PROTECTIVE ROLE OF THE POLYSACCHARIDES FROM SEA CUCUMBER, ACAUDINA MOLPAADIOIDEA, IN CECAL LIGATION AND PUNCTURE-INDUCED SEPSIS

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ABSTRACT: Sea cucumber, possessing various active compounds, is a traditional food in Asia. Acaudina molpadioidea is a kind of sea cucumber widespread in Eastern Asia. Sepsis is the systemic inflammatory response to infection predominantly from gram-negative bacteria. Here, we investigated the effect of active compounds of sea cucumber (A. molpadioidea) on the sepsis. Our results found the polysaccharides, but not polypeptides, from sea cucumber improved the survival in CLP induced septic mice. After polysaccharides oral administration, the colony-forming units (CFU) were all decreased in liver, spleen, and blood samples of septic mice compared with controls. The pro-inflammatory factors, IL-1β and TNFα, were both down regulated in the plasma of polysaccharides fed mice. There were similar plasma levels of IL-10 in polysaccharides and saline fed mice. The peritoneal macrophages from polysaccharides fed mice exhibited stronger phagocytosis and bacterial killing capabilities than controls. This study provides a kind of new potential food to possibly improve sepsis-related mortality in human.

KEYWORDS: Bacterial Burden, Macrophages, Polysaccharides, Sea Cucumber, Sepsis

INTRODUCTION

Sea cucumber, soft-bodied worm-like echinoderms, is a traditional tonic food in China and other Asian countries. Acaudina molpadioidea is a kind of low value sea cucumber widespread in Eastern Asia. A peptide isolated from A. molpadioidea gelatin hydrolysate shows antihypertensive effect (Zhao et al., 2007). The polysaccharides of A. molpadioidea have been reported anticoagulant activities (Ye et al., 2012). The extracts from other sea cucumbers, such as Holothuria and Cucumaria genera have shown a variety of biological activities such as antifungal, anticancer, hemolytic, cytostatic, antioxidant and immuno-modulatory effects (Dang et al., 2007; Han et al., 2008; Mamelona et al., 2007; Zhang et al., 2006; Zhong et al., 2007). It is necessary to reveal the other biological activities of A. molpadioidea for converting it to highly valued economic products.

Sepsis is the systemic inflammatory response to infection that can be elicited by bacteria, mycobacteria, parasites, fungus, and viruses but predominantly by bacteria (Annane et al., 2005). It is a major and increasing cause of mortality around the world with an annual incidence of 2.4-3.0 cases per 1,000 in the population (Angus and Wax, 2000; Martin et al., 2003). Despite the advances in medicine, sepsis mortality rates are still increasing and survivors suffer poor quality of life (Winters et al., 2010). Sepsis is characterized by overexpression of inflammatory cytokines and inefficient bacterial clearance (Cohen, 2002). Macrophages play an important role in sepsis because of its pivotal functions in both cytokine production and bacterial clearance (Sica and Mantovani, 2012). Dietary nutritional supplementation has shown to protect rodents from sepsis (Shapiro et al., 2009; Volman, 2009), providing a potential prevention of sepsis.

In this study, body wall polysaccharides and peptides were isolated from the A. molpadioidea. We further investigated the effect of A. molpadioidea extracts oral administration on cecal ligation and puncture (CLP) induced sepsis. The A. molpadioidea polysaccharides improved the survival of septic mice. Moreover, the polysaccharides down-regulated pro-inflammatory cytokines in sepsis and enhanced phagocytosis and bacterial killing in peritoneal macrophages.
MATERIALS AND METHODS

Sample Collection And Animals

Sea cucumbers (A. molpadioides) were collected from the bottom layer of the near shore water of Zhejiang Province in China and the body wall was frozen at -70 °C.

Adult male ICR mice (6-8 weeks) were purchased from the Zhejiang Province Experimental Animal Center and bred in our animal room. Animals were maintained with their mothers on a 12 h light/dark cycle (lights on 07:00-19:00) at 22 ± 1°C, and had free access to food and water. The experimental conditions and procedures were approved by the Local Institutional Animal Care and Use Committee and were carried out in close agreement with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Polysaccharides Preparation

The sea cucumbers were cut into small pieces and digested 0.4% pepsin and 0.45% trypsin (Sangon, Shanghai, China) and polysaccharides were preparation as previously described (Zhang et al., 2010). Briefly, the digestion solution was mixed with three volumes of ethanol and stored at 4 °C overnight. The precipitate was collected by centrifugation, re-dissolved in deionised water. Then the Sevag method was used to remove protein components of the crude polysaccharides. The resolution was applied to a DEAE anion-exchange column (Bio-Rad), eluting at a flow rate of 1.0 ml/min successively with distilled water and a gradient of 0–2 M NaCl. The yielded fractions were combined, desalted, and lyophilized to generate a purified sea cucumber polysaccharide. The polysaccharides were quantified using the anthrone-sulfuric acid method (Laurentin and Edwards, 2003). A glucose standard curve was generated with glucan and used to calculate the sugar content.

