes into account the patients' hematocrit. Compared to conventional algorithms, this enables a higher accuracy, since citrate does not enter erythrocytes and the achieved citrate concentration depends on the hematocrit of the patients' blood (17).

Results

Endotoxin inactivation

The activity of endotoxins decreased with increasing PMB concentration, as shown by determination of cytokine induction at different PMB concentrations using ELISA. The results of our in vitro experiments suggest a PMB concentration as low as 50-200 ng/mL for an effective endotoxin inactivation (Fig. 1). These promising effects of PMB were published recently (18).

Cytokine adsorption

Compared to commercially available cytokine adsorbents, the CG161c adsorbent offers a more efficient removal of cytokines (Fig. 2), especially for the one with the highest molecular mass, TNF-α.

Citrate anticoagulation

With increasing citrate concentration and the resulting decrease of Mg^{2+} and Ca^{2+}, complement activation can be increasingly suppressed (Fig. 3). The results show that cell adhesion to the materials was significantly lower when anticoagulation was carried out with citrate. Furthermore, it could be shown that activation of leukocytes as well as thrombocytes was lower when citrate was used (Fig. 4).

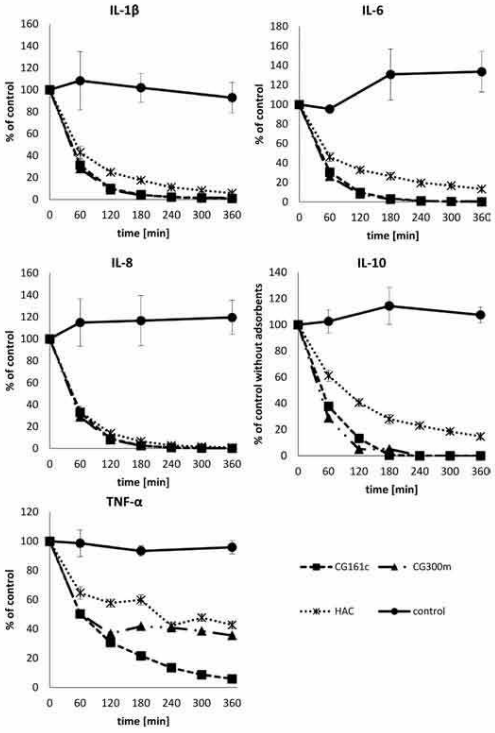

Fig. 2 - Comparison of cytokine removal for different adsorbents, n = 3 (19). HAC (dotted lines) = hemoadsorption column.

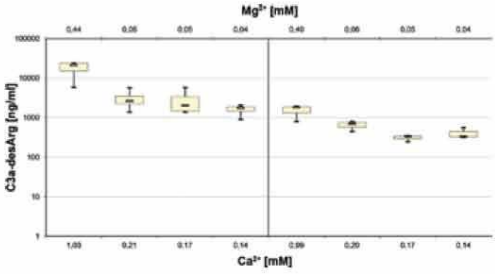

Fig. 3 - Dependency of the complement activation on the Mg^{2+} (Ca^{2+}) concentration. **Left:** Blood activated with cellulose microspheres. **Right:** Control without activation. n = 4 for activated samples and n = 3 for control (20).

Conclusions

The activity of bacterial endotoxins can be significantly reduced by PMB. The recommended concentration of 50-200 ng/mL in order to decrease the endotoxin activity to about 50% is far below the minimum inhibitory concentration (MIC) of this antibiotic and, therefore, its nephrotoxicity can probably be considered neglibile. Although polymyxins can significantly reduce the cytokine storm in endotoxemia, it must be taken into consideration that patients developing or even suffering from sepsis already show high cytokine levels before starting any therapy. Since polymyxins cannot reduce pre-existing cytokine levels, the administration of polymyxins should be supported by effective approaches for cytokine removal, such as adsorption or hemofiltration. The newly developed polystyrene-divinylbenzene based adsorbent offers optimal pore size distribution in order to remove cytokines efficiently. Citrate anticoagulation can support the extracorporeal treatment of severe sepsis or septic shock by reducing complement activation as well as cytokine induction. Since complement activation in blood purification mainly takes place in the extracorporeal circuit, citrate can especially support the treatment of inflammatory episodes in extracorporeal blood purification. The above-mentioned technologies can be combined into a multi-component system that potentially offers a new approach for the highly challenging treatment of sepsis or septic shock (Fig. 5). Caused by impaired function of the reticulo-endothelial system in liver failure, endotoxins from the portal vein can pass the liver and enter the systemic circulation, followed by induction of cytokines and systemic inflammation. Therefore, the technologies described here can also be applied in the treatment of acute liver failure.

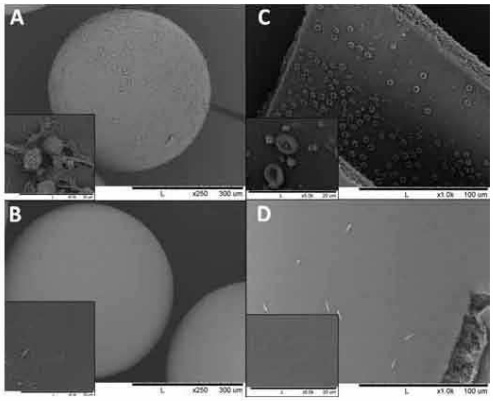

Fig. 4 - SEM images after in vitro experiments using a hemoperfusion adsorbent and a dialyzer with blood anticoagulated with 3 IU heparin (**A** and **C**) compared to blood anticoagulated with 6 mM citrate (**B** and **D**).

The multi-component system offers several advantages compared to other commercially available devices. Since the Albuflow® filter separates blood cells as well as the high molecular-weight (lipo)protein fraction from the adsorbent circuit, it highly improves the efficiency of the adsorbent. The new adsorbent offers high biocompatibility and removes

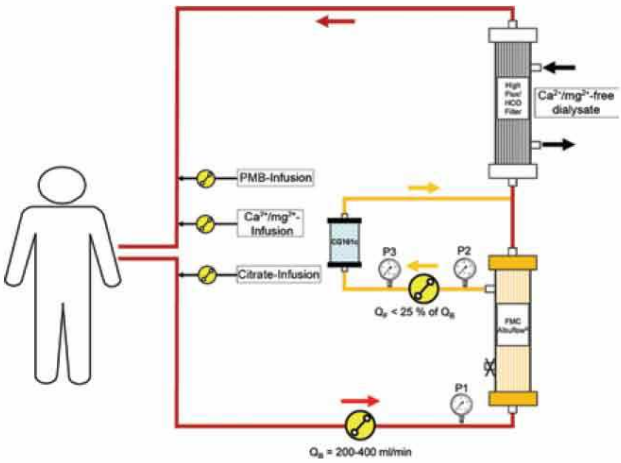

Fig. 5 - Scheme of the technology for supportive sepsis treatment.

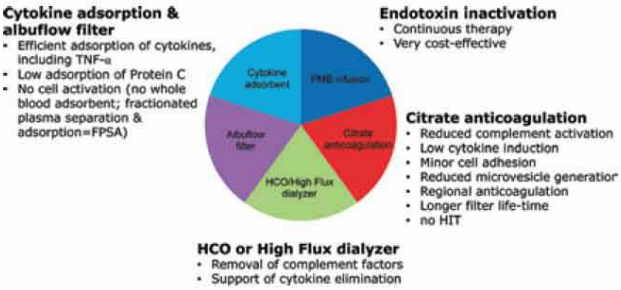

Fig. 6 - The multi-component system for the supportive treatment of sepsis.

target toxins in liver failure as well as in sepsis. In the case of Gram-negative inflammation, the low-dose PMB infusion inactivates endotoxins and offers a cost-effective, continuous method to avoid rebound of cytokines. The citrate anticoagulation module improves the biocompatibility of the whole system and could help to reduce the rebound of cytokines. Furthermore, citrate anticoagulation prolongs the filter lifetime, which supports long treatment times without interruptions or change of some components. Using a high cut-off (HCO) filter can further reduce the cytokine level as well as complement factors in the patient's blood (Fig. 6).

The new aspects presented here offer a potential new technology for the extracorporeal treatment of sepsis and liver failure. However, although the in vitro results are very promising, the efficacy of this newly developed, multi-component system has not yet been validated in vivo.

Acknowledgments

The authors thank Ute Fichtinger and Claudia Schildböck for their excellent technical work.

Fig. 7 - Dieter Falkenhagen in the laboratory, July 2011.

In memory of Dieter Falkenhagen

This paper is dedicated to the former head of our research group, Dieter Falkenhagen (Fig. 7), a multifaceted pioneer in the

field of extracorporeal blood purification (21, 22). Dieter Falkenhagen always had a friendly ear for new ideas, and his open-minded, sparkling mentality motivated us in our daily work in the laboratory. The new aspects and promising approaches for the supportive treatment of inflammation or sepsis that we present here result mainly from ideas that were generated during our inspiring discussions during the years 2012-2014.

Disclosures

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Conflict of interest: None of the authors has financial interest related to this study to disclose.

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2.1.9 Die Rolle von ionisiertem Calcium und Magnesium in der regionalen Zitrat-Antikoagulation und ihre Auswirkungen auf Entzündungsparameter

Karin Strobl, Stephan Harm, Viktoria Weber, Jens Hartmann: *The role of ionized calcium and magnesium in regional citrate anticoagulation and its impact on inflammatory parameters*. Int J Artif Organs. 2017; 40(1):15-21, DOI:10.5301/ijao.5000558

Kurzfassung:

Einleitung/Ziel: Die Blut- und Biokompatibilität in extrakorporalen Blutreinigungsverfahren kann durch die Verwendung von Zitratantikoagulation anstatt Heparin deutlich verbessert werden. Es gibt zahlreiche Protokolle zur Zitratantikoagulation, jedoch gibt es keinen Konsens über die Zielkonzentration von ionisiertem Kalzium (Ca^{2+}) im extrakorporalen Kreislauf. Mit Hilfe dieser *in vitro* Studie soll geklärt werden, welcher Ca^{2+}-Wert in der extrakorporalen Blutreinigung angestrebt werden soll, um einerseits ein sicheres und andererseits ein blutverträgliches Verfahren zu ermöglichen.

Methoden: Ziel dieser *in vitro* Studie war es, den Einfluss verschiedener Zitratkonzentrationen auf die Gerinnung, Komplementaktivierung und Zytokinsekretion zu untersuchen. Weiters wurde der Einfluss von ionisiertem Magnesium (Mg^{2+}) auf die Gerinnung und Zytokinaktivierung von Leukozyten untersucht.

Ergebnisse: Die Gerinnung, Komplementaktivierung und Zytokinsekretion wird in Abhängigkeit von der erreichten Ca^{2+} -Konzentration während der Zitratantikoagulation im Blut reduziert. Für eine effektive Antikoagulation war in den *in vitro* Versuchen eine Reduktion der Ca^{2+}-Konzentration auf 0,2 - 0,25 mM erforderlich. Mg^{2+} hat vor allem bei der Komplementaktivierung und Interleukin (IL)-1β Sekretion einen größeren Einfluss als Ca^{2+}. Zusätzlich wurde festgestellt, dass Mg^{2+} die aktivierte Gerinnungszeit (ACT) in Blut beeinflusst.

Schlussfolgerung: Unsere *in vitro* Daten zeigen, dass die Zitrat-Antikoagulation zu einer verminderten Freisetzung von Entzündungsmediatoren im extrakorporalen Kreislauf führt und dass sowohl Ca^{2+} als auch Mg^{2+} die Gerinnung, Komplementaktivierung und Zytokinsekretion beeinflussen.

Mein Beitrag für diese Publikation:

- Planung der Versuche
- Durchführung der Tests

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ORIGINAL RESEARCH ARTICLE

The role of ionized calcium and magnesium in regional citrate anticoagulation and its impact on inflammatory parameters

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ABSTRACT

Introduction: Regional anticoagulation with citrate has been found to be superior to heparin in terms of biocompatibility, and numerous protocols for regional citrate anticoagulation have been published, while a consensus on the target concentration of ionized calcium (Ca^{2+}) in the extracorporeal circuit has not been reached so far.
Methods: The aim of this in vitro study was to assess the impact of different citrate concentrations on coagulation as well as on complement activation and cytokine secretion and to investigate the impact of ionized magnesium (Mg^{2+}) on these parameters.
Results: We found that citrate effectively reduced coagulation, complement activation, and cytokine secretion in a dose-dependent manner and that a target Ca^{2+} concentration of 0.2-0.25 mM was required for efficient anticoagulation. Mg^{2+} triggered complement activation as well as interleukin (IL)-1β secretion in lipopolysaccharide (LPS)-stimulated whole blood in a dose-dependent manner and independently of Ca^{2+}. Additionally, it was found to reduce activated clotting time (ACT) in samples with low Ca^{2+} levels, but not at physiological Ca^{2+}.
Conclusions: Taken together, our data support the notion that regional citrate anticoagulation results in decreased release of inflammatory mediators in the extracorporeal circuit, requiring the depletion of both, Ca^{2+} and Mg^{2+}.

Keywords: Coagulation, Complement, Cytokines, Extracorporeal blood purification, Regional citrate anticoagulation

Introduction

Regional citrate anticoagulation is widely applied in acute dialysis, especially in patients with high risk of bleeding (1-5) or with risk of heparin-induced thrombocytopenia (6, 7). Citrate anticoagulation has been found to be associated with longer filter lifetimes compared to standard heparin anticoagulation (8-10) as well as with reduced complement activation (11, 12), and has been suggested to attenuate neutrophil degranulation (11, 13-15) as well as release of interleukin (IL-)1β (16). Two randomized controlled clinical trials comparing citrate versus heparin anticoagulation indicated reduced mortality in critically ill patients under citrate anticoagulation (17, 18), but these results could not be confirmed by others (19). Controversial results were obtained for cytokine secretion during citrate anticoagulation as well (17, 20).

Citrate acts via the chelation of Ca^{2+}, an essential factor in the coagulation cascade. Numerous protocols using different target concentrations of Ca^{2+} in the extracorporeal circuit have been proposed, with suggested target Ca^{2+}concentrations of <0.4 mM (4, 8, 21, 22), <0.5 mM (23, 24), or <0.65 mM (25). Most protocols, however, adjust the level of Ca^{2+} in the extracorporeal circuit to 0.25-0.35 mM (9, 26-29) or even lower (30). Beyond the chelation of Ca^{2+}, citrate also acts on Mg^{2+} and reduces its concentration in the extracorporeal circuit. While the influence of Mg^{2+} on coagulation is controversial (31-35), it is well established that Mg^{2+} represents an essential cofactor in the complement cascade, in particular in the alternative complement pathway, which is activated upon contact of blood with biomaterials, but the impact of Mg^{2+} substitution in dialysis fluid has not been studied in detail so far.

Here, we assessed the impact of different citrate concentrations on 2 global coagulation parameters, activated clotting time (ACT), and activated partial thromboplastin time (aPTT) as well as on the release of cytokines and on complement activation upon stimulation of citrated whole blood with lipopolysaccharide (LPS). We also investigated the effect of Ca^{2+} and Mg^{2+} substitution on coagulation, cytokine release, and complement activation.

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Materials and methods

Blood

Blood collection was approved by the Institutional Review Board of Danube University Krems and written informed consent was obtained from all donors. Human whole blood was freshly drawn from healthy volunteer donors into standard blood collection tubes (Vacuette; Greiner Bio-One) and anticoagulated as stated for the individual experiments.

Chemicals and reagents

Trisodium citrate solution (0.5 M; Provobispharm), ethylene diamine tetraacetic acid (EDTA) dihydrate (Sigma Aldrich), and unfractionated heparin (5000 IU/mL; Ebewe Pharma) were used as anticoagulants. $CaCl_2$ solution (0.5 M; Provobispharm) and $MgCl_2$ (Riedel-de Haën) were used to substitute Ca^{2+} and Mg^{2+}. LPS from *E. coli* 055:B5 was purchased from Sigma Aldrich.

Quantification of biochemical parameters

Ca^{2+} and Mg^{2+} were determined using an ion-selective electrode (Nova CRT 8; Nova Biomedical Waltham). Activated clotting time (ACT) was analyzed using the Hemochron Response or the Hemochron 401 coagulation monitors (International Technidyne Corporation) with kaolin containing tubes or with glass containing tubes. Activated prothrombin time (aPTT) was determined using the CL4 coagulometer (Behnk-Elektronik) and the actin FS reagent (Dade Behring). To test for complement activation, C3a-desArg, the stable metabolite of complement factor C3a, was quantified by ELISA (Progen). IL-1β, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) were determined by bead array (Bio-Plex Pro Cytokine Assay; Biorad).

Influence of citrate on global coagulation parameters

Freshly drawn whole blood was immediately anticoagulated with citrate at final concentrations of 2.75, 3.1, 3.65, 4.3, 5.5, and 6.5 mM to reach Ca^{2+} target concentrations of 0.4, 0.35, 0.3, 0.25, 0.2, and 0.15 mM. ACT was determined after activation of the samples with glass particles in the order of decreasing Ca^{2+} concentration. Samples containing 0.2 and 0.15 mM Ca^{2+} were additionally analyzed for aPTT.

Influence of citrate on cytokine secretion and complement activation

Freshly drawn whole blood was anticoagulated with 2 IU/mL heparin, and trisodium citrate was added to adjust Ca^{2+} to target concentrations of 0.5, 0.4, 0.3, 0.2, and 0.1 mM. Aliquots of 1.5 of citrated whole blood were stimulated with LPS (1 ng/mL) or incubated with saline solution at 37°C with gentle rolling. Samples were drawn after 15 minutes to quantify complement activation and after 4 hours to determine cytokine release. EDTA was immediately added to all samples at a final concentration of 6.7 mM to terminate stimulation, and plasma obtained by centrifugation of the samples at 2000 g (4°C, 15 minutes) was stored at -80°C until further analysis of C3a, IL-1β, IL-6, IL-8, and TNF-α, which was performed as described above.

