**Using Allium sativum (Garlic) as an Inhibitor of HMGB1 Stimulated Inflammation in RAW 264.7 Cells**

**Abstract:**

Sepsis is a serious global, public health problem that causes 225,000 deaths annually in the United States, and there are currently no vaccines or drugs to treat sepsis. The pathogenesis of sepsis involves a hyper-response by the body to an infection of the blood. This hyper-response is caused by the excessive release of pro-inflammatory cytokines. A relatively new anti-inflammatory cytokine named HMGB1 has been a target for the treatment of sepsis since; its release coincides with the death of septic patients. Garlic extract has been proven in multiple studies to be able to reduce LPS stimulated inflammation. For this reason, garlic extract was chose as a possible inhibitor of HMGB1. Garlic extract, trypsinized garlic extract (only reduced TNF), and boiled garlic extract successfully inhibited HMGB1 inflammation by reducing the levels of TNF and IL-10. Also, garlic extract and boiled garlic extract increased the levels of IL-6 and IL-1β. Additionally, Garlic extract, trypsinized garlic extract, and boiled garlic extract inhibited NF-kB activation at NLS p50. Furthermore, the anti-inflammatory molecule/molecules in the garlic extract were heat stable and not proteins. These results demonstrate that garlic extract inhibits HMGB1 inflammation and therefore could be tested as a novel treatment for sepsis.

**Introduction**

In April 2012, Rory Staunton, a sixth grader from Queens, suffered a minor cut while playing basketball. Nobody, not even the doctors at NYU Langone Medical Center expected what happened next; Rory was taken to the emergency room after experiencing severe discomfort in his abdominal region. He was sent home after being diagnosed with a stomachache. Days later Rory died. He died from the complications of sepsis (Dwyer, 2012). The death of Rory Staunton galvanized nationwide awareness of sepsis and the effort to stop sepsis, as Rory’s case is not an isolated incidence. About 750,000 people in the U.S. each year get sepsis, and about 225,000 of them die from it (Wang et al., 2004). The condition is an infection of the bloodstream, and it can arise from a number of infectious bugs that attack the body, such as meningitis, pneumonia and infections of the skin or bladder, to name a few. The blood poisoning is caused not by the germs themselves, but by the body's hyper-response (to release excessive amounts of pro-inflammatory cytokines) to those germs, when it releases a barrage of chemicals that can lead to organ failure (Mayo Clinic, 2014). The lethality of sepsis is apparent considering it is the third leading cause of death in the ICU (Wang et al., 2004). Unfortunately, even with the best antibiotics and supportive care, a third of these patients will die and no current drugs are able to treat the infection.

 Aimed to find a treatment for this deadly syndrome sepsis, in recent years, research involving the inhibition of pro-inflammatory cytokines to treat sepsis has been conducted. Cytokines are small molecules that allow signaling between cells, their interactions control chemotaxis, cellular growth, and cytotoxicity (Heath, 2002). Additionally, cytokines regulate the immune system and the body’s inflammatory response (Heath, 2002). This makes them a target for treatment of inflammatory diseases. The cytokines predominantly targeted in sepsis studies are tumor necrosis factor (TNF) and interleukin 1 (IL-1). Inhibition of TNF and IL-1 treatment was shown to be very successful in improving sepsis survival rates in animal models. Despite success in animal models, clinical trials failed to improve survival rates in septic patients (Wang, 1999). The reason for this is the early release of TNF and IL-1 during sepsis; TNF and IL-1 are released at peak levels 1 to 2 hours into sepsis (Wang et al., 2001). This early release creates a very small therapeutic window and does not address downstream cytokines that cause death. A late mediator of sepsis has been discovered and identified, high mobility group box 1 (HMGB1) protein. HMGB1 was originally thought to be a nuclear DNA-binding protein. It was believed that HMGB1 functioned only as a cofactor in transcription regulation, but it was later discovered that HMGB1 has many different roles including being an intercellular messenger and a pro-inflammatory cytokine when released extracellularly. HMGB1 release reaches its peak at about 20 hours after the infection (Wang et al., 1999) and it was also discovered that HMGB1 release coincides with death in sepsis (Yang et al., 2013). In recent years it has been shown that anti-HMGB1 treatment has been successful in mice models of sepsis, studies have shown treating for HMGB1 significantly increases survival rates in mice suffering from sepsis induced by bacterial toxin LPS (Wang et al., 1999). These fore mentioned facts make HMGB1 a very intriguing therapeutic target, since treating for HMGB1 would create a substantially larger window for the treatment of sepsis and theoretically eliminate the mediator of death in sepsis. For these reasons, my project aimed at finding a better treatment for sepsis and using Allium sativum (Garlic) as a possible natural inhibitor of HMGB1.

