In Vitro Reduction of Mycotoxins and Other Inflammatory Mediators from Whole Blood using Hemocompatible Porous Polymer Beads

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ABSTRACT

Opportunistic fungal infections account for up to 25% of all hospital-acquired blood infections, which are often highly fatal. The production of mycotoxins by certain types of fungi partially explains the pathogenicity of these infections. Aflatoxin and tricothecene T-2 toxin, produced by *Aspergillus* and *Fusarium* species, respectively, are two examples of mycotoxins that are both easily obtained and highly toxic to humans. Exposure via injection, inhalation or dermal penetration of inadvertently or intentionally contaminated food, water, or air can result in systemic toxicity. CytoSorbents' porous polymer technology utilizes hemocompatible, highly porous polymer beads to reduce inflammatory mediators and toxins in blood based on size exclusion and surface adsorption via extracorporeal hemoperfusion therapy. In this study, it was demonstrated that CytoSorbents' polymers are capable of efficiently reducing levels of aflatoxin B1 and epoxomicin, a non-toxic surrogate for T-2 toxin, as well as a broad range of other inflammatory mediators.

Keywords: mycotoxins, inflammatory mediators, hemoperfusion, blood purification, porous polymers

1 INTRODUCTION

Opportunistic fungal infections account for up to 25% of all hospital-acquired blood infections. Patients at highest risk are those with weakened immune systems, and include elderly patients, patients with cancer or HIV infection, and patients taking immunosuppressive drugs due to chronic autoimmune disease or recent organ transplant. Despite the use of anti-fungal medications, these insidious persistent infections are often highly fatal, with a mortality of more than 90% in many studies [1]. Over 20 *Aspergillus* species are known to cause human disease. Invasive aspergillosis (IA) is a devastating infectious disease that mainly affects critically-ill and immunocompromised patients. *Aspergillus fumigatus* is the most prevalent and is largely responsible for the increased incidence of invasive aspergillosis in the immunocompromised patient population [2]. IA is a devastating illness, with mortality rates in some patient groups reaching as high as 90%. *Aspergillus* species produce a variety of mycotoxins, such as aflatoxin, that contribute to pathogenicity by host immunosuppression. *Fusarium* species cause a broad spectrum of infections in humans, including superficial, locally invasive, and disseminated infections. *Fusarium* species possess several virulence factors, including T-2 toxin, a trichothecene mycotoxin, which suppresses humoral and cellular immunity and may also cause tissue breakdown. The production of mycotoxins partially explains the pathogenicity of these fungal infections.

There are numerous types of mycotoxins, many of which are different both structurally and chemically [3]. Contamination of food, water, and air can rapidly lead to severe illness and potentially death. Two mycotoxins of increased significance are aflatoxin and trichothecene T-2 toxin because they are easily obtained and are highly toxic to humans. Inadvertent exposure most commonly occurs via ingestion of contaminated foods; however, toxicity can also occur through inhalation or dermal penetration, and any route of exposure can result in systemic toxicity [4, 5]. In addition, these toxins have been weaponized as biowarfare agents in the past [6]. Aflatoxin in the bloodstream, for example, can cause acute liver failure, neurologic coma, and death [7]. T-2 toxin has been implicated as a possible cause of Gulf War syndrome, and can cause fatal lung and liver injury, bone marrow destruction, nerve damage and bleeding [5].

Human pathogens of all types - fungal, bacterial and viral - are known to release a daunting array of virulence factors that modulate the immune response and influence the severity of the disease. The host response to toxins and pathogen-associated molecular pattern molecules (PAMPs) involves multiple sequential and concurrent processes that produce both exaggerated inflammation and immune suppression. PAMPs, which also include bacterial endotoxins and exotoxins, can either cause direct damage to tissues, or trigger a systemic inflammatory response syndrome (SIRS) in the host, driven by the production of high levels of cytokines and the release of damage-associated molecular pattern molecules (DAMPs) [8]. This milieu of circulating inflammatory mediators can subsequently induce a maladaptive SIRS response that can lead to multiple organ dysfunction syndrome (MODS), organ failure, and patient death.