Influence of Ca^{2+} and Mg^{2+} on activated clotting time

To investigate the effect of Mg^{2+}-free versus Mg^{2+}-containing dialysis fluid on ACT, freshly drawn whole blood anticoagulated with 6 mM citrate was dialyzed against physiological saline solution containing 0.5 mM Mg^{2+} or against Mg^{2+}-free saline solution using FX 80 dialysis filters (Fresenius Medical Care) to deplete Ca^{2+}, citrate, and, in the case of dialysis against Mg^{2+}-free saline, also Mg^{2+}. Thereafter, Ca^{2+} levels were restored by addition of $CaCl_2$ to yield Ca^{2+} target concentrations of <0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 1.2 mM.

Influence of Ca^{2+} and Mg^{2+} on cytokine secretion and complement activation

Freshly drawn whole blood was anticoagulated with 3 IU/mL heparin, and Ca^{2+} or Mg^{2+} was complexed by addition of trisodium citrate (6 mM). Thereafter, physiological concentrations of Ca^{2+} (1.2 mM) or Mg^{2+} (0.6 mM) were either fully or partly restored by addition of $CaCl_2$ or $MgCl_2$. Whole blood without citrate was used as positive control, and citrated blood without substitution of Ca^{2+} or Mg^{2+} served as negative control. All samples were stimulated with LPS and analyzed for cytokine secretion and complement activation as described above. The higher heparin concentration was used to prevent clotting after ion restoration.

Statistical analysis

Statistical analysis was performed with SigmaStat version 2.03 (SPSS). One-way analysis of variances (ANOVA) was used for comparing groups regarding the influence of Ca^{2+} on cytokine secretion and complement activation. Data are given as mean ± standard deviation.

Results

Influence of citrate on global coagulation parameters, cytokine release, and complement activation

ACT increased with decreasing Ca^{2+} concentrations as shown in Figure 1A. At a Ca^{2+} level of ≤0.2 mM, ACT increased steeply to values of 1500 seconds, corresponding to the upper detection limit of the device. Consistent with ACT data, aPTT increased with decreasing Ca^{2+} concentrations as well, as shown in Figure 1B. The release of cytokines and complement activation in whole blood stimulated with LPS decreased significantly with increasing citrate concentrations (Fig. 2, $p<0.001$).

Influence of Ca^{2+} and Mg^{2+} on cytokine release, complement activation, and coagulation

Samples containing 6 mM citrate in addition to the basal anticoagulation with 3 IU/mL heparin showed a reduction of cytokine release and complement activation by more than

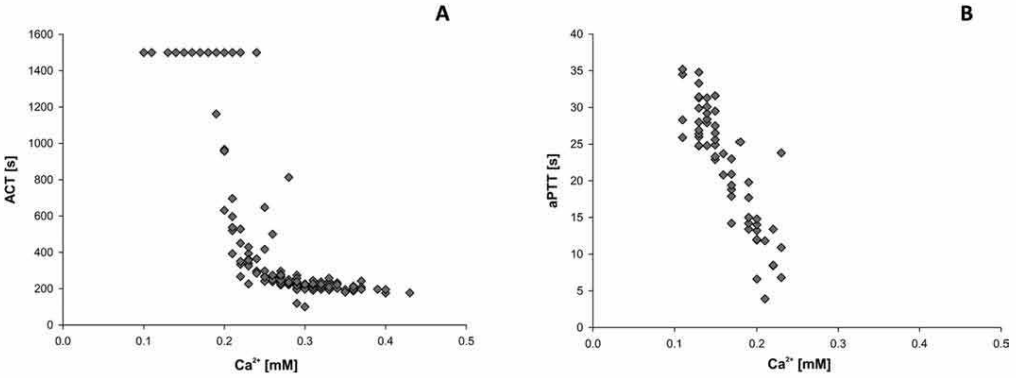
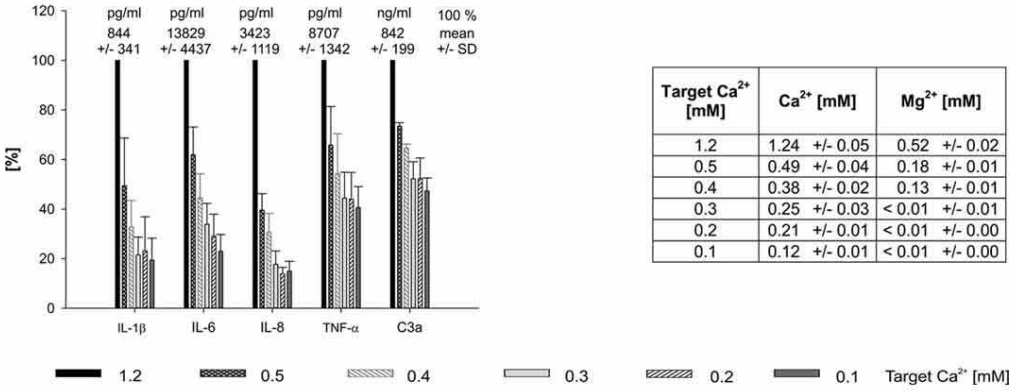


Fig. 1 - Influence of Ca^{2+} on the global coagulation parameters ACT (A; n = 10) and aPTT (B; n = 7).



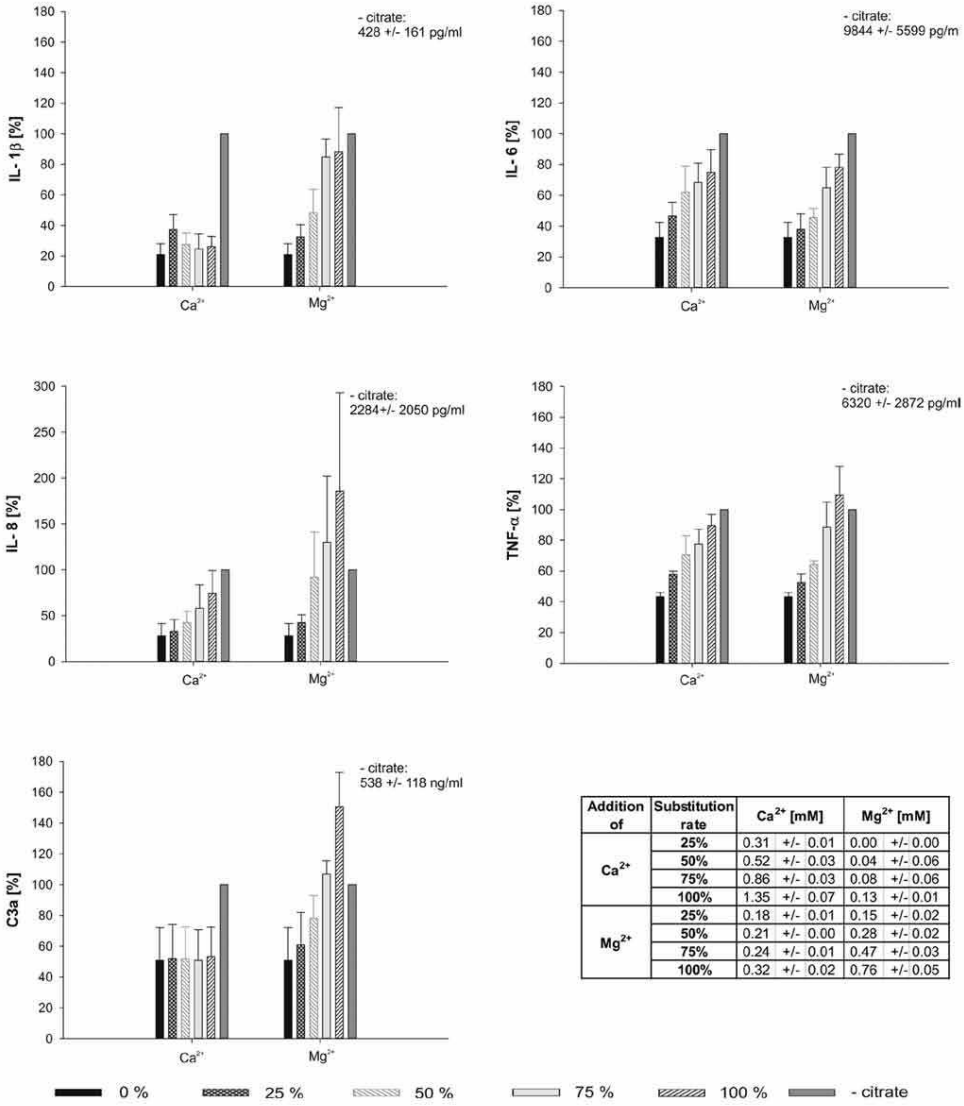
Target Ca^{2+} [mM]	Ca^{2+} [mM]	Mg^{2+} [mM]
1.2	1.24 +/- 0.05	0.52 +/- 0.02
0.5	0.49 +/- 0.04	0.18 +/- 0.01
0.4	0.38 +/- 0.02	0.13 +/- 0.01
0.3	0.25 +/- 0.03	< 0.01 +/- 0.01
0.2	0.21 +/- 0.01	< 0.01 +/- 0.00
0.1	0.12 +/- 0.01	< 0.01 +/- 0.00

Fig. 2 - Reduction of cytokine secretion and complement activation in LPS stimulated whole blood anticoagulated with different amounts of citrate ($p<0.001$). Freshly drawn whole blood was anticoagulated with different amounts of citrate to reach Ca^{2+} target concentrations ranging from 0.1 mM to the physiological level of 1.2 mM, stimulated with LPS, and tested for cytokine release and generation of complement factor C3a as described in materials and methods (n = 3). Concentrations indicated in the graph correspond to the mean ± SD of physiological samples (100%). The target concentrations of Ca^{2+} as well as the mean ± SD of measured concentrations of Ca^{2+} and Mg^{2+} are summarized in the table.

50% as compared to samples anticoagulated with 3 IU/mL heparin alone, as shown in Figure 3. Restoration of physiological Ca^{2+} concentrations by addition of $CaCl_2$ resulted in a dose-dependent increase of IL-6, IL-8, and TNF-α release up to levels equivalent to the control without citrate. Notably, however, IL-1β release was insensitive to Ca^{2+} substitution, while Mg^{2+} triggered IL-1β secretion in a dose-dependent manner. Likewise, complement activation was found to be dependent on the substitution of Mg^{2+}, as evidenced by increasing levels of C3a upon substitution of Mg^{2+}, while C3a remained unaffected by addition of Ca^{2+}. At physiological Ca^{2+} (1.22 mM), ACT was not influenced by Mg^{2+} (Fig. 4). At low Ca^{2+} concentrations, however, Mg^{2+} free blood showed an increasingly prolonged ACT as compared to blood containing Mg^{2+}.

Discussion

Regional citrate anticoagulation is increasingly applied in extracorporeal therapies due to its safety and effectiveness (1-6, 8-18, 36, 37). Citrate exerts its anticoagulant effect by chelation of Ca^{2+} and Mg^{2+}, and has been reported to exhibit effects beyond anticoagulation, such as reduction of complement activation and cellular activation (38). Exogenous citrate is metabolized to bicarbonate by the liver and by the skeletal muscle with a half-life of 30 to 60 minutes (39), whereas



Addition of	Substitution rate	Ca^{2+} [mM]	Mg^{2+} [mM]
Ca^{2+}	25%	0.31 +/- 0.01	0.00 +/- 0.00
	50%	0.52 +/- 0.03	0.04 +/- 0.06
	75%	0.86 +/- 0.03	0.08 +/- 0.06
	100%	1.35 +/- 0.07	0.13 +/- 0.01
Mg^{2+}	25%	0.18 +/- 0.01	0.15 +/- 0.02
	50%	0.21 +/- 0.00	0.28 +/- 0.02
	75%	0.24 +/- 0.01	0.47 +/- 0.03
	100%	0.32 +/- 0.02	0.76 +/- 0.05

Fig. 3 - Influence of Ca^{2+} and Mg^{2+} substitution on cytokine release and complement activation in LPS stimulated whole blood. Freshly drawn whole blood was anticoagulated with 3 IU/mL heparin and 6 mM citrate, Ca^{2+} and Mg^{2+} were partially (25-75%) or fully 100% restored, and cytokine release as well as complement activation following stimulation with LPS were analyzed as described in materials and methods (n = 3 for C3a, n = 4 for cytokines). Concentrations indicated in the graphs correspond to mean ± SD of controls without citrate (- citrate).

approximately 20% of infused citrate remains unmetabolized and is excreted by the kidney. While citrate has to ensure adequate anticoagulation of the extracorporeal circuit, the rate of citrate infusion into the patient should be kept as low as possible to limit potential systemic effects of citrate, such as hypocalcemia or metabolic alkalosis.

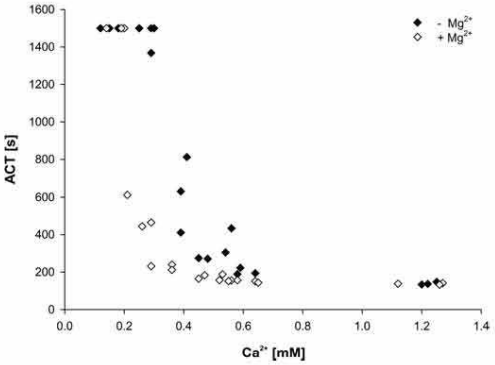


Fig. 4 - Impact of Mg^{2+} on activated clotting time in whole blood (n = 3). The presence of Mg^{2+} is associated with reduced ACT at low levels, but not at physiological levels of Ca^{2+}.

The aim of this in vitro study was 2-fold: first, to investigate impact of citrate on LPS-induced cytokine release and on complement activation as well as on global coagulation parameters in whole blood; and second, to assess the relative contributions of Ca^{2+} and Mg^{2+}, which are both affected by citrate anticoagulation, in the context of cytokine induction and complement activation in the extracorporeal circuit. Regarding global coagulation parameters, our data show a clear correlation of ACT and Ca^{2+} levels, confirming clinical data (40, 41). We found that Ca^{2+} levels of 0.2 mM or lower inhibit coagulation almost completely, whereas Ca^{2+} concentrations above 0.4 mM did not sufficiently suppress coagulation. Our findings are in good accordance with previous reports showing no indications of prolonged ACT at post-filter Ca^{2+} levels slightly below 0.4 mM (22). James et al described Ca^{2+} levels below 0.33 mM to be required to prevent coagulation as shown by thromboelastography (42), while other studies identified thresholds of 0.23 mM (43) or 0.25 mM (44) for thrombin generation. Combining the in vitro data presented here with our previous clinical data in chronic dialysis patients (41) and with the reports described above (43, 44), a Ca^{2+} concentration of 0.2 to 0.25 mM should be targeted to assure sufficient anticoagulation of the extracorporeal circuit. A further reduction of Ca^{2+} would hardly offer further benefits and would increase the risk of citrate accumulation.

Beyond the inhibition of coagulation, we found that citrate resulted in reduced cytokine secretion and complement activation in LPS-stimulated whole blood as compared to anticoagulation with unfractionated heparin, confirming previous results by others (16, 17, 45).

To address the relative contributions of Ca^{2+} and Mg^{2+} on LPS-induced cytokine release and complement activation, we gradually substituted Ca^{2+} and Mg^{2+} and observed an increase of C3a only upon substitution of Mg^{2+}, while restoration of Ca^{2+} had no impact on complement activation. Similarly, release of IL-1β was found to be dependent on substitution of Mg^{2+}. But the pronounced increase of IL-1β in samples substituted with Mg^{2+} compared to Ca^{2+} has not been described in other studies (46) and Mg^{2+} deficiency has been found to increase IL-1β secretion after LPS stimulation (47). Complement activation via the alternative pathway depends on Mg^{2+} (48). This is in agreement with our findings.

Determination of the global coagulation parameter ACT in the presence and absence of Mg^{2+} indicated that the impact of Mg^{2+} on coagulation seems to be low at physiological Ca^{2+} concentrations, but may be relevant at low Ca^{2+} levels, which is in accordance with the reduced clotting time observed in chronic hemodialysis patients dialyzed with Mg^{2+}-containing as compared to Mg^{2+}-free and Ca^{2+}-free dialysis fluid (34). A procoagulant effect of Mg^{2+} in vitro was also described by Messer et al, who suggested that Mg^{2+} may promote the binding of phospholipids to vitamin K-dependent clotting factors (49), which is in line with reports suggesting that Mg^{2+} might facilitate Ca^{2+} binding to coagulation factors VII, X, and IX (33). This would imply that dialysis fluid containing Mg^{2+} might support the activation of coagulation in the extracorporeal circuit.

In conclusion, the reduction of Ca^{2+} and Mg^{2+} during regional citrate anticoagulation is associated with reduced cytokine release and complement activation in the extracorporeal circuit, and our data support a target Ca^{2+} concentration of 0.2-0.25 mM in the extracorporeal circuit.

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Disclosures

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Conflict of interest: None of the authors has financial interest related to this study to disclose.

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2.1.10 *In vitro* Studie zur Entfernung von Gallensäuren durch extrakorporale Therapien

Jens Hartmann, Stephan Harm: *Removal of bile acids by extracorporeal therapies: an in vitro study*. Int J Artif Organs. 2018; 41(1):52-57, DOI:10.5301/ijao.5000643

Kurzfassung:

Einleitung/Ziel: Gallensäuren (BA), die bei Patienten mit Leberversagen im Blutkreislauf akkumulieren, werden für den auftretenden Juckreiz mitverantwortlich gemacht und beeinträchtigen sehr stark die Lebensqualität der betroffenen Patienten.

Ziel dieser *in vitro* Studie war es, die Entfernung von unterschiedlichen BA aus dem Plasma durch Adsorber und Dialyse zu untersuchen. Weiters wurde die Proteinbindung der einzelnen BA untersucht.