Polypeptides Preparation

Polypeptides preparation was performed as previously described (Lu et al., 2010). Briefly, sea cumumbers were defatted with 10% isopropyl alcohol at a solid to solvent ratio of 1:10 (w/v) for 24 h. The matter was extracted with acetic acid for 3 days and digested with pepsin. The supernatant of viscous solution was collected to solvent ratio of 1:10 (w/v) for 24 h. The matter was defatted with 10% isopropyl alcohol at a solid to solvent ratio of 1:10 (w/v) for 24 h. The matter was extracted with acetic acid for 3 days and digested with pepsin. The supernatant of viscous solution was collected to serum-starved for 8 h before treatment in all experiments.

Phagocytosis And Bacterial Killing

E. coli strain (DH5α) was labeled with FITC (Amresco, Cleveland, OH, USA) according to Saresella et al. (Saresella et al., 1997) (here after designated as E. coli-FITC). For phagocytosis, the bacteria were opsonized with sera obtained from syngeneic mice at 37°C for 30 min. In each sample, 1 × 10⁶ macrophages were cocultured with E. coli-FITC at 37°C for 1 h at an MOI of 10. The plates were covered in a dark container on ice, and cells were washed extensively with PBS to remove extracellular E. coli-FITC. Then, the fluorescence that was outside of the cells or sticking to the surface of the cells was quenched by trypan blue. The uptake of the bacteria into cells was determined by trypan blue.
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captured by a microscope and quantified by measuring fluorescence intensity using Image software (US National Institutes of Health).

For bacterial killing assay, the extracellular bacteria were removed by two washes with PBS containing 12.5 μg/ml gentamicin (Invitrogen) after incubation with *E. coli* for 30 min. One set of samples (uptake group) was lysed in 0.1% Triton X-100 solution and plated onto LB plates for colony counting to provide bacterial uptake values. The remaining set (kill group) was incubated for additional 2 h at 37 °C to allow bacterial killing to occur, lysed, and then plated onto LB plates. Surviving bacteria were grown for 18 h and subsequently the colony-forming units (CFU) were measured, and bacterial survival was determined by dividing the number of colonies in the kill group by those in the uptake group.

**Statistical Analysis**

Results are presented as mean ± SEM. The Kaplan-Meier methods were used to analyze mortality with SPSS (Version 13.0, Chicago, IL, USA). Other data were analyzed by one-way ANOVA. In all cases, *P* < 0.05 was considered statistically significant.

**RESULTS**

**The Effect Of Polysaccharides And Polypeptides On Survival After CLP Induction**

We first extracted polysaccharides and polypeptides from sea cucumbers. The yield rate of polysaccharides was 2.8%, and the yield rate of polypeptides was 11.9%. The gains of body weight were identical in mice intake of saline (SA), low concentration of polysaccharides (PS1), high concentration of polysaccharides (PS2), low concentration of polypeptides (PE1), and high concentration of polypeptides (PE2) (Fig. 1). After 30 days on test diets, animals received an CLP experiments were conducted in these mice. 24 h after CLP induction, mice clearly displayed the sepsis symptoms, such as decreased motor activities, ocular exudates, and ruffled fur. The postoperative deaths occur 3 days after CLP surgery. Mice were followed up for 8 d and monitored every 24 h for various signs of sickness. PS2 provided significant protection against CLP-induced lethality (*P* < 0.05). The overall survival rate of PS1 treated mice tended to be higher than that of SA treated mice after CLP. However, this difference did not reach statistical significance (*P* = 0.078). The survivals in PE1 and PE2 groups were similar with SA one (fig. 2).

**Oral Administration Of Polysaccharides Decreases Bacterial Burden After CLP Infection**

Following CLP induction, the host employs a broad immune defense strategy to eliminate invading microorganisms. Impaired immune responses can predispose the host to induce septic shock. We further examined the effects of PS2 oral administration on bacterial burden. Tissue homogenates were evaluated for bacterial CFU at 2 days after CLP surgery. Homogenates of liver and spleen in PS2 treated mice produced less CFU than samples from SA treated controls (Fig. 3). The CFU of blood samples in PS2 treated mice were 27% of controls (Fig. 3). In summary, these data indicate that PS2 treated mice have increased bacterial clearance, which could contribute to the increased survivals seen after CLP infection.

**Oral Administration Of Polysaccharides Alters Cytokine Levels In Septic Mice**

Plasma cytokines levels in mice orally administrated with PS2 were evaluated 24 h after CLP induction, including pro-inflammatory factors IL-1β and TNFα,
anti-inflammatory factor IL-10. As shown in Figure 4, PS2 treated mice shown an decrease in IL-1β and TNFα levels in relation to SA treated ones. The IL-10 levels appeared to be similar in PS2- and SA-fed mice.