CytoSorb® (CytoSorbents Corporation, USA) is an extracorporeal hemoadsorption therapy that utilizes hemocompatible, highly porous polymer beads to reduce cytokines and other inflammatory mediators in blood based on size and surface adsorption. Hemoadsorption technologies can provide therapeutic benefit through the removal of toxins and PAMPs that directly cause tissue injury as well as the removal of cytotoxic levels of cytokines and other inflammatory mediators (DAMPs) to

reduce the inflammatory cascade and redirect immune cells to the actual site of infection, limiting subsequent organ damage [9]. Cytokine reduction has been correlated with improvement in survival and clinical outcomes in experimental studies and clinical reports using CytoSorb® [10-15]. CytoSorb® is also capable of removing small hydrophobic molecules from blood and, therefore, it is postulated that CytoSorb will be effective against a broad range of mycotoxins.

Furthermore, this porous polymer bead technology has been expanded upon through the development of advanced sorbents with capture and adsorption capabilities tailored for specific target molecules. Specialized small pore sorbents designed to target the removal of small toxic molecules, such as aflatoxin and other mycotoxins, have been generated. The targeted molecular weights ranges for selected sorbents are illustrated in Figure 1, with particular toxins and inflammatory mediators positioned according to molecular weight.

The purpose of this study is to 1) further characterize the ability of CytoSorbents' specialized small pore polymers to remove aflatoxin in a single compartment, *in vitro* blood recirculation model, 2) investigate the potential ability of CytoSorbents' small and medium pore polymers to remove T-2 toxin by first evaluating the removal of a non-toxic surrogate with a similar chemical structure to T-2 toxin from whole blood and 3) examine the ability of CytoSorbents' medium pore polymer beads to remove a broad range of PAMPs and cytokines in a single compartment, *in vitro* blood recirculation model.

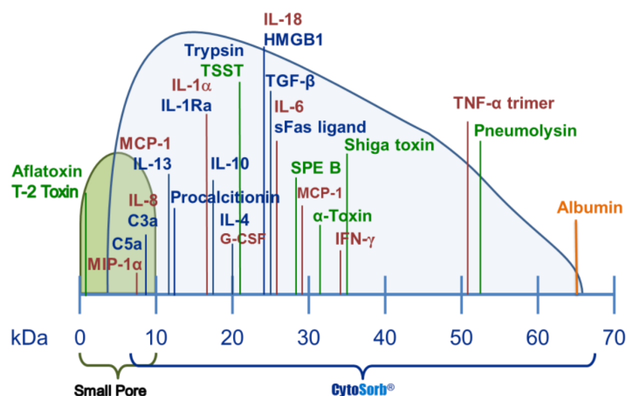

Figure 1: Polymer pore optimization strategy. Small pore polymers are optimized for removal of small toxins, such as mycotoxins, less than 10 kDa in molecular weight (green balloon). Medium pore polymers are optimized for removal of medium molecular weight range (~5-55 kDa) cytokines, DAMPS and PAMPS (blue balloon).

2 MATERIALS AND METHODS

All experiments were carried out in a single compartment, *in vitro,* citrated whole bovine blood recirculation model. Hemoperfusion devices containing CytoSorbents' polymers were scaled to size based on expected clinical conditions. For each experiment, a negative control was included in which blood was recirculated through an empty hemoperfusion device.

2.1 Aflatoxin Blood Recirculation

Aflatoxin B1 was added to 1L whole blood to achieve a starting concentration of 10 μg/mL. Citrated (3.8%) whole blood was recirculated at 140 mL/min through a hemoperfusion device packed with 70 mL CytoSorbents porous polymer beads for five hours. Plasma was analyzed using an enzyme-linked immunosorbent assay (ELISA) as per manufacturer instructions (Helica).

2.2 T-2 Toxin Surrogate Blood Recirculation

Epoxomicin was selected as a nontoxic surrogate molecule for T-2 toxin based on the similarity of the chemical structures. T-2 toxin has a molecular weight of 467 Da, while epoxomicin is 554 Da, and both molecules have epoxide group functionality. Epoxomicin starting concentration of 18μM was selected, based above the expected LD_{50} for T-2 toxin in pigs [16]. 150mL of citrated whole blood was recirculated through a hemoperfusion device packed with 10mL polymer at a flow rate of 20 mL/min for four hours. Epoxomicin concentration was quantified from plasma using a kinetic, fluorogenic enzyme-based assay via 20S Proteosome inhibition (Enzo Life Sciences). The assay sensitivity ranged from 9nM to 180nM, and post-hemoperfusion concentrations were extrapolated from the standard curve.