Methode: Dialyseexperimente wurden mit Hilfe von pädiatrischen Kreisläufen mit BA angereicherten Humanplasmen durchgeführt. Für die Adsorptionexperimente wurden Batch-Tests mit 10 % Adsorberanteil in BA-angereichertem Humanplasma durchgeführt. Die Bindung von BA an Plasmaproteine wurde mit Hilfe von Membranfiltrationssäulen ermittelt. Die Siebkoeffizienten der einzelnen BA wurden mit einem Albuminfilter und einem High-Flux-Dialysator ermittelt.

Ergebnisse: Während alle getesteten BA durch Adsorption entfernt wurden, konnten mittels High-Flux-Dialyse nur hydrophile BA wie Glykochol- und Taurocholsäure effizient abgereichert werden.

Schlussfolgerungen: Die Entfernung von BA durch Dialyse oder Ultrafiltration ist stark von deren Hydrophobizität abhängig. Stark hydrophobe BA sind stärker an Plasmaproteine gebunden und können somit schwieriger über Filtrationsverfahren entfernt werden. Adsorptionsbasierte Systeme bieten somit die Möglichkeit auch stark hydrophobe BA aus dem Blut zu entfernen.

Mein Beitrag für diese Publikation:

- Planung der Versuche
- Durchführung der Tests
- Datenauswertung

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ORIGINAL RESEARCH ARTICLE

Removal of bile acids by extracorporeal therapies: an in vitro study

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ABSTRACT

Introduction: Bile acids (BAs) accumulating in the circulation in patients with liver failure are considered to be responsible for pruritus, which strongly impairs quality of life of the affected patients. The aim of this study was the in vitro characterization of different BAs regarding their removability with high-flux dialysis as well as with different adsorbents, and the evaluation of their binding to plasma proteins.

Methods: Dialysis experiments were conducted in pediatric circuits with human plasma. For the adsorption studies, batch tests using 10% adsorbent in spiked human plasma were carried out. The binding of BAs to plasma compounds was determined by centrifugation of spiked plasma through spin columns. Sieving coefficients were determined using an albumin filter and a high-flux dialyzer.

Results: With high-flux dialysis, only hydrophilic BAs such as glycocholic and taurocholic acid could be removed efficiently, while all tested BAs were removed by adsorption.

Conclusions: In conclusion, the hydrophilicity of BAs plays a major role in their removability using extracorporeal approaches. Adsorption-based systems offer particular advantages for the removal of hydrophobic BAs.

Keywords: Adsorption, Bile acids, Dialysis, Hydrophobic interactions, Liver failure, Pruritus

Introduction

Bile acids (BAs) are cholesterol derivatives synthesized in the liver. After their conjugation to glycine and taurine, they are secreted into the bile duct and transported into the gall bladder, from where they are released into the duodenum. They are amphipathic molecules that assume various functions, such as emulsifying lipids and vitamins to enable their resorption in the intestine, but they also act as signaling molecules, as they bind to the nuclear hormone receptors and, therefore, can activate genes that play major roles in BA synthesis, BA transport, or lipid metabolism (1). During the digestion process, about 90% of the BAs are reabsorbed from the intestine, partly as primary BA and partly after conversion by intestinal microbiota into secondary BA. Finally, the uptake of BA from the portal vein into the hepatocytes closes the loop of the so-called enterohepatic circulation to ensure BA preservation. This uptake is highly efficient and leads to a BA concentration downstream of the liver that is 5-6 times lower (2-3 µmol/L) as compared to the portal vein (14 µmol/L). Due to this recycling, the healthy liver produces only about 500 mg BA per day (1, 2).

In disorders with decreased liver function, such as acute or chronic liver failure, cholestasis, or genetic defects causing enzyme deficiencies, specific BA can accumulate in the patients' blood to reach concentrations up to 100 times higher than the reference value (3, 4), or in case of enzymatic disorders, unusual BA and metabolites are produced and accumulate (5). Furthermore, the gut microbiome has a profound impact on the chemical diversity of BA (6, 7). In hepatobiliary diseases, mainly the conjugated BA are elevated in the circulation (3). BA accumulation leads to severely compromised quality of life caused by itching or unbearable pruritus (8), and although individual BA or even particular BA ratios or patterns (9) are considered to differ in their ability to provoke pruritus (10, 11), detailed investigations regarding BA patterns and the corresponding pruritus are still pending.

Although available extracorporeal blood purification systems (12-17) as well as administration of anion exchangers such as cholestyramine (3) have been shown to efficiently decrease total BA concentration and to reduce pruritus, there is still a lack of knowledge regarding the efficiency of the removal of specific BA. Since especially unconjugated BAs are very hydrophobic and, therefore, strongly bound to albumin and lipoproteins (18, 19), the positive reports regarding improvement of pruritus and removal of BA by dialyzers may either be caused by BA adsorption to the membrane or by the removal of only the most hydrophilic BA (20).

The aim of this study was the in vitro characterization of 6 BAs with different hydrophobicity regarding their removability

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with high-flux dialysis as well as different adsorbents and the evaluation of their binding to plasma proteins.

Methods

Plasma and plasma fractions

Human serum albumin solution (200 g/L) was purchased from Baxter. Fractionated plasma was generated from fresh frozen plasma (Red Cross, Linz, Austria) by filtration through an Albuflow filter (Fresenius Medical Care).

Bile acids

Lithocholic acid (LCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), cholic acid (CA), glycocholic acid (GCA) and taurocholic acid (TCA) were purchased from Sigma Aldrich. Plasma was spiked by dissolving the BAs in 0.3 M NaOH and adding this solution to plasma. After 1 hour of mixing at 37°C, the spiked plasma was neutralized with the same amount of 0.3 M HCl. The plasma concentration of each BA was 100 µmol/L.

Binding to plasma compounds

In order to identify the non-protein-bound fraction of the different BAs, the spiked medium (plasma, fractionated plasma, and human serum albumin [HSA]) was centrifuged in spin columns with a molecular weight cut-off of 10 kDa (Corning Spin-X UF concentrator) for 20 minutes at 15,000 g. The free fraction of each BA was calculated from the concentration of the BAs in the spiked plasma and in the filtrate of the spiked plasma.

Adsorption

The characterization of the adsorbents was carried out in batch experiments with 10% v/v adsorbent (9 mL spiked and citrated plasma, 1 mL adsorbent) with gentle shaking at 37°C. Amberchrom CG161c as well as the clinically approved products Cytosorb, diaMARS AC250, Hemosorba, prometh 01 and prometh 02 were used (Tab. I). Samples were taken at 5, 15, 30, 60 and 120 minutes and stored at -20°C until analysis.

Dialysis, clearance and sieving coefficient

Dialysis experiments were carried out using stand-alone pumps (Ismatec MCP Process with Pro 380AD pump head) in miniaturized in vitro setups at 37°C using pediatric high-flux filters (FX paed; Fresenius Medical Care) and 500 mL citrated plasma (Red Cross, Linz, Austria). To avoid uncontrolled filtration, fluid balance was controlled using 2 pumps in the dialysate circuit, 1 upstream and another 1 downstream of the dialyzer. The flow rates were Q_P = 50 mL/min and Q_D = 2000 mL/h for the plasma and the dialysate flow, respectively. Samples were taken upstream (c_{in}) and downstream (c_{out}) of the dialyzer over 180 minutes (n = 8). The clearance (C) in mL/min was calculated using the formula $C = Q_P \cdot (c_{in}-c_{out})/c_{in}$, the extraction rate (R) in % of plasma flow was calculated using the formula $R = 100 \cdot (c_{in}-c_{out})/c_{in}$. From the clearance data for each BA, the trend of BA decrease over time for a patient with 3360 mL plasma was calculated using the formula $C = V/t \cdot \mathrm{Ln}(c_0/c_t)$, where V = plasma volume (mL), t = treatment time (min), c_0 = BA concentration at the beginning of the treatment (µM), c_t = BA concentration at time t (min).

For the most hydrophilic and the most hydrophobic BA used in this study, the sieving coefficients were determined using pediatric Albuflow filters as well as pediatric high-flux dialyzers (Albuflow paed and FX paed; Fresenius Medical Care). The experiments were conducted in citrated plasma at plasma and filtrate flow rates of 50 and 5 mL/min, respectively. Samples were taken after 10, 30 and 60 minutes, and the sieving coefficient (SC) in percent was calculated using the formula $SC = (2 \cdot c_f/(c_i + c_o)) \cdot 100$, where c_f, c_i and c_o are the BA concentration in the filtrate, upstream and downstream of the dialyzer, respectively.

Analysis

BA were analyzed using a colorimetric test kit (Trinity Biotech) with a Roche/Hitachi 902 auto-analyzer. Since this test kit does not differentiate between the individual BA, only 1 BA was spiked into each batch in order to distinguish the adsorbent characteristics for each BA.

Statistics

Means and standard deviations were calculated using Microsoft Excel 2010. In order to compare the 2 filters regarding SC, data (n = 12) were checked for normal distribution before Mann-Whitney rank sum test for significance was conducted using SigmaPlot 13.0 for Windows (Systat Software).

Results

Binding of BA to plasma compounds

Only a small fraction of BA is present in free form and not bound to plasma compounds (Fig. 1). Compared to plasma, lower ratios of BA are protein bound in HSA solution, which

TABLE I - Characteristics of the investigated adsorbents

	Amberchrom CG161c	Cytosorb	diaMARS AC250	Hemosorba	prometh 01	prometh 02
Associated blood purification system	-	-	MARS	-	Prometheus	Prometheus
Material	PS-DVB, neutral	DVB-PVP, neutral	AC, neutral	AC, neutral	PS-DVB, neutral	PS-DVB, anion exchanger

PS-DVB = poly(styrene-divinylbenzene); DVB-PVP = crosslinked divinylbenzene/polyvinylpyrrolidone; AC = activated carbon.

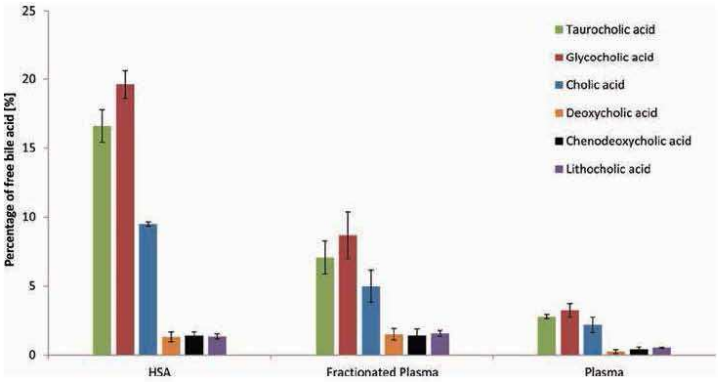

Fig. 1 - Fraction of free BA (mean ± standard deviation) in different matrices, n = 3. LCA = lithocholic acid; CDCA=chenodeoxycholicacid; DCA= deoxycholic acid; CA = cholic acid; GCA = glycocholic acid; TCA = taurocholic acid.

indicates that in plasma, BAs are not only bound to albumin, but also to other proteins or lipoproteins. In fractionated plasma, which contains practically no lipids but mainly albumin and other proteins or lipoproteins in the size range of albumin or below, the ratio of bound BA was between the ratio found for HSA and plasma.

Depletion of BA by adsorption

Figure 2 shows the results of the adsorption batch tests. The anion exchanger prometh 02 removed the different BA according to their hydrophilicity. Within 2 hours, the most hydrophobic and the most hydrophilic BA, namely LCA and TCA, were adsorbed by 24% and 61%, respectively. The removal of BA by neutral adsorbents followed the same pattern: the more hydrophilic the BA, the more effective was the adsorption. However, there were differences between the adsorbents. Hemosorba, Cytosorb, prometh 01, and diaMARS AC250 removed the 3 more hydrophilic BA more effectively than the hydrophobic ones. Amberchrom CG161, a strongly hydrophobic adsorbent, was capable of effectively removing the hydrophobic BA, as well.

Depletion of BA by dialysis

Figure 3 shows the removal for BA by high-flux dialysis. The removal correlates with the hydrophilicity of the BA and confirms the findings of the binding study (Fig. 1). The progressions of the BA concentration for TCA, GCA, CA and DCA over time were calculated for a virtual treatment of a patient with a plasma volume of 3,360 mL and are shown in the insert of Figure 3. While TCA and GCA are removed by about 50% during 24 hours of treatment, CA and DCA can only be removed by 35% and 7%, respectively. However, it should be taken into account that this model ignores the rebound of BA during the treatment and that the decrease would be significantly lower under clinical conditions.

Sieving coefficient of BA

The sieving coefficient (SC) for TCA was 48.3% ± 1.7% and 8.2% ± 1.5% for the Albuflow and the FX paed filter, respectively, and 50.2% ± 12.1% and 0.8% ± 0.7% for LCA (n = 12). The SC for both BAs was significantly higher with the Albuflow filter than with the FX filter ($p<0.001$). Since the Albuflow filter offers a high SC for albumin, the albumin-bound as well as the free fraction of BA can pass the filter. Therefore, there is no significant difference between the SC of the 2 BAs for the Albuflow filter ($p = 0.436$). However, due to the fact that a high-flux dialyzer has much smaller pores, only the free fraction of BA can pass the high-flux filter, and the SC is significantly higher for TCA than for the strongly albumin-bound LCA ($p = 0.001$).

Discussion

Our data show that BAs in plasma are not only bound to albumin, but also to other proteins and lipoproteins, confirming the findings by other authors (4, 21).

Our results indicate that adsorptive removal of BA is possible with anion exchangers as well as with neutral resins. This may be explained by the fact that BA are amphiphilic molecules offering 2 different moieties for adsorption. The hydrophobicity of BA increases with decreasing number of hydroxyl groups. Although TCA, GCA and CA have the same amount of hydroxyl groups, TCA and GCA are more hydrophilic, since they are conjugated to taurine and glycine, respectively. This is manifested in the lower pKa of TCA and GCA (Tab. II).

While hydrophilic BA can be removed by anion exchangers as well as by moderately hydrophobic neutral adsorbents, hydrophobic BA can only be removed at high efficiency by strongly hydrophobic neutral resins since these BA such as LCA, CDA and DCA are strongly albumin bound. This was shown in this study for the Amberchrom CG161c, which has, in addition to its hydrophobicity, a pore size that is optimal for the depletion of albumin-bound metabolites (22). The other adsorbents tested showed limited removal rates for strongly hydrophobic BA.

The pKa (23, 24) and the number of hydroxyl groups correlate with the hydrophilicity and the removability of bile acid with commercially available extracorporeal blood purification systems.

Although anion exchangers are applied in extracorporeal blood purification devices to remove bilirubin, hydrophilic BA

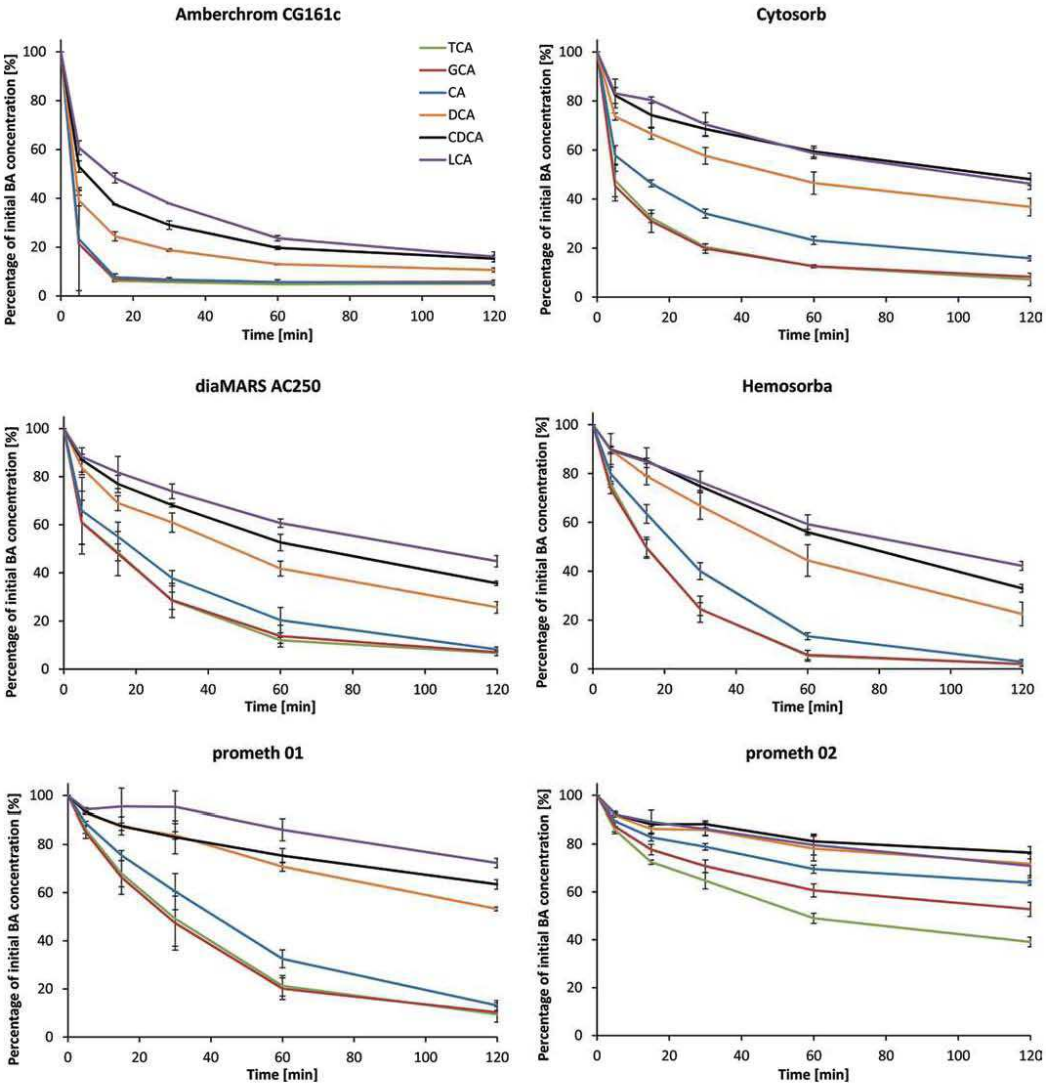

Fig. 2 - Adsorption of BA to different adsorbents. Batch experiments in plasma with 10 % v/v adsorbent, initial BA concentration 100 µM; n = 3. LCA = lithocholic acid; CDCA = chenodeoxycholic acid; DCA = deoxycholic acid; CA = cholic acid; GCA = glycocholic acid; TCA = taurocholic acid.

and other anionic compounds, it should be noted that anionic anticoagulants such as heparin and citrate are removed by these adsorbents. Therefore, strongly hydrophobic resins might be a better choice for the removal of amphiphilic substances.