**FIGURE 3. Enhanced bacterial clearance in mice treated with PS2.** Liver, spleen, and blood were excised aseptically. Liver, spleen, and blood homogenates were cultured on LB plates. CFU were normalized to volume (for blood) and tissue weight (for liver and spleen). Data represent the bacterial burden in tissue and blood. n = 5 mice/group. *P < 0.05, **P < 0.01, ***P < 0.001.

**FIGURE 4. Plasma cytokine levels in SA and PS2 treated mice after CLP induction.** Mice were euthanized 2 days following CLP induction. Blood was collected and cytokines measured by ELISA. n = 4 mice/group, *P < 0.05.

**FIGURE 5. The phagocytosis and bacterial killing of peritoneal macrophages in SA and PS2 treated mice.** Mice were orally administrated with SA and PS2 for 30 days and the peritoneal macrophages were isolated. A) For phagocytosis, macrophages were incubated with E. coli-FITC at an moi of 10. B) For bacterial killing, the cells were lysed, and then plated onto LB after incubation with E. coli. *P < 0.05.

**Phagocytosis And Bacterial Killing Of Peritoneal Macrophages From PS2- And SA-fed Mice**

Phagocytosis by macrophages is the key component of the host defense against bacterial infections during sepsis. We next measured the phagocytic activity of peritoneal macrophages from PS2 treated mice to elucidate the effect of PS2 on phagocytic ability of macrophages. Under the microscope, E. coli-FITC was monitored as the indices of macrophage activation. Total fluorescence intensity of E. coli-FITC taken into the cells was quantified as phagocytic activity. As shown in Figure 5A, control cells had moderate activity to take up E. coli-FITC, while PS2 treatment led to a dose-dependent increase of the phagocytosis of E. coli-FITC. Previous results had shown that PS2 treated mice have increased bacterial clearance, we hypothesized that the macrophages from PS2 treated mice may be more efficient at bacterial killing. The direct measurement of intracellular CFU showed that survival of bacteria in PS2 group was less than control (Fig. 5B). These results strongly suggest...
that PS2 can enhance the macrophage functions, which may contribute to higher survival in septic mice.

DISCUSSION

This study shows for the first time that the polysaccharides from sea cucumber improved survival in septic mice and that the effect of polysaccharides may result from modulation of macrophage functions to reduce tissue bacterial burden and cytokine secretion.

The severity of sepsis can be influenced by the nutritional status of humans and animals. In recent years, more and more polysaccharides have been investigated and reported to exhibit a variety of biological activities, including anti-tumor (Jiao et al., 2009), anti-oxidation (Li et al., 2003) and anti-apoptosis (Hu et al., 2010). Administration of polysaccharides has also shown immunomodulation effect on mammals in vivo. The NK cells are activated in a dose-dependent manner after orally administrated with polysaccharides from rice bran (Kim et al., 2007). Administration of polysaccharides derived from the Acai berry before or after infection enhances mice survival against type A F. tularensis (Skyberg et al., 2012). Modulation of the macrophage function by polysaccharides has been shown to increase phagocytosis, microbicidal activity, chemotaxis, and antigen presentation to T cells, thus helping in preventive and therapeutic strategies against diseases (Desai et al., 2007). In the present study, the beneficial effects of consuming polysaccharides from sea cucumber on sepsis were observed, supporting the conception that natural polysaccharides possess broad biological activities.

TNFα, a important early mediator in the innate host inflammatory response, attributes a critical role in sepsis (Lorente and Marshall, 2005). IL-1β may inflict tissue injury and contribute to multiple organ dysfunction and cell death during sepsis (Jean-Baptiste, 2007). In contrast, sepsis also induced anti-inflammatory pathways to releases of anti-inflammatory cytokines, including IL-10, that serve as counter-regulatory mechanisms to dampen the inflammatory response. What must be emphasized, however, is that, the development of septic shock result from the predominant state of pro-inflammatory pathways (Liu and Malik, 2006). The results in this study clearly reveal that mice orally administrated with polysaccharides from sea cucumber noticeably decreased the production of pro-inflammatory cytokines, TNFα and IL-1β in septic mice. Our results also show that the dietary uptake of the polysaccharides enhance bacterial clearance in liver, spleen and blood. The ability of the polysaccharides to reduce pro-inflammatory production in inflammatory conditions may come from the enhancement of tissue bacterial clearance. Sepsis is characterized by overexpression of inflammatory cytokines and inefficient bacterial clearance. Macrophage function is pivotal to the both two aspects during sepsis. Our results show the abilities of phagocytosis and bacterial killing are enhanced in the macrophages of polysaccharides fed mice compared with controls, partly explaining the reason for the enhancement of tissue bacterial burden.

Analysis of these data leads us to speculate that the beneficial effect of dietary polysaccharides from sea cucumber (A. molpadioidea) against sepsis may be associated with the enhancement of macrophage functions. It is a kind of potential functional food to improve survival in sepsis.

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CONFLICT-OF-INTEREST STATEMENT

We declare that we have no conflict of interest.

REFERENCES


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