2.3 Bacterial PAMPs and Cytokine Blood Recirculation

Purified proteins, or toxins, were added to 265-300mL blood to achieve expected clinical concentrations. Blood was recirculated at 140 mL/min through a hemoperfusion device packed with 20mL CytoSorbents' medium pore polymer beads for five hours. Plasma for bacterial toxins was analyzed using ELISA reagents from Toxin Technologies and cytokines using R&D Systems Duosets as per manufacturer instructions.

3 RESULTS

The small pore polymers SP7 and SP9 exhibited highly efficient removal of aflatoxin reducing the toxin concentration in blood by more than 99% after 2 hours (Figure 2). In addition, small polymer SP9 and medium pore polymer MP1 beads reduced the concentration of epoxomicin, the T-2 toxin surrogate molecule by more than 97% after 2 hours (Figure 3). While specific removal of

mycotoxins is postulated to provide substantial therapeutic benefit, cytokine removal may also be required afterwards should a "cytokine storm" ensue following mycotoxin exposure. Therefore, the ability of CytoSorbents' polymer beads to remove cytokines and larger protein toxins from whole blood were also evaluated. Inflammatory cytokines MIP1-α, IFN-γ, IL-6, and TNF-α, as well as toxic bacterial proteins *Staph* toxic shock syndrome (TSST-1) and *S. aureus* α-toxin were analyzed in whole blood adsorption experiments. Levels of these inflammatory mediators and toxic proteins were greatly reduced after five hours of hemoperfusion through the device filled with CytoSorbents' medium pore polymer MP1 beads (Table 1). In negative control experiments conducted with a device without polymer beads (data not shown) reduction of each of the analytes was less than 20% by the five hour time point.

Protein, $[A_0]$	% Removal
Staph TSST-1, 2 μg/mL	97 ± 4.5
S. aureus α-toxin, 1.5 μg/mL	83 ± 3.2
MIP1-α, 400 pg/mL	98 ± 3.1
IFN-γ, 400 pg/mL	79 ± 13
IL-6, 3000 pg/mL	82 ± 14
TNF-α, 800 pg/mL	41 ± 10

Table 1: *In vitro* removal of bacterial PAMPs and cytokines using hemoperfusion devices packed with CytoSorbents' medium pore polymer MP1 after 5 hours.

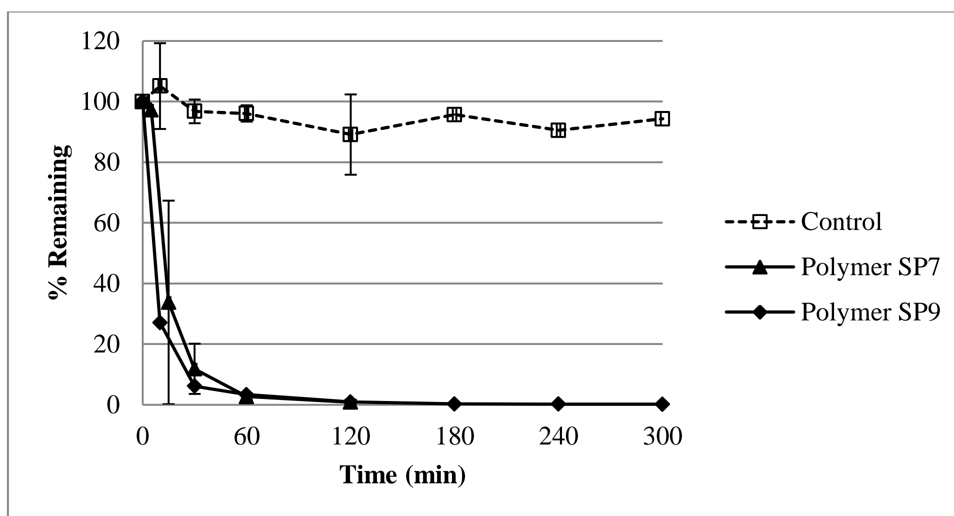

Figure 2: *In vitro* removal of aflatoxin B1using hemoperfusion devices packed with CytoSorbents' small pore polymers SP7 and SP9 or a control (no bead) device.