Our results show that the removability of BA, both by dialysis as well as by adsorption, decreases with their hydrophobicity and, therefore, with the affinity of BA to albumin or to other proteins and lipoproteins in plasma. These findings match the hepatic reabsorption of the different BA by the healthy liver at the end of the enterohepatic cycle (25). The poor removal of strongly hydrophobic BA by dialysis suggests that for an efficient removal of these BAs, especially under clinical conditions where patients produce

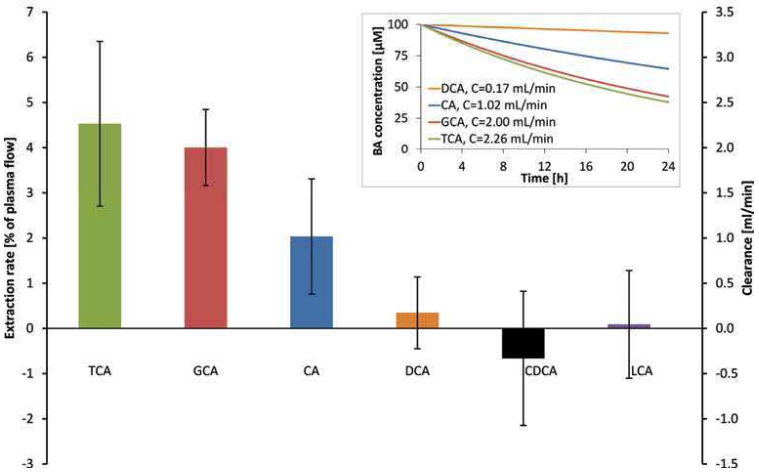

Fig. 3 - Removal of BA (bile acid) with a high-flux dialyzer in mL/min and in percent of plasma flow, n = 8 **Insert:** Concentrations of BA in a virtual treatment of a patient with a plasma volume of 3,360 mL; calculated data based on the clearance of each BA. LCA = lithocholic acid; CDCA = chenodeoxycholic acid; DCA = deoxycholic acid; CA = cholic acid; GCA = glycocholic acid; TCA = taurocholic acid.

TABLE II - The pKa and number of hydroxyl groups

Bile acids	pKa	Number of OH groups on cholesterol ring
TCA	<2	3
GCA	3.8	3
CA	5.2	3
DCA	6.2	2
CDCA	6.2	2
LCA	6.7	1

BA during treatment, hydrophobic adsorbents should be used.

Studies by many authors compare different technologies such as adsorption, albumin dialysis, or conventional dialysis, or even compare dialyzer materials regarding the removability of BA. Here, we show that the removability of BAs strongly depends on their hydrophobicity. While mainly the conjugated BA, which can be removed more easily from blood, show increased levels in patients with hepatobiliary diseases, hydrophobic BAs are especially cytotoxic due to their ability to destabilize cell membranes and can trigger apoptosis or induce necrosis (16, 26). They should, therefore, be considered important targets for removal. For these BAs, the hydrophobic adsorbent CG161c offers promising adsorption characteristics for all tested BAs. Our results suggest that among the available membrane/adsorption based systems, the Prometheus system is superior by combining the highly permeable Albuflow filter with efficient adsorbents. Cytosorb as well as Hemosorba are whole blood adsorbents without the need for prior plasma separation and are, therefore, particularly promising for the removal of albumin-bound BA.

It should be noted that membrane/adsorption-based systems are mainly based on polysulphone membranes, and there has been controversial discussion about these membranes regarding their impact on the development of pruritus. While some authors showed that hemodialysis patients treated with polysulphone membranes suffered more commonly from pruritus than patients treated with hemophane or cuprophane membranes (27), others found that hemophane and cellulose membranes promote pruritus more than polysulfone (28, 29), or that high-flux polyacrylonitrile and polymethylmethacrylate membranes can reduce pruritus, the latter due to their ability to remove larger uremic toxins and ions by a combination of convection and adsorption (20, 30). Therefore, further observations are needed to determine the preferred membrane material for the treatment of patients suffering from pruritus.

Since various diseases, including conditions without apparent liver disease (31), may be associated with pruritus based on significantly altered BA patterns, this may influence the choice of adequate adsorbents. However, compared to dialysis, the application of adsorption technology in clinical practice is expensive and complex. Since the pathogenesis and the pathophysiology of bile acid patterns which are responsible for pruritus are not yet completely understood, it cannot be guaranteed that adsorption technology will improve the patients' condition.

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Disclosures

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2.1.11 Vergleich der Clearance von ausgewählten Plasma Zytokinen mit kontinuierlicher veno-venöser Hämodialyse zwischen Ultraflux EMiC2 und Ultraflux AV1000S

Tanja Eichhorn, Jens Hartmann, Stephan Harm, Ingrid Linsberger, Franz König, Gerhard Valicek, Georg Miestinger, Christoph Hörmann, Viktoria Weber: *Clearance of Selected Plasma Cytokines with Continuous Veno-Venous Hemodialysis Using Ultraflux EMiC2 versus Ultraflux AV1000S*. Blood Purif. 2017; 44(4):260-266, DOI:10.1159/000478965

Kurzfassung:

Einleitung: High Cutoff Hämofilter werden in der extrakorporalen Sepsistherapie verwendet, um Entzündungsmediatoren aus dem Blutkreislauf zu entfernen und so die Immunhomöostase wieder herzustellen

Methoden: Bei 30 Sepsis-Patienten mit akutem Nierenversagen wurde die Interleukin (IL)-6, IL-8, IL-10 und Tumornekrose Faktor-Alpha (TNF-α) Abreicherung durch eine kontinuierliche veno-venöse Hämodialyse zwischen einem High Cut off-Filter (EMiC2, CVVHDHCO) und einem Standardfilter (CVVHD-STD) verglichen. Die Behandlungszeit betrug jeweils 48 Stunden.

Ergebnisse: Es konnte gezeigt werden, dass die Entfernung von IL-6 und IL-8 bei Verwendung von CVVHD-HCO signifikant höher war. Die mittleren Plasma-Zytokin-Konzentrationen sanken während der Behandlungsdauer bei beiden Filtern gleich stark. Bei CVVHD-HCO konnte kein signifikant höherer Albumin-Verlust nachgewiesen werden. C-reaktives Protein blieb bei beiden Filtern während der Behandlung stabil, während Procalcitonin durch beide Behandlungsmodalitäten signifikant verringert werden konnte.

Schlussfolgerung: Von allen gemessenen Parametern konnte die CVVHD-HCO im Vergleich zu CVVHD-STD nur eine verbesserte IL-6 und IL-8 Abreicherung im Blut bewirken.

Mein Beitrag für diese Publikation:

- Planung der *in vitro* Versuche
- Durchführung der *in vitro* Dialyse-Versuche

Original Paper

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Clearance of Selected Plasma Cytokines with Continuous Veno-Venous Hemodialysis Using Ultraflux EMiC2 versus Ultraflux AV1000S

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Keywords
High cutoff filter · Cytokine clearance · Continuous veno-venous hemodialysis · Interleukin-6

Abstract
Background: High cutoff hemofilters might support the restoration of immune homeostasis in systemic inflammation by depleting inflammatory mediators from the circulation. ***Methods:*** Interleukin (IL)-6, IL-8, IL-10, and tumor necrosis factor-alpha depletion was assessed in 30 sepsis patients with acute renal failure using continuous veno-venous hemodialysis with high cutoff versus standard filters (CVVHD-HCO vs. CVVHD-STD) over 48 h. ***Results:*** The transfer of IL-6 and IL-8 was significantly higher for CVVHD-HCO, as shown by increased IL-6 and IL-8 effluent concentrations. The mean plasma cytokine concentrations decreased over time for all cytokines without detectable differences for the treatment modalities. No transfer of albumin was observed for either of the filters. C-reactive protein remained stable over time and did not differ between CVVHD-HCO and CVVHD-STD, while procalcitonin decreased significantly over 48 h for both treatment modalities. ***Conclusion:*** CVVHD-HCO achieved enhanced removal of IL-6 and IL-8 as compared to CVVHD-STD, without differentially reducing plasma cytokine levels.

Introduction

Sepsis, severe sepsis, and septic shock encompass a spectrum of clinical syndromes caused by a dysregulated host response to infection [1–3]. Sepsis affects 18 million people worldwide each year, with mortality rates of 30–50% even in state-of-the-art intensive care units, and its incidence is increasing, mainly due to the emergence of antibiotic-resistant microorganisms and an aging population. Despite substantial advances in our understanding of the host response to infection and its dysregulation in sepsis, the development of targeted therapeutic approaches has been severely restricted by the considerable heterogeneity of sepsis patients [4–6] and the lack of accurate diagnostic methods to (1) prospectively identify those patients who would benefit from a specific therapy and (2) stratify patients into biologically homogenous subgroups, for example, with respect to plasma cytokine levels.

Acute renal failure, a frequent consequence of sepsis, has been identified as an independent risk factor for mortality in sepsis patients and has been shown to increase the complexity and cost of care [7]. Extracorporeal therapies are mainly applied to support or replace kidney function and to ameliorate uremic complications

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in sepsis, but there is evidence that these approaches might have effects beyond renal support by depleting inflammatory mediators, such as cytokines, chemokines, and complement factors from the circulation [8–11]. A significant portion of cytokines in the circulation, however, are bound to plasma proteins or associated with their target cells [12], and are therefore not directly accessible to depletion by renal replacement therapies. Still, the depletion of unbound portions of these factors from the circulation may support the restoration of immune homeostasis by inducing re-equilibration of free and bound cytokine fractions.

Unlike drugs targeting single factors, extracorporeal therapies can influence a wide range of molecules, and the concept of blood purification has evolved towards the depletion of a broad spectrum of inflammatory mediators. High cutoff hemofilters, in particular, are characterized by an increased pore diameter of around 0.008–0.01 μm [13, 14], and have been reported to exhibit more efficient depletion of inflammatory cytokines as compared to standard high-flux membranes [15–18]. In the majority of these studies, high cutoff filters were applied in convective settings, such as hemofiltration or hemodiafiltration, where enhanced cytokine depletion was reported to be associated with a reduction of albumin, in particular at increased effluent rates [19]. Here, we compared the removal of selected cytokines under predominantly diffusive conditions using continuous veno-venous hemodialysis with high cutoff hemofilters (CVVHD-HCO) versus standard hemofilters (CVVHD-STD), both in vitro and in sepsis patients with acute renal failure. Our primary hypothesis was that the two treatment modalities would mainly differ with respect to the depletion of cytokines in a molecular weight range of 20–25 kDa, such as IL-6.

Materials and Methods

Human Plasma, Chemicals and Reagents

Fresh frozen human plasma was obtained from the Red Cross, Linz, Austria. Recombinant human interleukin (IL)-6, IL-8, IL-10, and tumor necrosis factor-alpha (TNF-α) were purchased from R&D Systems, Minneapolis, MN, USA. Unfractionated heparin was obtained from Gilvasan Pharma, Vienna, Austria. Dialysis fluid (Fresenius Medical Care, Bad Homburg, Germany) contained 33 mM bicarbonate, 138 mM Na^+, 2 mM K^+, and 0.5 mM Mg^{++}.

Hemodialysis Filters

The high cutoff filter Ultraflux EMiC2 and the standard filter Ultraflux AV1000S (Fresenius Medical Care) were used in this study. Both are polysulfone filters with a surface area of 1.8 m^2 and a molecular weight cutoff of approximately 30 kDa for Ultraflux AV1000S and approximately 45 kDa for Ultraflux EMiC2, respectively.

Sieving Coefficient and Cytokine Clearance in vitro

Fresh frozen human plasma (2 L) was thawed, filtered to remove cryo-precipitates, supplemented with 5 IU/mL of unfractionated heparin, and spiked with recombinant human IL-6 (target concentration 1,000 pg/mL), IL-8 (500 pg/mL), IL-10 (300 pg/mL), and TNF-α (800 pg/mL). The cytokine clearance for both filters was determined at a flow rate of 300 mL/min in the blood and dialysate circuits (Fig. 1a). Samples were drawn from the blood circuit at the filter inlet (S_{in}) and outlet (S_{out}) as well as from the dialysate circuit at the filter outlet (S_f) after 0, 15, 30, 60, 120, 180, and 240 min, and stored at –80 °C until further analysis. Three independent sets of experiments were performed. For each set of experiment, a separate batch of plasma was spiked with recombinant human cytokines, and new filter sets were used.

The sieving coefficient (SC) was determined using the experimental set up shown in Figure 1b with flow rates of 300 mL/min in the blood circuit and a filtration rate of 30 mL/min. Samples were drawn from the blood circuit at the filter inlet (S_{in}) and outlet (S_{out}) after 0, 15, 30, 45, and 60 min as well as from the filtrate (S_f). All samples were stored at –80 °C until further analysis. IL-6, IL-8, IL-10, and TNF-α were quantified with enzyme-linked immunosorbent assays (R&D Systems, Minneapolis, MN, USA). The clearance (K) was calculated as

$$K = (V/t) \cdot \ln (C_0/C_t)$$

with V denoting the plasma volume, t denoting the time of sampling, and C_0 and C_t denoting the plasma concentration of a given cytokine at 0 min and at the respective sampling time. The SC was determined as the ratio of cytokine concentrations (C) in the filtrate and plasma, and calculated as

$$SC = 2 \cdot C_f/(C_{in} + C_{out})$$

Cytokine Depletion in vivo

To assess the cytokine depletion in vivo, we performed a single-center, randomized, controlled clinical study comparing continuous veno-venous hemodialysis using EMiC2 (CVVHD-HCO) versus AV1000S (CVVHD-STD). The study protocol was in accordance with the principles of the Declaration of Helsinki. Informed consent and approval by the Ethics Committee of the University Hospital St. Pölten, Austria (GS4-EK-3/082-2012) were obtained from all donors before the onset of the study. In total, 30 patients suffering from sepsis with acute renal failure were randomized to either CVVHD-HCO or CVVHD-STD and treated for 48 h using a blood flow (Qb) of 200 mL/min. Patients aged below 18 years or pregnant patients were excluded from the study.

Anticoagulation was performed with citrate according to the Ci-Ca protocol (Fresenius Medical Care). Simplified Acute Physiology Score (SAPS III) and Therapeutic Intervention Scoring System (TISS) score, leukocyte counts, comorbidities, as well as the sites of infection were recorded as baseline characteristics. Blood samples were drawn pre- and post-filter at baseline, that is, at the start of CVVHD, and at 1, 24, and 48 h. All samples were immediately centrifuged at 2,000 *g* (15 min, 4 °C), and the resulting plasma samples were stored at –80 °C until quantification of IL-6, IL-8, IL-10, TNF-α, and albumin. In addition, samples were drawn from the effluent at the indicated time points and analyzed as described above to trace cytokine transfer across the membrane. Cytokines

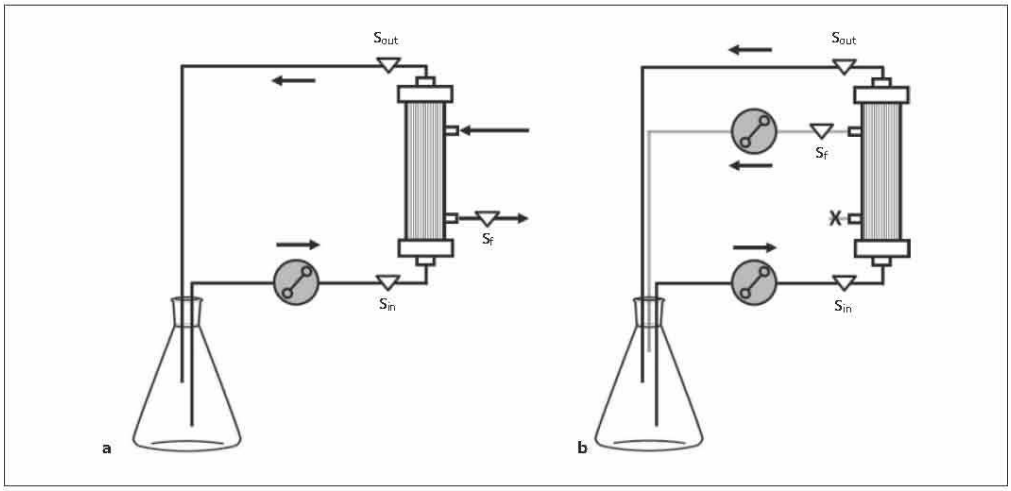

Fig. 1. Experimental set up to determine the clearance and sieving coefficient in vitro. Human plasma was spiked with recombinant human IL-6, IL-8, IL-10, and TNF-α, and clearances (**a**) as well as sieving coefficients (**b**) for individual cytokines were determined as described in Materials and Methods. S_{in}, S_{out}, and S_f denote sampling points at the filter inlet, the filter outlet, and at the filtrate side.

were quantified with a bead array using the Bio-Plex 200 system (Bio-Rad, Vienna, Austria). Albumin was quantified using the colorimetric bromocresol green albumin assay (Sigma-Aldrich, St. Louis, MO, USA). The hybcell antibody microarray (CubeDX, St. Valentin, Austria) was used to quantify C-reactive protein (CRP) and procalcitonin (PCT). Plasma was mixed with conjugate solution at a ratio of 1:1, and 100 µL of the prepared mixture was loaded into the hybcell and analyzed, according to the manufacturer's instructions.