 Garlic is a known anti-microbial and anti-inflammatory substance (Keiss et al., 2003). Garlic also has many active compounds including allicin and thiacremone. Thiacremone is a sulfur based compound that has been proven to inhibit nuclear factor-kB (NF-kB) activity when RAW 264.7 cells were stimulated with lipopolysaccharide (LPS), an exogenous toxin from the cell wall of bacteria (Ok Ban et al., 2009). In other studies garlic has also been proven to significantly reduce production of TNF, IL-1α, interleukin 8 (IL-8), interferon-gamma (IFN-γ) and interleukin 2 (IL-2) in peripheral blood mononuclear cells (PBMC’s) when stimulated with LPS (Hodge et al., 2002). Garlic’s proven inhibition of LPS stimulated NF-kB activity and cytokines, has led me to hypothesize that garlic could possibly also inhibit HMGB1, leading to improved treatment for sepsis.

**Materials and Methods**

**Materials**

LPS (E.coli. 0111:B4) and toluene (99.8%) were purchased from Sigma (St. Louis, MO, USA). Trypsin-EDTA (0.5%), Dulbecco's Modified Eagle Medium (DMEM, Fetal Bovine Serum (FBS), Penicillin-Streptomycin (P/S), and Opti-MEM (OP) were purchased from Gibco by Life Technologies (Grand Island, NY, USA). Raw 264.7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Garlic was purchased at Waldbaums (Island Park, NY, USA). Mouse TNF enzyme-linked immunosorbent assay (ELISA) kits were purchased from eBioscience (San Diego, CA, USA). Mouse IL-6, IL-10, and IL-1β ELISA kits were purchased from R&D Systems (Pittsburgh, PA, USA). NF-kB ELISA kit and Nuclear Extract Kit were purchased from Active Motif (Carlsbad, CA, USA). Bio-Rad Protein Assay Dye Reagent was purchased from Bio-Rad Laboratories Ltd (Hemel Hempstead, UK). HMGB1 was made in the lab according to standard protocol (Li J et al., 2004).

**Cell Culture and Treatment**

 Murine macrophage-like RAW 264.7 cells were cultured in DMEM, FBS and P/S. Cells were cultured in sterile 96 and 6 well plates purchased from Fisher Scientific (Waltham, MA, USA).

**Cytokine Measurements**

 TNF, IL-6, IL-10, IL-1 β released in the supernatants of RAW 264.7 cell cultures were measured using commercially available ELISA kits according to the instructions of the Manufacturer (eBioscience and R&D Systems).