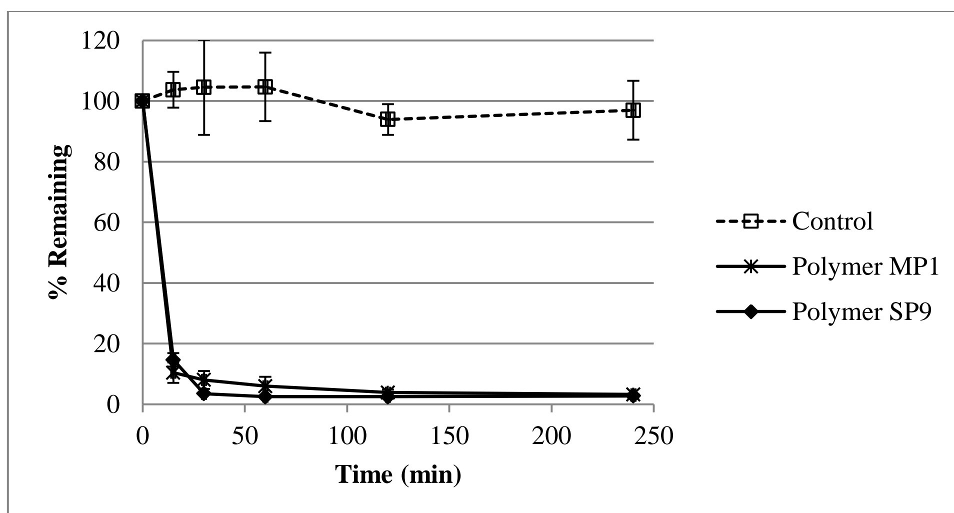

Figure 3: *In vitro* removal of epoxomicin (T-2 toxin surrogate) using hemoperfusion devices packed with CytoSorbents' small pore polymer SP9 and medium pore polymer MP1 or a control (no bead) device.

4 CONCLUSIONS

This study demonstrates CytoSorbents' hemoadsorbent polymer beads are capable of reducing clinically significant concentrations of mycotoxins and toxin surrogates from whole blood. The excellent capability of the adsorbent polymer beads to rapidly reduce aflatoxin and dangerous levels of pro-inflammatory cytokines supports the potential future novel clinical use of CytoSorbents polymer beads to treat deadly fungal infections, such as Aspergillosis, and reduce the risk of SIRS and MODS, when used with standard of care therapy. Potential also exists to use this hemoperfusion approach as a medical countermeasure for acute mycotoxicin exposure. *In vivo* studies to identify the optimal polymer material and treatment regimen for therapeutic efficacy are warranted.

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REFERENCES

[1] Richardson MD. 2005. *Journal of Antimicrobial Chemotherapy.* 56(Suppl. S1):i5-i11.

[2] Sales-Campos H, et al. 2013. "The Immune Interplay between the Host and the Pathogen in Aspergillus fumigatus Lung Infection." *BioMed Research International*. 2013:1-14.

[3] Turner NW, et al. 2009. "Analytical methods for determination of mycotoxins: A review." *Analytica Chimica Acta*. 632:168-180.

[4] Boonen J, et al. Human skin penetration of selected model mycotoxins. *Toxicology*, 2012. 301: 21-32.

[5] Park CW. 2016. "CBRNE – T-2 Mycotoxins." *Medscape.*

[6] "Biological Warfare." *Central Intelligence Agency*. Central Intelligence Agency, 23 Apr. 2007.

[7] "BBB - Aflatoxins." *FDA*. U.S. Food and Drug Administration, 5 Aug. 2013.

[8] Bianchi ME. 2007. "DAMPS, PAMPS and alarmins: all we need to know about danger." *J Leuk Biol.* 81:1-5.

[9] Reiter K, et al. 2002. "In vitro Removal of Therapeutic Drugs with a Novel Adsorbent System." *Blood Purif Blood Purification*, 20(4), 380-8.

[10] Kellum JA, et al. 2004. *Crit Care Med.* 32(3):801-5.

[11] Peng ZY, et al. 2008. *Crit Care Med.* 36(5):1573-7.

[12] Hinz B., et al. 2015. *Int J Artif Organs.* 38(8):461-4.

[13] Basu R, et al. 2014. *Indian J Crit Care* Med. 18(12):822-824.

[14] Hetz H, et al. 2014. *Int J Artif Organs.* 37(5):422-6.

[15] Mitzer SR, et al. 2013. *Blood Purif.* 35:314-315.

[16] Sun et al. 2012. *J of Animal and Veterinary Advances*. 11(12):1977-1981.