Statistical Analysis

Data were analyzed using the SPSS version 22 (SPSS Inc., Chicago, IL, USA). The unpaired, non-parametric Wilcoxon rank sum test was applied to compare groups (i.e., high cutoff vs. standard filters at a given time point). The paired, non-parametric Wilcoxon signed-rank test was used to analyze changes in CRP and PCT levels over time in clinical samples for a given filter. p values of ≤0.05 were considered as statistically significant.

Results

Sieving Coefficient and Cytokine Clearance for CVVHD-HCO versus CVVHD-STD

The in vitro clearance of IL-6 (M_r = 26 kDa) and IL-10 (M_r = 17 kDa) was significantly higher for CVVHD-HCO as compared to CVVHD-STD (4.7 ± 0.2 vs. 0.8 ± 0.4 mL/min for IL-6; 5.8 ± 0.6 vs. 2.9 ± 0.8 mL/min for IL-10) at 240 min. The low molecular weight cytokine IL-8 (M_r = 8 kDa) was cleared equally well by both filters with 16.9 ± 3.1 mL/min for CVVHD-HCO and 17.0 ± 3.0 mL/min for CVVHD-STD, while the clearance of TNF-α (M_r = 51 kDa) was 2.4 ± 0.5 vs. 0.6 ± 0.3 mL/min for CVVHD-HCO and CVVHD-STD, respectively. In vitro SCs were significantly higher for EMiC2 as compared to AV1000S for all tested cytokines, as summarized in Table 1. The decrease of cytokine concentrations in the plasma pool over time is shown in Figure 2.

In vivo Cytokine Depletion

We performed a single-center, randomized, controlled study in patients with sepsis and acute renal failure to assess the depletion of IL-6, IL-8, IL-10, and TNF-α using two treatment modalities. Of the 30 patients included in the study, 14 patients were treated with CVVHD-HCO, while 16 patients received CVVHD-STD. Patient baseline characteristics are summarized in Table 2, and mean baseline cytokine concentrations as well as the range for individual cytokines are displayed in Table 3. Baseline levels of IL-6, IL-8, IL-10, and TNF-α were elevated in the majority of patients and ranged widely within the study population.

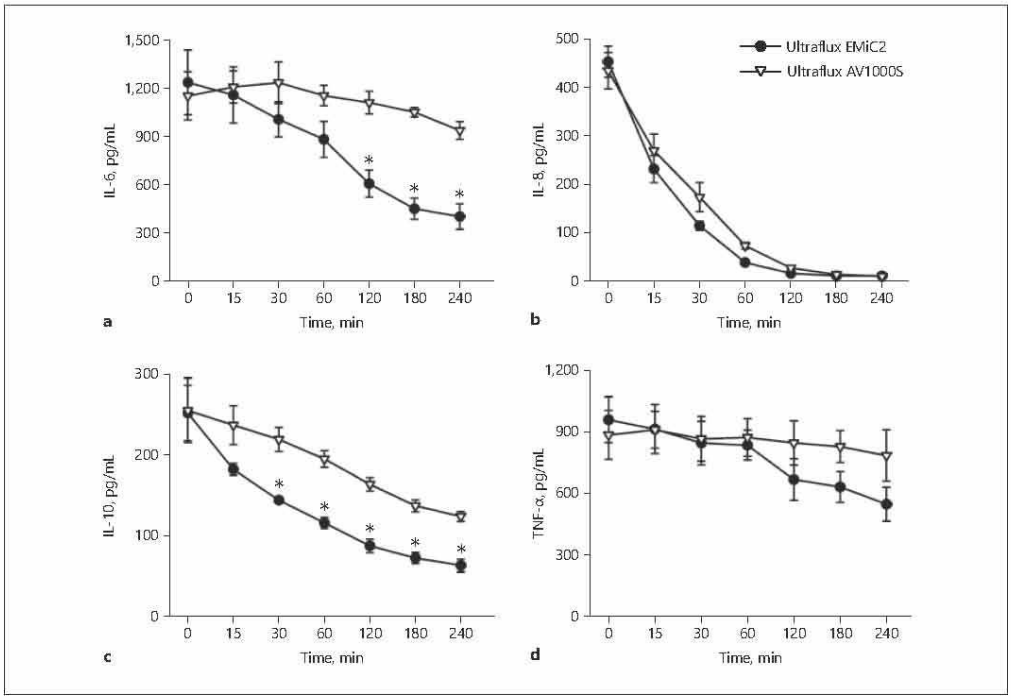

Fig. 2. Cytokine depletion from spiked human plasma using Ultraflux EMiC2 and Ultraflux AV1000S in vitro. Human plasma was spiked with recombinant human IL-6 (**a**), IL-8 (**b**), IL-10 (**c**), and TNF-α (**d**) as described in Materials and Methods and subjected to continuous veno-venous hemodialysis using the high cutoff filter Ultraflux EMiC2 or the standard filter Ultraflux AV1000S. Samples were taken from the plasma pool at the indicated time points to determine cytokine concentrations. Data are given as mean ± SEM for 3 independent experiments. The difference in IL-6 and IL-10 depletion for EMiC2 and AV1000S reached statistical significance ($p \leq 0.05$) from 120 min (IL-6) and 30 min (IL-10) onwards, respectively, as indicated by the asterisks (*).

To assess the transfer of IL-6, IL-8, IL-10, and TNF-α across the EMiC2 and AV1000S membranes during CVVHD-HCO and CVVHD-STD, the concentration of these cytokines was determined in the effluent and is displayed in Table 4 as percentage of the respective plasma cytokine concentrations. In agreement with the in vitro data, EMiC2 depleted IL-6 more efficiently than AV1000S, as patients treated with EMiC2 showed a significantly higher percentage of IL-6 in the effluent relative to plasma concentrations at 1, 24, and 48 h. High amounts of the low molecular weight cytokine IL-8 were detected in the effluent for both filters, while the concentrations of IL-10 and TNF-α in the effluent remained below 10 and 1% of the respective plasma concentrations. Mean plasma levels of all cytokines tested decreased over time in both groups as summarized in online supplementary Table 1 (see www.karger.com/doi/10.1159/000478965).

Depletion of Albumin, CRP, and PCT

CVVHD-HCO and CVVHD-STD did not differ significantly with respect to albumin removal. The albumin concentration in the effluent relative to the plasma concentration was 1.8 ± 0.3% for EMiC2 and 1.4 ± 0.2% for AV1000S, respectively. CRP levels showed no significant alterations over the treatment period for both filters, while PCT decreased significantly over time for both treatment modalities (Table 5).

Table 1. In vitro sieving coefficients of selected cytokines for Ultraflux EMiC2 and Ultraflux AV1000S

	IL-6, %	IL-8, %	IL-10, %	TNF-α, %
Ultraflux EMiC2	14.7±1.6	45.7±5.8	6.8±2.1	3.9±0.9
Ultraflux AV1000S	1.6±0.8	17.5±5.6	0.1±0.1	0.4±0.1

Data are given as mean ± SEM (n = 3). The difference in sieving coefficients was statistically significant for all four parameters.

Table 2. Baseline characteristics of study patients

Characteristics	Data
Age, years	62±3 (26–89)
Sex, male, n (%)	19 (63)
SAPS III	68.4±2.3 (45–107)
TISS	39.5±1.3 (26–57)
Leukocyte count, $\times 10^3$/μL	15±1.6 (2–35)
Comorbidities, number (% of total)	
Cardiovascular	18 (60)
Pulmonary	7 (23)
Hepatitis/pancreatitis	1 (3)
Inflammatory	7 (23)
Neurological	9 (30)
Renal	5 (17)
Diabetes	9 (30)
Primary site of infection, number (% of total)	
Lung	1 (3)
Abdomen	10 (33)
Blood	7 (23)
Urinary tract	3 (10)
Other	7 (23)
Unknown	2 (7)

Data are given as mean ± SEM (range) or n (% of all patients included).

SAPS III, simplified acute physiology score III; TISS, therapeutic intervention scoring system.

Table 3. Baseline plasma cytokine concentrations of the study patients

IL-6	3,562±1,525 (26–40,611)
IL-8	253±95 (29–2,746)
IL-10	69±23 (0–631)
TNF-α	126±23 (6–557)

Concentrations are given in pg/mL as mean ± SEM. The range is indicated in brackets.

Discussion

The application of high cutoff hemofilters encompass clinical conditions requiring an effective removal of substances in the molecular weight range of 20–50 kDa, such as middle molecules, myoglobin, free light chain immunoglobulins, or cytokines. Enhanced cytokine depletion with high cutoff filters has been reported in a number of studies in vitro and in vivo [20–22]. It has been proposed that the concurrent removal of multiple mediators from the circulation can contribute to restoring immune homeostasis by eliminating the peaks of cytokine blood concentrations in the early phase of sepsis (peak concentration hypothesis) [23], by effecting the depletion of cytokines due to re-equilibration of blood and tissue cytokine levels (threshold immunomodulation hypothesis) [24], by inducing increased lymphatic flow and a displacement of mediators to the blood compartment (mediator delivery hypothesis) [25], or by directly acting on inflammatory cells, such as monocytes or neutrophils (cytokinetic model) [26].

In patients with sepsis-induced acute kidney injury, high cutoff hemofiltration was found to be associated with improvement of physiological parameters, reduction in vasopressor requirements [16], as well as restored monocyte proliferation capacity [15]. Due to their increased porosity, however, the application of high cutoff filters may be associated with albumin loss, in particular, under convective conditions and at increased effluent rates [27]. We, therefore, used CVVHD to assess the depletion of IL-6, IL-8, IL-10, and TNF-α, covering a molecular weight range from 8 to 51 kDa, by high cutoff versus standard hemofilters under predominantly diffusive conditions. SCs and cytokine clearances were initially determined in vitro in spiked human plasma to circumvent intra-experimental cytokine generation, showing that the high cutoff filter was significantly more efficient in clearing IL-6 and IL-10, which are in the middle molecular weight range, whereas the low molecular weight cytokine IL-8 was cleared equally well, and the removal of TNF-α was negligible for both filters.

To confirm these findings in vivo, we performed a single-center, randomized, controlled clinical study involving 30 sepsis patients with acute renal failure. Baseline plasma cytokine concentrations ranged widely across the study population and were in the upper range of those reported for severe sepsis, substantiating previous data and confirming the heterogeneity of sepsis patients [17, 28–31].

Only insignificant amounts of albumin were detected in the effluent for both filters, demonstrating the safety of

Table 4. Cytokine concentrations in the effluent relative to cytokine plasma concentrations

	IL-6		IL-8		IL-10		TNF-α	
	EMiC2	AV1000S	EMiC2	AV1000S	EMiC2	AV1000S	EMiC2	AV1000S
1 h	49.1±15.6 (n = 13)	16.1±4.9 (n = 16)	68.0±11.3 (n = 13)	39.3±5.0 (n = 16)	0.4±0.4 (n = 13)	3.1±2.6 (n = 16)	0.0±0.0 (n = 13)	1.5±0.8 (n = 16)
24 h	31.4±10.0 (n = 12)	7.9±2.5 (n = 14)	98.7±20.7 (n = 12)	44.4±5.7 (n = 14)	10.5±4.6 (n = 12)	5.1±3.0 (n = 14)	1.1±0.9 (n = 12)	1.0±0.5 (n = 14)
48 h	29.9±7.9 (n = 12)	6.7±2.6 (n = 10)	106.1±21.6 (n = 12)	39.8±5.7 (n = 10)	3.9±2.0 (n = 12)	0.5±0.3 (n = 9)	0.1±0.1 (n = 12)	1.3±0.6 (n = 10)

Cytokine concentrations were determined in plasma samples and in the effluent at the indicated time points as described in Materials and Methods. The concentration of each cytokine in the effluent is given as percentage of the respective cytokine concentration in plasma for each time point. Data are expressed as mean ± SEM. The difference in effluent cytokine concentrations relative to plasma concentrations was statistically significant between EMiC2 and AV1000S for IL-6 and IL-8 at all time points.

Table 5. Plasma concentrations of CRP and PCT in sepsis patients

	CRP, mg/L		PCT, ng/mL	
	EMiC2	AV1000S	EMiC2	AV1000S
0 h	143.8±12.6 (n = 14)	170.5±14.1 (n = 16)	2.8±0.5 (n = 14)	3.9±0.8 (n = 16)
1 h	135.4±14.8 (n = 14)	161.3±12.7 (n = 16)	2.5±0.5 (n = 14)	3.6±0.7 (n = 16)
24 h	153.8±15.7 (n = 13)	161.6±14.3 (n = 14)	1.9±0.5 (n = 13)	3.3±0.8 (n = 14)
48 h	138.8±13.2 (n = 12)	154.0±16.9 (n = 10)	1.7±0.5 (n = 12)	2.7±0.8 (n = 10)

Data are given as mean of n experiments ± SEM.

The decrease of PCT levels at 48 h was statistically significant for both filters. CRP, C-reactive protein; PCT, procalcitonin.

the high cutoff filter with regard to albumin loss. The quantification of cytokines in the effluent confirmed the transfer of IL-6 and of IL-8 across both filters, with significantly higher IL-6 and IL-8 effluent concentrations for the high cutoff filter. Cytokine transfer remained stable over the treatment period of 48 h without being substantially affected by fouling or secondary membrane formation due to plasma protein deposition, and there was no indication of coagulation with either of the filters. While these results confirm the in vitro data with respect to the depletion of IL-6, the transfer of IL-8 was less efficient as compared to the in vitro experiments for the standard filter. We assume that this reduced transfer in vivo may be due to the association of IL-8 with plasma proteins or blood cells. Likewise, only minor amounts of IL-10 were detected in the effluent for both filters. IL-10 has been shown to be associated with heparan sulfate shed from the activated endothelium in sepsis, which may decrease its transfer across the filter in vivo. Overall, plasma levels of all tested cytokines decreased over the treatment period of 48 h with both treatment modalities. The significantly enhanced removal of IL-6 and IL-8 with the high cutoff filter as compared to the standard filter did, however, not result in significantly lower IL-6 and IL-8 plasma concentrations at 48 h. This is in agreement with recent data from a retrospective study using CVVHD-HCO in 28 patients with septic shock and acute kidney injury, where the average IL-6 serum concentrations across the study population were even found to increase despite enhanced IL-6 clearance, apparently due to an intradialytic release of IL-6 in some patients, exceeding its elimination by dialysis [17].

Conclusions

In conclusion, CVVHD-HCO exhibited a significantly higher clearance for IL-6 and IL-10 as compared to CVVHD-STD in vitro. We could, however, not confirm a differential effect on plasma cytokine levels for CVVHD-HCO after 48 h in vivo, highlighting differences between cytokine depletion in vitro using human plasma spiked with recombinant cytokines and the in vivo situation. This may be due to de novo cytokine release depending on the status of the patient, as well as to the association of cytokines with blood cells, plasma proteins, or circulating glycosaminoglycans in vivo, resulting in their reduced transfer to the effluent.

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Disclosure Statement

The authors have no conflict of interest to declare.

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2.1.12 Entfernung von Stabilisatoren aus pharmazeutischem humanem Serumalbumin mittels Adsorption und Dialyse

Stephan Harm, Claudia Schildböck, Jens Hartmann: *Removal of stabilizers from human serum albumin by adsorbents and dialysis used in blood purification*. PLoS One. 2018; 13(1):e0191741, DOI:10.1371/journal.pone.0191741

Kurzfassung:

Einleitung: Humanserumalbumin (HSA) ist ein monomeres Protein, ist mengenmäßig das häufigste Plasmaprotein, besitzt viele Bindungsdomänen und hat somit eine außerordentlich hohe Bindungs- und Transportkapazität. Im menschlichen Kreislauf spielt es eine wichtige Rolle bei der Speicherung und beim Transport von körpereigenen Abbauprodukten, Metaboliten und Medikamenten. In der Klinik wird HSA bei Krankheiten wie Schock, Verbrennungen, Blutungen und Trauma verwendet. Um das Protein für die Lagerung und während der Hitzebehandlung für die Virusinaktivierung zu stabilisieren, wird das pharmazeutische HSA mit Natriumcaprylat und N-Acetyltryptophanat versetzt.

Methoden: Ziel dieser Studie war es, zu ermitteln, ob die beiden Stabilisatoren adsorptiv entfernt bzw. abgereichert werden können. Mehrere Adsorber, von denen einige im klinischen Einsatz sind, wurden im Batchtest und in einem dynamischen Setup getestet. Darüber hinaus wurde die Clearance der Stabilisatoren mit einem pädiatrischen High-Flux-Dialysator bestimmt.

Ergebnisse: Die Ergebnisse zeigen, dass Aktivkohle effektiver bei der Entfernung von N-Acetylthryptophanat ist, während Polystyrol-basierte Adsorber besser für die Entfernung von Caprylat aus HSA-Lösungen geeignet sind. Eine Mischung aus beiden Adsorbern wäre demnach die effektivste Möglichkeit, Stabilisatoren adsorptiv aus der HSA-Lösung zu entfernen. Nach einer 4-stündigen Behandlung mit einem High-Flux-Dialysator wurde N-Acetyltryptophanat vollständig und Caprylat zu 80 % entfernt.

Schlussfolgerung: Extrakorporale Blutreinigungssysteme, bei welchen Aktivkohle, Polystyrol basierende Adsorber oder Dialysatoren zum Einsatz kommen, sind in der Lage, HSA-Stabilisatoren zu entfernen.

Mein Beitrag für diese Publikation:

- Planung der Versuche
- Verfassen der Publikation
- Durchführung der Tests
- Datenauswertung

PLOS ONE

RESEARCH ARTICLE

Removal of stabilizers from human serum albumin by adsorbents and dialysis used in blood purification

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Abstract

Introduction

Human serum albumin (HSA) is a monomeric multi-domain protein that possesses an extraordinary binding capacity. It plays an important role in storing and transporting endogenous substances, metabolites, and drugs throughout the human circulatory system. Clinically, HSA is used to treat a variety of diseases such as hypovolemia, shock, burns, hemorrhage, and trauma in critically ill patients. Pharmaceutical-grade HSA contains the stabilizers sodium caprylate and N-acetyltryptophanate to protect the protein from oxidative stress and to stabilize it for heat treatment which is applied for virus inactivation.

Material and methods

The aim of this study was to determine if the two stabilizers can be depleted by adsorbent techniques. Several, adsorbents, some of them are in clinical use, were tested in batch and in a dynamic setup for their ability to remove the stabilizers. Furthermore, the removal of the stabilizers was tested using a pediatric high flux dialyzer.

Results

The outcome of this study shows that activated charcoal based adsorbents are more effective in removal of N-acetylthryptophanate, whereas polystyrene based adsorbents are better for the removal of caprylate from HSA solutions. An adsorbent cartridge which contains a mix of activated charcoal and polystyrene based material could be used to remove both stabilizers effectively. After 4 hours treatment with a high flux dialyzer, N-acetyltryptophanate was totally removed whereas 20% of caprylate remained in the HSA solution.

Introduction

Human serum albumin (HSA) is a protein consisting of 585 amino acids with a molecular mass of 66.7 kDa, is highly water soluble and has a strong negative charge. Albumin makes up half the normal intravascular protein mass, is synthesized in the liver and is responsible for 75–85% of the plasma colloid osmotic pressure [1]. The reference range for albumin concentrations in human blood is between 35 and 50 g/l in healthy individuals and the total intravascular mass is about 120 g. Albumin represents the most important plasma transport molecule in the human organism. It is responsible for the transport of hormones, fatty acids, amino acids (notably tryptophan and cysteine), steroids, metals (calcium, copper and zinc), numerous pharmaceutical drugs, bile acids, bilirubin and many others through the blood stream. The interaction and binding of these small molecules to HSA is important in determining their transport, distribution, metabolism and elimination in human body. Furthermore, albumin acts as important extracellular antioxidant and mediates protection from free radicals and other harmful chemical agents [2].

The structure and binding sites of the HSA molecule was first reported by He and Carter [3]. The heart-shaped protein consists of three homologous helical domains (I, II and III) whereas each domain is divided into two subdomains (IA, IB, IIA, IIB, IIIA, IIIB). While the domains have similar structure, each domain has different ligand-binding affinities and functions. Two most important and well characterized binding sites on human serum albumin are Sudlow sites I and II. Sudlow site I is located in subdomain IIA and Sudlow site II is located in subdomain IIIA [4]. It became evident from many ligand binding studies in the past that the principal binding regions on HSA are located in subdomains IIA and IIIA. Representative drugs which bind to site I are sulphonamides, coumarin anticoagulants and salicylate, to mention just a few [5]. The Sudlow site II is the most active binding site. Many ligands were found to bind preferentially to this site, for example ibuprofen and tryptophan [3, 6, 7]. As presented earlier [8], poor or strong binding to native HSA or infused pharmaceutical HSA is believed to affect half-life of drugs and, therefore, has an influence on their half-life and bioavailability in plasma. Due to the fact that pharmaceutical HSA shows significantly lower drug binding capacity compared to native HSA [9] and since many drugs are highly albumin-bound at therapeutic concentrations, drug pharmacokinetics may be influenced after large amounts of albumin infusion. Moreover, pharmaceutical HSA does not have the oxidative properties of native albumin [10]. In addition to its drug binding capabilities, HSA has also catalytic activities. One of the most investigated catalytic activities is the hydrolysis of acetyl salicylic acid to salicylic acid which is an esterase-like activity of the HSA molecule [11]. Because HSA can form a stable HSA-heme complex, the catalytic properties and ligand binding of HSA are strictly dependent on heme concentration in plasma which can be increased in haematological diseases [11]. Another major function of serum albumin is to protect from the toxic effects of bilirubin and other catabolic products of the human metabolism. Bilirubin is transported to the liver by albumin where it is transformed into a water soluble form by conjugation to glucuronic acid in order to enable the elimination via the bile into the duodenum. Other toxins which are shuttled by albumin through the blood stream to the liver for their detoxification are bile acids and certain amino acids. In disorders related to the liver, such as acute or chronic liver failure, cholestasis or genetic defects causing enzyme deficiencies, these liver toxins can accumulate in the patients' blood up to 100 times higher than in healthy individuals. Based on this fact, the removal of lipophilic, albumin-bound substances such as bilirubin, bile acids, metabolites of aromatic amino acids, medium-chain fatty acids and cytokines should be beneficial to the clinical course of a patient in liver failure. This led to the development of filtration and absorption based blood purification devices. Although the use of extracorporeal liver support systems

didn´t show an improvement of the 28 day survival rat they are used to prolong the survival time of the liver patient. However, it needs to be pointed out that without liver transplantation, the mortality is not decreased [12]. Another clinical application where albumin solutions were used is the therapeutic plasma exchange for sepsis treatment. Fresh frozen plasma, diluted with 5% albumin in different ratios are used to replace plasma loss during continuous flow plasmapheresis but none of the clinical studies which were carried out showed a beneficial outcome regarding survival rate [13–15].

Since in the case of liver diseases the production and detoxification of albumin by the liver is strongly reduced, albumin infusion is often part of the therapy. Also some extracorporeal blood purification systems for liver support therapy include albumin as detoxification agents in their circuits. These are the Molecular Adsorbent Recirculation System [16] (MARS) and the Single Pass Albumin Dialysis (SPAD) [17]. Further indications for which albumin therapy is considered include hypoalbuminemia, shock, hypovolemia, burns, surgery or trauma, acute respiratory distress syndrome, cardiopulmonary bypass and hemodialysis [18, 19] whereas albumin solution is mainly used in patients with cirrhosis who undergo paracentesis or are treated for hepatorenal syndrome. HSA for clinical application is separated from donated human plasma. Therefore, the risk of transmission of pathogenic viruses such as those causing hepatitis, HIV and others not yet identified exists. The viruses are usually heat-inactivated at 60˚C for 10 hours. To avoid protein denaturation during this pasteurization process, caprylate and N-acetyl-tryptophanate (NAT) are widely used as stabilizers [20, 21]. Furthermore, caprylate ligand binding to the HSA molecule may protect HSA from aggregation during storage by increasing the electrical double layer that surrounds the protein [22]. However, the stabilizers are bound to Sudlow site II and therefore the transport function of pasteurized albumin is impaired. It is known from the literature that both stabilizers have additionally to their primary binding site in Sudlow site II other binding sites located on the protein with weak binding constants [23–26]. The common molar ratio for each of the two stabilizers is >5:1 (stabilizer: albumin). Because caprylate and NAT are metabolized in healthy humans, the use of high concentrations to stabilize albumin became state of the art until today but both stabilizers have been identified as vasodilators and may contribute to reduced renal perfusion [12]. Reduced albumin binding to drugs and other ligands can be observed in liver diseases with impaired liver functions. This is mostly the consequence of reduced albumin concentration and accumulation of drugs and metabolites (benzodiazepines, tryptophan, fatty acids, and bile acids) which are normally cleared by the liver [27–29]. It would be an advantage to remove the stabilizers from the HSA solution to administer albumin molecules with full native transport functions to patients. The Hepalbin adsorbent is a commercially available medical device which enables the bed-side removal of unwanted contaminations of human albumin solutions with caprylate and NAT and provides thereby a product which resolves the conflict between the need for stabilizers during production and storage and the desire for clean albumin with available binding sites after infusion or for albumin dialysis into liver disease patients.

The aim of this study was to determine if the industrial stabilizers can be depleted by adsorbent techniques. Several adsorbents were tested in batch and in a dynamic setup for their ability to remove the stabilizers and were compared to the commercially available Hepalbin filter. Additionally, the clearance of caprylate and NAT was evaluated with *in vitro* dialysis experiments.

Material and methods

Materials

For this study, two different human serum albumin solutions were used. The 200 g/L solution for infusion which contains 16 mmol/L sodium caprylate and 16 mmol/L N-acetyltryptophanate

Table 1. Adsorbents evaluated for their characteristics to remove caprylate and NAT from HSA.

adsorbent	provider	characteristics	clinical use	mean diameter [µm]
Prometh01	Fresenius Medical Care Adsorber Tec GmbH, Austria	polystyrene-divinylbenzene	plasma perfusion (Prometheus, liver support system)	550
diaMARS AC 250	Baxter, USA	activated charcoal	regenerate dialysate in MARS (liver support system)	900
Amberchrom CG161c	Dow Chemical, USA	polystyrene-divinylbenzene	no	120
Amberchrom CG161s	Dow Chemical, USA	polystyrene-divinylbenzene	no	35
Amberchrom HPR-10	Dow Chemical, USA	polystyrene-divinylbenzene	no	10
Hepalbin	Albutec GmbH, Germany	activated charcoal	remove stabilizer from pharmaceutical-grade HSA	filter plate
Hemosorba	Asahi Medical Co.Ltd, Japan	activated charcoal	hemoperfusion (liver support device)	650
Cytosorb	CytoSorbents GmbH, Germany	polystyrene-divinylbenzene coated with polyvinylpyrrolidone	hemoperfusion (sepsis and liver support device)	450

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as stabilizers was obtained from Kedrion Biopharmaceuticals (Gallicano, Italy). The second one was a 97% lyophilisate without any stabilizers from Sigma Aldrich (St. Louis, Mo., USA). Bi-distillated water and physiological sodium chloride solution were purchased from Fresenius Kabi (Graz, Austria). Methanol, acetonitrile, water (all HPLC grade), n-acetyl-L-tryptophane, ethanol and phosphate buffered saline (PBS) were ordered from Sigma Aldrich (St. Louis, Mo., USA). Adsorbents which were used for this study and their providers are listed in Table 1.

Albumin was measured with a Hitachi/cobas c311 automated analyzer with a corresponding test kit, both purchased from Roche (Penzberg, Germany). The adsorbents which were tested in this study are listed in Table 1.

Adsorbent pre-treatment

Prior to use for adsorption experiments, the non-clinically used polystyrene-divinylbenzene (PS-DVB) based adsorbents (CG161c, CG161s and HPR-10) were washed with ethanol, water and 0.9% saline solution for 60 min each, using a ratio of one volume part adsorbent and four volume parts liquid for each washing step. This pre-treatment is necessary to wet the inner nano-porous surface of the dry adsorbent particles. Activated charcoals were washed with physiological sodium chloride solution.

Removal of stabilizers in batch tests

The determination of the adsorption capacity for caprylate and N-acetyltryptophanate of each adsorbent was carried out in batch tests using 10% (w/v) of wet adsorbent (9 ml 20% HSA + 1 g adsorbent). Hepalbin, which is a charcoal based filter plate, was cut with a scalpel into small pieces with a maximum diameter of 2 mm. HSA without adsorbents was used as positive control and HSA without stabilizers was included as negative control in all batch tests. The incubation was carried out on a lab shaker for 24 hours at 37˚C. Samples were taken after 0, 30, 60, 120, 240, 360 minutes and after 24 hours and were frozen at -20˚C until quantification of caprylate, N-acetyltryptophanate, albumin and albumin binding capacity for Sudlow site II (ABiC II) was performed. To verify if the HSA without stabilizers contains some impurities which reduce the albumin binding capacity, additional batch tests were conducted where the HSA was incubated with Hemosorba and Prometh01.

Removal of stabilizers in a dynamic model

To check if an adsorbent cartridge can be used during HSA infusion therapy for stabilizer depletion, a dynamic model was used (Fig 1). An empty cartridge (Rezorian, Sigma, St. Louis, Mo., USA) was packed with 5 ml of adsorbent material and flushed with 15 ml physiological sodium chloride solution. In case of the Hepalbin filter, circular plates with the same diameter as the cartridge were punched to fit them into the column. Afterwards, 50 ml 20% HSA solution was pumped through using a roller pump, with a flow rate of 0.5 ml/min. One milliliter fractions were collected post cartridge with a fraction collector Model 2110 (BIO-RAD, Hercules, California, USA). Every third fraction was used for quantification of caprylate, NAT, albumin and ABiC II.

Removal of stabilizers by dialysis

Dialysis experiments were carried out in miniaturized *in vitro* setups (Fig 2) using pediatric tubing and a polysulfone based pediatric high-flux dialyzers (PX paed, Presenius Medical Care, Bad Homburg, Germany) with an effective membrane surface of 0.2 m^2. 200 ml 5% HSA-solution was dialysed against 0.9% sodium chloride solution. The flow rates were Q_{HSA} = 100 ml/min for the HSA-solution and Q_D = 50 ml/min for the dialysis fluid. The experiments were carried out at 37˚C and HSA-samples were taken after 0, 30, 60, 120, 180 and 240 minutes. Samples were stored at -20˚C until quantification of caprylate, NAT, albumin and albumin binding capacity for Sudlow site II (ABiC II).

The clearance (C) was calculated using the formula

$$C = \frac{V}{t} \times \ln \frac{c_0}{c_t} \quad (1)$$

where V = plasma volume [ml], t = treatment time [min], C_0 = concentration before starting treatment [μM], C_t = concentration at time t [μM].

Determination of the albumin binding capacity

The albumin binding capacity for binding site II (ABiC II) was estimated using the method described previously [29–31] with minor modifications. For the determination of the binding capacity on Sudlow´s site II, the fluorescent dye Dansylsarcosine (DS) (Sigma Aldrich, St. Louis, Mo., USA) was used. The binding of DS to the albumin molecule on Sudlow´s site II leads to an increase of the fluorescence intensity of the chromophore. Consequently, albumin without stabilizers results in a stronger fluorescence signal since it is able to bind higher amounts of DS. For ABiC II measurement a 10 mM DS stock solution, solved in acetonitrile, was prepared and stored in the dark at 4˚C. Immediately before usage a 1 mM DS working solution, diluted with 10 mM PBS was prepared. The measurements were performed in 96-well plates (cellstar, PS, F-bottom, μclear, black) purchased from Greiner Bio-One GmbH (Kremsmünster, Austria). 100 μl of each 0.1 mM albumin sample was mixed with 10 μl DS working solution and the fluorescence signal was measured with a multi detection microplate reader (BioTec Instruments Inc., Winooski, VT, USA; excitation wavelength 360 nm, emission wavelength 460 nm). The binding capacity was calculated in percent, where the stabilizer-free HSA from Sigma was set to 100% (positive control), according to the following equation:

$$ABiCII(\%) = \frac{fluorescence\ \ of\ \ adsorbent\ \ treated\ \ HSA}{fluorescence\ \ of\ \ HSA\ \ without\ \ stabilizer} \times 100 \quad (2)$$

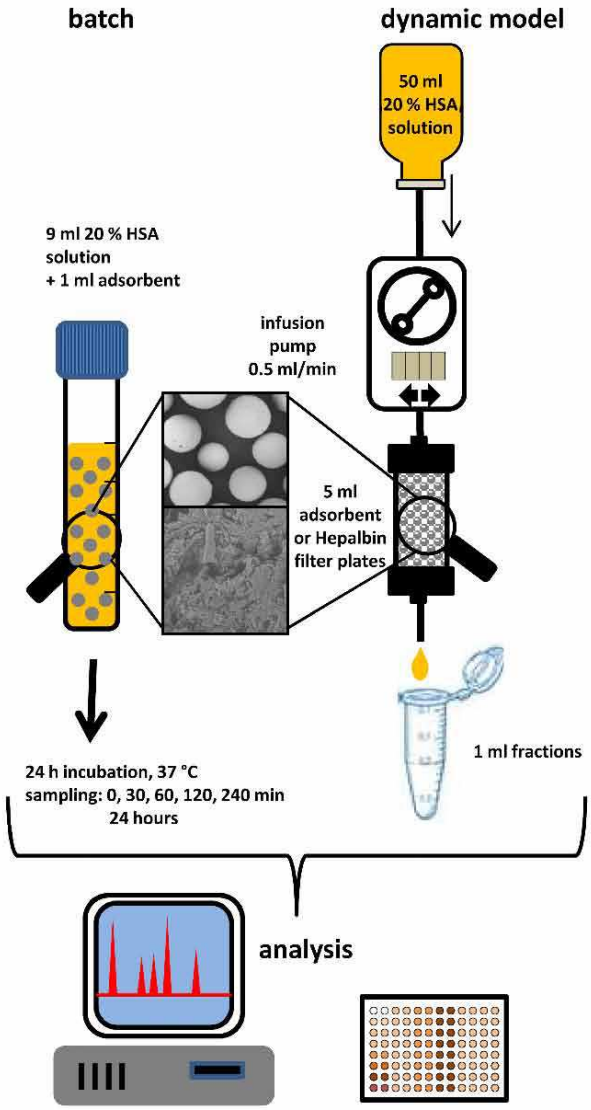

Fig 1. Schematic illustration of the batch tests and the dynamic model. The batch tests were performed with 10% (w/v) adsorbent in 20% HSA solution for 24 hours to determine the adsorption capacity of NAT and caprylate of each adsorbent. The dynamic model was used to simulate the removal rates of stabilizers from HSA solution before intravenous administration into the patient. 50 ml 20% HSA solution was pumped (0.5 ml/min) through a 5 ml adsorbent cartridge and 1 ml HSA fractions were collected post cartridge for determination of the NAT, caprylate level and ABiC.

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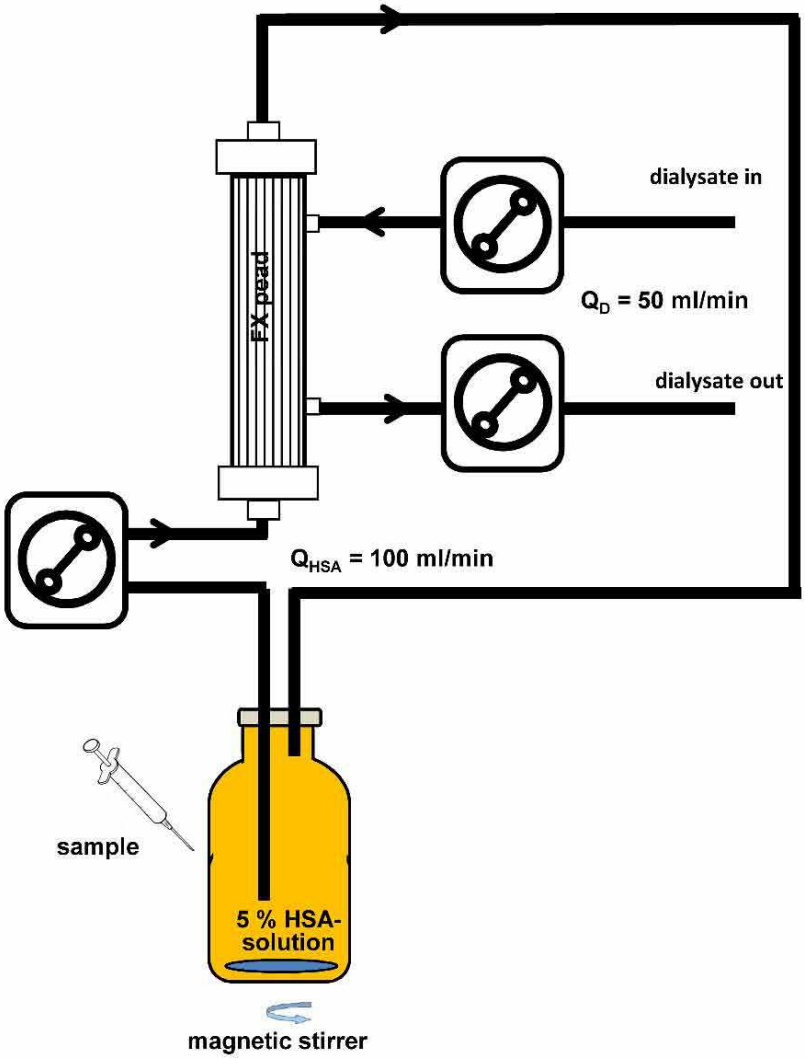

Fig 2. Setup of the dialysis circuit to determine the removal of the stabilizers by a high flux dialyser. 200 ml 5% HSA solution was dialysed against 0.9% NaCl solution using a pediatric high flux dialyser. Flow rates for the HSA solution was 100 ml/min and the dialysate flow was set to 50 ml/min. Samples were taken during 4 hours treatment time.

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As negative control, the untreated 20% HSA-solution for infusion was used. Both controls were included in each analytical run. The albumin concentration of each sample was checked with the Hitachi/cobas 311c automated analyser. Each sample was diluted with physiological sodium chloride solution to yield a 0.1 mM albumin solution and a molecular weight of 66400 Da for albumin was assumed.

Determination of N-acetyltryptophanate level

The quantification of N-acetyltryptophanate was performed with a high performance liquid chromatography method. Samples were precipitated by adding 50 μl HSA sample to 250 μl methanol, vortexed, and cooled at -80˚C for 20 minutes. After centrifugation (14000 g, 5 min) 20 μl of the supernatant was injected into the HPLC column (150 x 4.6 mm Nucleosil 100–5 C18 column combined with a 4 x 3 mm Nucleosil 100–5 C18 guard column; Macherey-Nagel, Düren, Germany). The temperature of the column oven was set to 35˚C. The elution consisted of a linear gradient program from 30 to 50% methanol in water over 6.5 minutes, maintained at 50% methanol for 1 minute and returned to 30% methanol for 1 minute. The flow rate was 0.5 ml/min and absorbance detection at 280 nm was performed for NAT quantification. The amount of N-acetyltryptophanate was calculated in percent of the untreated 20% HSA-solution (Kedrion Biopharmaceuticals, Italy), and as negative control the stabilizer-free HSA (Sigma Aldrich, USA) was analyzed. The recovery of NTA using this HPLC procedure is 72.3 ± 0.5% (S1 Fig). It was determined by comparing the peak areas between NTA diluted in methanol and NTA spiked in stabilizer-free albumin solution.

Determination of caprylate level

The quantification of caprylate was performed with a free fatty acid quantification assay kit (abcam, Cambridge, UK). This kit provides a sensitive, enzyme-based method for colorimetric detection of long- and middle chain free fatty acids in biological samples. Concentrations of free fatty acids in the samples were calculated from standards which are included in the kit. To obtain a signal in the standard range (0 to 200 μM), the samples were diluted 1:100 with dilution reagent which was provided with the kit. The amount of caprylate was calculated in percent of the untreated 20% HSA-solution (Kedrion Biopharmaceuticals, Italy), and as negative control the stabilizer-free HSA (Sigma Aldrich, USA) was used.

Statistics

All experiments were carried out at least thrice and data are expressed as mean ± SD which was calculated using Microsoft Excel 2010. Significances were calculated with the one-tailed t-test using SigmaPlot for Windows Version 13.0. Area under the curve and area above the curve calculations were conducted with Microsoft Excel for Windows 2010.

Results and discussion

Removal of stabilizers in batch tests

The adsorption capacity of different adsorbents regarding caprylate and NAT removal was tested in batch. The results show that larger adsorbent particles (diaMARS AC 250, Cytosorb, Prometh01 and Hemosorba) reach the equilibrium concentration after several hours compared to the smaller adsorbent particles (CG161c, CG161s, HPR10) which show very fast adsorption kinetics (Fig 3). The maximum time (t_{max}) which was needed to reach an equilibrium concentration for caprylate and NAT and the adsorption capacity (mg caprylate or NAT per g adsorbent) for the two stabilizers in the batch tests are listed in Table 2. Particle size and

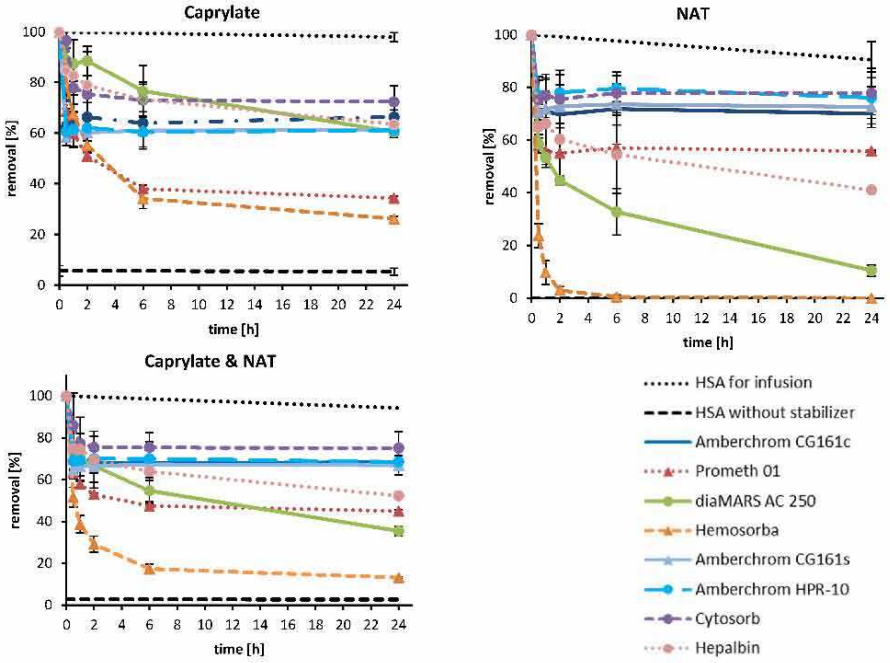

Fig 3. Percental removal rates of caprylate, N-acetyltryptophanate and of both from 20% HSA infusion solution by different adsorbents tested in batch. HSA solution was incubated with 10% (v/v) adsorbent for 24 hrs at 37˚C. Samples for NAT and caprylate quantification were taken after 30, 60, 120, 240, 360 min and 24 hrs. The stabilizer levels of HSA solution without adsorbent (dotted line) were set to 100%. The caprylate and NAT levels of stabilizer free lyophilized HSA act as negative control (dashed lines).

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Table 2. The maximum time [h] to reach the equilibrium concentration for caprylate and NAT removal and adsorption capacity [mg/g] for caprylate and NAT of each adsorbent in the batch tests.

	t_{max} to reach the equlibrium for caprylate [h]	t_{max} to reach the equlibrium for NAT [h]	adsorption capcity [mg caprylate /g adsorbent]	adsorption capacity [mg NAT /g adsorbent]
Amberchrom CG161c (120 μm)	0.5	0.5	7.3	10.6
Prometh01	> 24	1	14.2	15.7
diaMars AC 250	> 24	> 24	8.5	31.7
Hemosorba	> 24	24	15.9	35.5
Amberchrom CG161s (35 μm)	0.5	0.5	8.4	9.8
Amberchrom HPR-10	0.5	0.5	8.4	8.5
Cytosorb	6	0.5	6.0	7.9
Hepalbin	> 24	> 24	7.9	20.9

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pore size are geometric parameters of the adsorbent material which influence the adsorption kinetic of target molecules. The outer pores of the adsorbent surface act as a molecular sieve and prevent the entry of molecules that are larger than the molecular cut-off of the pores. In case of adsorbent materials with defined pore structure, the viscosity radius of molecules, also known as hydrodynamic radius, is essential for adsorption because it determines if the molecule can come in contact with the inner adsorbents surface or not [32]. A high dependence of the diffusion time ($t_{\Delta x}$) on the diffusion path (Δx) can be found using the Einstein-Smoluchowski equation, which predicts that:

$$t_{\Delta x} = \frac{\Delta x^2}{2D} \quad (3)$$

where D is the diffusion coefficient. Consequently, smaller adsorbent particles show faster adsorption kinetics.

Fig 3 shows that NAT is removed more efficiently by charcoal based adsorbents (diaMARS AC 250, Hepalbin and Hemosorba) compared to polystyrene based adsorbents. Only Hemosorba was able to remove all NAT from the HSA solution. A possible reason for this could be that Hemosorba has, compared to the other charcoal based adsorbents (diaMARS AC 250. Hepalbin) the largest accessible inner pore surface. From the polystyrene based adsorbent only Prometh01 shows good NAT adsorption properties (44 ± 1%). The adsorbents which show the highest caprylate removal are Hemosorba (73 ± 3%) and Prometh01 (66 ± 1%). Because both stabilizers were added in the same molar ratio (16 mM), we were able to calculate the mean removal rate for both stabilizers (Fig 3). The ranking regarding removal rate of both stabilizers reads as follows: Hemosorba (87 ± 1%) > diaMARS AC 250 (65 ± 2%) > Prometh01 (55 ± 1%) > Hepalbin (48 ± 1%) > CG161c (32 ± 2%) > CG161s (33 ± 5%) > HPR-10 (32 ± 6%) > Cytosorb (25 ± 8%).

Albumin binding capacity for binding site II (ABiC II) is a simple method for characterizing the site II-specific binding functions of the albumin molecule [33]. After diluting the different albumin samples from the batch tests to the same albumin concentration (0.1 mM) and incubation with the binding site II-specific fluorescent marker, the amount of bound marker can be determined by fluorescence detection. The ABiC II of HSA without any stabilizer was set to 100% binding capacity. As it is shown in Fig 4A the binding capacity of commercially available HSA for infusion is highly reduced by the stabilizers (26 ± 7%) compared to the HSA without stabilizer. The results indicate that the increase of ABiC II correlates more with the removal of caprylate than with NAT. Hemosorba was able to raise the ABiC II value from 26 ± 7% to 57 ± 7% whereas the CG161c treatment caused only an increase of 2 ± 1%. All adsorbent treatments caused a significant ($p \leq 0.05$) increase of ABiC II. Also the ABiC II of the stabilizer free albumin was increased using Hemosorba (116 ± 1%) and Prometh01 (111 ± 1%). This indicates that the stabilizer free HSA includes some impurities which influence the ABiC II and can be removed by adsorption treatment (Fig 4). The albumin level was determined to evaluate the influence of each adsorbent on the albumin level of the HSA solution after treatment. The highest loss of albumin was caused by CG161s treatment which shows an albumin binding of 210 mg per ml adsorbent. With the exception of Hepalbin, the charcoals showed low albumin adsorption whereas the lowest reduction on the albumin level was achieved by the diaMARS AC 250 adsorbent (Fig 4B).

Removal of stabilizers in the dynamic model

For the practical application, the adsorbents were tested in a dynamic model, where the HSA solution for infusion was pumped through a cartridge filled with adsorbent and the removal

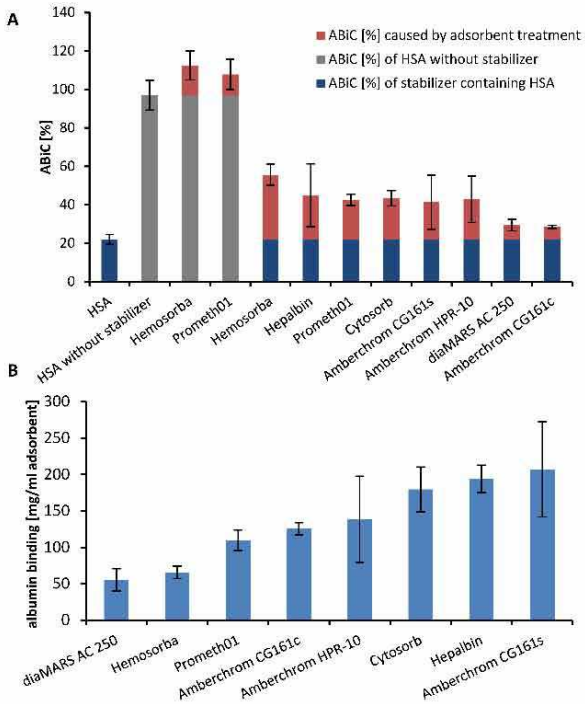

Fig 4. The adsorbent treatment increases the ABiC II levels. The albumin binding capacity (ABiC II) after adsorbent treatment was compared to untreated HSA and HSA without stabilizers, which was set to 100% (A). All adsorbent treatments caused a significant ($p < 0.05$) increase of ABiC II. The albumin concentration was measured after 24 hours adsorbent treatment in batch to calculate the albumin binding in mg per ml adsorbent volume (B).

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rate of stabilizers was measured downstream from the cartridge. The aim of this setup was to simulate an intravenous HSA infusion where the HSA is depleted of stabilizers using an adsorption column before it is infused into the blood stream (Fig 1). To save material and costs, the model was downscaled using 50 ml 20% HSA solution which was pumped in single pass through a 5 ml adsorbent filled cartridge. Based on the flow rate of 0.5 ml/min, the contact time of HSA to the adsorbent material was calculated to be 10 min. The relatively short contact time between adsorbent surface and the HSA solution, results in a shorter diffusion distance. Consequently, large adsorbent particles are not as efficient in the dynamic model compared to the batch test where the contact time was 24 hours. To illustrate that the particle size of adsorbents influences the outer adsorbent surface we calculated the outer adsorbent surface of a 5 ml filled cartridge. Assuming that all adsorbent are spherical particles and follows the close-packing of equal spheres (adsorbent volume = 74% of cartridge volume) the outer adsorbent surface of HPR-10 was about 100 times larger than that of the diaMARS AC in the dynamic model (Table 3). As it is shown in Fig 3, the post cartridge level of stabilizers is very low in the first fractions and increases depending on the adsorbent. For mathematical evaluation of the

Table 3. Calculated outer adsorbent surface of an 5 ml filled cartridge, assuming that all adsorbent are spherical particles and follows the close-packing of equal spheres (adsorbent volume = 74% of cartridge volume).

	particle diameter [µm]	particle volume [cm^3]	particle/ cartridge	outer surface/ particle [cm^2]	outer particle surface/ cartridge [cm^2]
diaMARS AC 250	900	3.82×10^{-4}	0.97×10^4	2.5×10^{-2}	247
Hemosorba	650	1.44×10^{-4}	2.57×10^4	1.3×10^{-2}	342
Pormeth01	550	8.71×10^{-5}	4.25×10^4	9.5×10^{-3}	404
Cytosorb	450	4.77×10^{-5}	7.75×10^4	6.4×10^{-3}	667
Amberchrom CG161c	120	9.05×10^{-7}	4.09×10^6	4.5×10^{-4}	1850
Amberchrom CG161s	35	2.24×10^{-8}	1.65×10^8	3.8×10^{-5}	6343
Amberchrom HPR-10	10	5.24×10^{-10}	7.07×10^9	3.1×10^{-6}	22200

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dynamic model, the adsorbed amount of NAT and caprylate is shown as area above the curve in Fig 5. The level of removed stabilizers is shown in Table 4. In summary, it can be stated that in the dynamic model the Hepalbin (-60%) and Hemosorba (-60%) are the most effective adsorbents for both stabilizers. From the polystyrene based adsorbents, Prometh01 (-40%) and Cytosorb (-38%) showed the best removal rates of stabilizers in the dynamic model, whereby Cytosorb showed the highest efficiency regarding caprylate removal (Fig 5).

Due to the fact that the increase of ABiC II correlates more with the removal of caprylate than with the removal of NAT, the highest ABiC II values in the dynamic set up could be reached using Hepalbin and CG161s whereas only little increase of ABiC II was observed using diaMARS AC 250 or HPR10 adsorbent (Table 1 and Fig 6). The reason for this finding is that NAT has a lower binding affinity to albumin than DS and, therefore, DS will bind to albumin independently from the NAT concentration. As a consequence, the ABiC II method is only suitable for strongly albumin-bound substances.

The quantification of ABiC II in the dynamic model was determined by calculating the area under the curve of Fig 6. The data are given in percent compared to HSA without stabilizer (Table 4). The results of the ABiC II determination show that the first post adsorbent fractions of Hepalbin, Cytosorb and CG161s achieve a higher ABiC II level than compared to HSA without stabilizers. This might indicate that also the HSA without stabilizer has not the full binding capacity and that the binding site II is occupied with some impurities originating from the manufacturing process. This was proven by the performed batch tests where we could show that the ABiC II of stabilizer free HSA increased after adsorbent treatment (Fig 4 and S2 Fig). The highest calculated ABiC II using the dynamic model was achieved using the Hepalbin adsorbent (82%) followed by CG161s (74%) and Cytosorb (59%). HPR10 and diaMARS AC 250 treatment caused the lowest increase of ABiC II. By comparing the removal rates of NAT and caprylate to the determined ABiC II it can be concluded that caprylate removal has more influence on the improvement of the ABiC II than the removal of weakly bound NAT.

Removal of stabilizers by dialysis

Albumin infusion is often applied in patients who suffer from liver failure. In some extracorporeal liver support systems such as MARS and Prometheus, a dialyzer is included to remove uremic toxins from patient's blood. Water soluble toxins as well as weakly albumin bound toxins can be removed by dialysis. To evaluate if the two stabilizers can be removed by dialysis, a downscaled dialysis setup was used, where 200 ml 5% HSA solution was dialyzed against physiological sodium chloride solution for 4 hours (Fig 2). Since the binding constant between HSA and caprylate ($K_a \leq 5.5 \times 10^5$) [20, 34] is higher than between HSA and NAT (tryptohan: $Ka \leq 1 \times 10^4$), NAT is removed easier by dialysis because of the higher rate of unbound

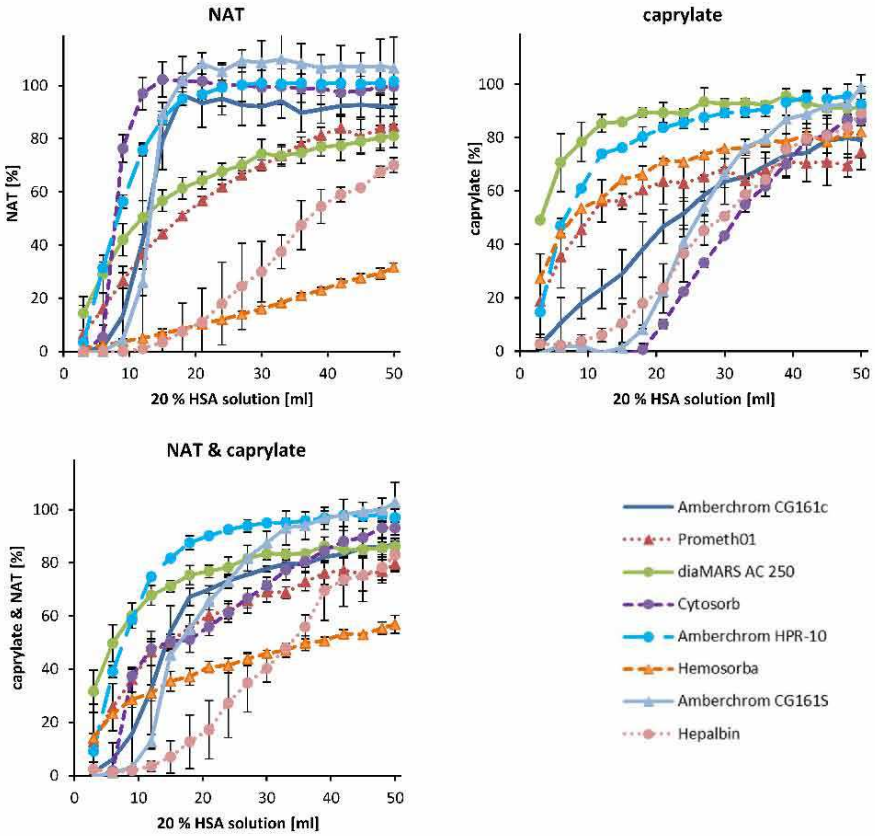

Fig 5. Pre cartridge concentrations of stabilizers from 20% HSA solution using different adsorbents in the dynamic model. 50 ml 20% HSA solution was pumped with 0.5 ml/min through a 5 ml adsorbent filled cartridge. 1 ml fractions were collected post cartridge for NAT and caprylate measurement.

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Table 4. ABiC II values compared to HSA without stabilizer in percent and removal rates of caprylate, N-acetyltryptophan and both in percent from a 20% HSA solution using different adsorbent in the dynamic model.

	Hepalbin	Cytosorb	CG161s (35 µm)	Hemosorba	CG161c (120 µm)	Prometh 01	diaMARS AC 250	HPR-10
removal NAT [%]	67 ± 11	11 ± 6	15 ± 4	85 ± 1	24 ± 3	40 ± 2	29 ± 16	19 ± 2
removal caprylate [%]	51 ± 12	64 ± 1	53 ± 2	31 ± 5	51 ± 5	40 ± 7	13 ± 2	13 ± 1
removal NAT & caprylate [%]	59 ± 12	38 ± 3	34 ± 3	59 ± 3	38 ± 4	40 ± 5	21 ± 9	16 ± 1
albumin loss [%]	3 ± 1	4 ± 1	4 ± 1	1 ± 1	9 ± 3	3 ± 1	3 ± 3	5 ± 1
ABiC II [%]after treatment	82 ± 24	59 ± 1	73 ± 5	44 ± 5	39 ± 3	25 ± 1	44 ± 16	26 ± 1

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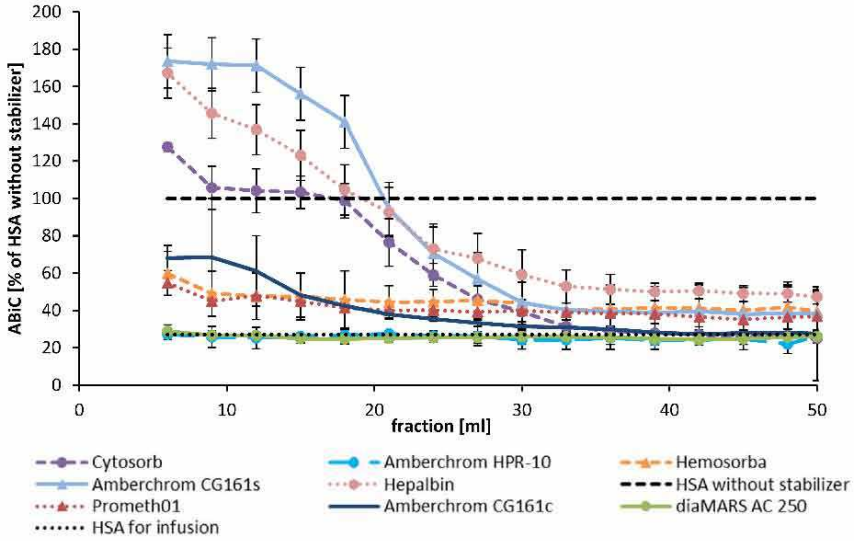

Fig 6. ABiC II levels of the post cartridge fractions from the dynamic model. ABiC II was determined from post adsorbent cartridges fractions. ABiC II of HSA without stabilizer (dotted line) was set to 100%. ABiC II of untreated HSA is shown in broken line.

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molecules (Fig 2). In our dialysis setup, NAT was totally removed after 60 min, whereas caprylate could be lowered after 4 hours to 17 ± 6% compared to the starting concentration (Fig 7). A 93 ± 24% recovery of the ABiC II was achieved by dialysis and a reduction of the albumin level was not observed which can be explained by the low sieving coefficient for albumin which is given < 0.001 for the FX paed in the product data sheet. The calculated clearance according to equation [1] is listed in Table 5. It is noticeable that the caprylate clearance decreases from 4 ± 1 at time point 30 minutes to 0.2 ± 0.6% after 4 hours. These results show that the binding sites for the five caprylate molecules have varying binding constants. At a molar ratio of one (HSA: caprylate) the removal rate of caprylate via dialysis reaches nearly zero which means that one caprylate binding site on the HSA molecule is comparably strong as the bilirubin binding site ($K_a = 3 \times 10^6\ M^{-1}$).

Conclusions

For patients suffering from hypoalbuminemia, hyperbilirubinemia or other illnesses causing an impaired transport capacity, the removal of the albumin-blocking compounds is of high importance. The same is true for patients who need albumin substitution. The removal of stabilizers from HSA could be achieved by using an adsorbent cartridge where the HSA solution passes through before it is administered intravenously to the patient, similar to the Hepalbin filter. The study shows that activated charcoals have better adsorption properties for NAT, whereas polystyrene based adsorbents are more suitable for caprylate removal. Because most of the pharmaceutical grade HSA includes NAT and caprylate in equal molar ratios, a combination of these two materials would be favourable to efficiently remove both stabilizers. The results from the dynamic setup revealed that the use of Hepalbin filter caused the highest ABiC

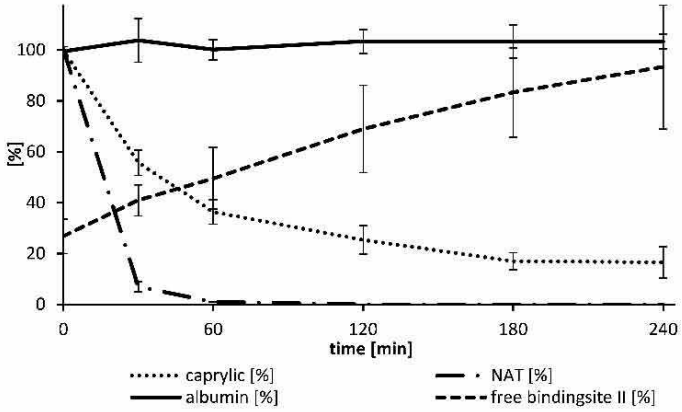

Fig 7. Removal of stabilizers during 4 hours dialysis. 200 ml 5% HSA solution was dialyzed against physiological sodium chloride solution using a pediatric high-flux dialyzer. The flow rate of the HSA solution was 100 ml/min and the dialysate flow was set to 50 ml/min. The treatment time was 4 hours and samples were taken after 30, 60, 120, 180 and 240 min, n = 4.

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Table 5. Calculated clearance rates using the formula (1) for NAT and caprylate from the dialysis experiment (n = 4).

time [min]	C_{NAT} *[%]*	$C_{caprylate}$ *[%]*
30	18 ± 2	4 ± 1
60	15 ± 0	3 ± 1
120	N/A	1.2 ± 0.3
180	N/A	1.5 ± 0.3
240	N/A	0.2 ± 0.6

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in Sudlow site II and is an efficient medical device for applying stabilizer reduced albumin infusion into the patient. We could further show that all NAT and more than 80% of caprylate can be removed by dialysis. These results suggest that an albumin therapy for liver patients could be combined with dialysis treatment to achieve stabilizer reduced albumin with almost completely restored transport capacity and, therefore, detoxifying capability. A combination of dialysis with an adsorbent based blood purification system, such as Prometheus or MARS, is able to reduce the level of stabilizers of human serum albumin. This implies that the albumin infusion should be given shortly before or at the beginning of the extracorporeal liver support treatment to reduce the level of stabilizers in patients' blood. Furthermore, the results confirm previous studies which showed that the Open Albumin Dialysis system (OPAL), which uses several Hepalbin filters in the dialyse circuit instead of the diaMARS AC 250 charcoal in the MARS system, is an efficient system to generate high albumin binding capacity in liver failure patients [35, 36].

Supporting information

S1 Fig. Recovery of the HPLC method for NAT quantification. The recovery was determined by comparing the peak areas between NTA diluted in methanol and NTA spiked in

stabilizer-free albumin solution. The tested NAT concentrations were 4 and 16 mM. All samples for HPLC injection were done in triplicates.
(DOCX)

S2 Fig. Adsorbent treatment of stabilizer free HSA increase the ABiC II value. To verify if the HSA without stabilizers contains some impurities which reduce the albumin binding capacity, additional batch tests were conducted where the HSA was incubated with Hemosorba and Promth01.
(DOCX)

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Funding acquisition: Stephan Harm, Jens Hartmann.

Investigation: Stephan Harm, Claudia Schildböck.

Methodology: Stephan Harm.

Project administration: Jens Hartmann.

Software: Stephan Harm.

Supervision: Jens Hartmann.

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2.2 Posterpräsentationen und Vorträge bei Kongressen

Stephan Harm & Jens Hartmann: *Influence of different anticoagulants on endotoxin activity in human serum.* Vienna, Austria, Conference: ESAO 2017 (Poster)

Stephan Harm & Jens Hartmann: *Binding of synthetic AMPs to human serum albumin.* Copenhagen, Denmark, Conference: IMAP 2017 (Poster)

Jens Hartmann & Stephan Harm: *Antimicrobial Peptides in Endotoxemia – an in vitro study.* Copenhagen, Denmark, Conference: IMAP 2017 (Poster)

Jens Hartmann & Stephan Harm: *Cytokine induction in human blood by fractionated bacterial lysates.* Krems, Austria, Conference: BioNanoMed 2017 (Poster)

Stephan Harm & Jens Hartmann: *Removal of stabilizers from human serum albumin by nanostructured adsorbents.* Krems, Austria, Conference: BioNanoMed 2017 (Poster)

Stephan Harm, Jens Hartmann: *Citrate anticoagulation improves blood compatibility in dialysis and apheresis - data form in vitro experiments.* Warsaw, Poland, Conference: ESAO 2016 (Vortrag)

Jens Hartmann, Stephan Harm: *Removal of Bile Acids in Extracorporeal Therapies: An In-Vitro Study*. Conference: ESAO 2016, Warsaw, Poland (Poster)

Jens Hartmann, Stephan Harm, Viktoria Weber: Intensive care medicine experimental ESICM, Milano 2016 (Poster)

Stephan Harm, Jens Hartmann: *Polymyxin coated cytokine adsorbent for supportive treatment of sepsis.* Intensive care medicine experimental ESICM, Milano 2016 (Presentation)

Stephan Harm & Jens Hartmann: *Polymyxin-coated Nanostructured Materials.* Krems, Austria, Conference: BioNanoMed 2016 (Poster)

Jens Hartmann & Stephan Harm: *Enhanced Middle Molecule Clearance with a High Cut-Off Filter.* Krems, Austria, Conference: BioNanoMed 2016 (Poster)

Stephan Harm & Jens Hartmann: *A blood purification system which combines cytokine removal and PMB release – an option for sepsis treatment.* London, Conference: International Meeting on Antimicrobial Peptides 2015 (Poster)

Stephan Harm & Jens Hartmann: *Extracorporeal Liver Support – A Balancing Act Between Efficiently and Biocompatibility.* Malta, Conference: World Conference on Targeting Liver Diseases 2015 (Vortrag)

Jens Hartmann, Karin Strobl & Stephan Harm: *Citrate vs Heparin Anticoagulation in Extracorporeal Liver Support: A Glimplse on Biocompatibility.* Krems, Austria, Malta, Conference: World Conference on Targeting Liver Diseases 2015 (Poster)

Stephan Harm & Jens Hartmann: *Polymyxin – long forgotten weapon against endotoxemia.* London, Conference: International Meeting on Antimicrobial Peptides 2014 (Poster)

Jens Hartmann, Dieter Falkenhagen & Stephan Harm: *Size matters - nanostructured polysufon membranes act as molecular sieve in blood purification.* Krems, Austria, Conference: Bionanomed 2014 (Poster)

Stephan Harm, Dieter Falkenhagen & Jens Hartmann*: Nanostructures – a key property of adsorbents in extracorporeal blood purification.* Krems, Austria, Conference: Bionanomed 2014 (Poster)

Jens Hartmann & Stephan Harm: *Effective Removal of Estrogens from Drinking Water and Waste Water by Adsorption Technology.* Porto, Conference: European Water Resources Association 2013 (Poster)

Stephan Harm & Jens Hartmann: *A new and simple method for simultaneous detection of native and synthetic estrogen.* Porto, Conference: European Water Resources Association 2013 (Poster)

2.3 Patente und Patenteinreichungen

Stephan Harm, Jens Hartmann, Dieter Falkenhagen: *DOSIERUNGSANLEITUNG FÜR ENDOTOXINBINDENDE LIPOPEPTIDE*. Ref. No: US-2015-0190461-A1, Year: 03/2015

Stephan Harm, Jens Hartmann, Dieter Falkenhagen: *EXTRAKORPORALE PERFUSIO APPARATUS*. Ref. No: US2015/0328387 A2, Year: 12/2014

Stephan Harm, Jens Hartmann, Dieter Falkenhagen: *Selektives Sorptionsmittel für die extrakorporale Blutreinigung*. Ref. No: EP 2 679 302 A1, Year: 06/2012

Stephan Harm, Jens Hartmann, Dieter Falkenhagen, Viktoria Weber: *NOVEL SORBENT FOR ENDOTOXINS*. Ref. No: PCT/AT2011/000273, Year: 06/2011

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Cirriculum Vitae

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seit 2009	Wissenschaftlicher Mitarbeiter am Department Gesundheitswissenschaften und Biomedizin an der Donau-Universität Krems
seit 2012	Mitglied der Europäischen Gesellschaft für künstliche Organe (ESAO)

Weitere Qualifikationen

Fremdsprache: Englisch in Wort und Schrift

EDV-Kenntnisse: Window 10, MS Office 2016, SPSS 20, Visual Designer, IMARIS (3D-Rekonstruktionssoftware), …

Publikationen in peer reviewed internationalen Journalen

Jakob Gubensek, Karin Strobl, Stephan Harm, Rene Weiss, Tanja Eichhorn, Jadranka Buturovic-Ponikvar, Viktoria Weber, Jens Hartmann: *Influence of citrate concentration on the activation of blood cells in an in vitro dialysis setup*. PLoS ONE 06/2018; 13(6), DOI:10.1371/journal.pone.0199204

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