**Production of Nuclear Extract**

 RAW 264.7 cells cultured in a six well plate were aspirated of their medium and frozen overnight. The next morning, 3 milliliters (mLs) of cold PBS/Phosphatase inhibitor was added to each well. The cells were removed from their wells using a cell scrapper. Once removed the cells were transferred to pre-chilled 15 mL conical tubes. The cells were then centrifuged for 5 minutes at 200 x g at 4 degrees Celsius. Next, the supernatant was discarded and the cell pellet was kept on ice. The pellet was then gently resuspended in 500 microliters 1x hypotonic buffer. The cells were transferred from the 15 mL conical tube to pre-chilled mircocentifurge tubes. The cells incubated on ice for 15 minutes. Next, 25 microliters of detergent were added to the cells and the cells were vortexed at the highest setting for 10 seconds. The cells were then centrifuged for 30 seconds at 14,000 x g at 4 degrees Celsius. The supernatant (cytoplasmic fraction) was transferred to pre-chilled mircocentifurge tubes and stored at -80 degrees Celsius. The pellet was then resuspended in 50 microliters of complete Lysis Buffer and vortexed for 10 seconds at the highest setting. The resuspended pellet incubated for 30 minutes on a rocker set to 150 RPM’s on ice. After incubation the resuspended pellet was vortexed for 30 seconds at the highest setting. The resuspended pellet was then centrifuged for 10 minutes at 14,000 x g at 4 degrees Celsius. The supernatant (nuclear fraction) was transferred to a pre-chilled mircocentifurge tube and stored at -80 Celsius till use. The remaining pellet and regents were discarded.

**NF-kB Measurements**

NF-kB in the nuclear extract was measured by a commercially available ELISA kit according to the instructions of the manufacturer (Active Motif, Carlsbad, CA, USA).

**General Protein Measurements**

Protein levels in the cell wells were measured using a commercially available Protein Assay Dye Regent according to the instructions of the manufacturer (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK).

**Garlic Extraction**

 I chopped 10 grams (g) of fresh garlic and mixed with 20 mL’s of toluene. Then, I incubated the mixture overnight on a rocker. Next, I filtered the mixture through Whatman no.1 filter paper. I added 10 mL’s of sterile water to the remaining mixture. Then, I stirred the mixture at room temperature for 24 hours. Next, I separated the aqueous phase from the organic phase of the mixture. Finally, I sterile filtered the aqueous phase of the mixture and made aliquots. Store at -20 degrees Celsius (Rasmussen et al., 2004).

**Preparation of Boiled Garlic Extract**

 Thaw an aliquot of garlic extract. Boil for 10 minutes at highest setting possible. Allow for the boiled garlic extract to cool and centrifuge it quickly at 8,000 RPM.

**Preparation of Trypsinized Garlic Extract**

 Thaw an aliquot of garlic extract. Add 1.0% trypsin-EDTA (0.5%) to the aliquot. Incubate overnight.

**Statistical Analysis**

Data is presented as means +SEM.Differences between groups were determined by a Two Tailed Student t test. P values less than 0.05 were considered significant.

**Results**

**TNF Release by RAW 264.7 Cells is Inhibited by Garlic Extract, Boiled Garlic Extract and Trypsinized Garlic Extract**

 TNF release was stimulated using HMGB1 or LPS. LPS stimulated TNF release acted as a control, since previous studies showed that garlic extract inhibited LPS stimulated TNF release (Hodge et al., 2002). Like other studies LPS stimulated TNF release was inhibited by garlic extract and HMGB1 stimulated TNF release was also inhibited by the garlic extract (Figure 1).



It was found that HMGB1 stimulated TNF release significantly decreased at ≥ .5 ug/mL garlic extract in dose dependent manner. The dose dependence of the garlic extract is promising, since it implies the garlic extract is inhibiting TNF release through a mechanism.

 The inhibitory effects of boiled and trypsinized garlic on HGMB1 and LPS stimulated TNF release were also tested. The purpose of boiling and adding trypsin was to see if the garlic extract would lose its anti-inflammatory properties when the proteins in the extract were denatured. It was discovered that the boiled and trypsinized garlic extract still inhibited HMGB1 and LPS stimulated TNF release (Figure 2).



Boiled garlic extract significantly inhibited HMGB1 and LPS stimulated TNF release at concentrations ≥ 0.1 ug/mL of boiled garlic extract. It was also found that at ≥ 0.5 ug/mL of boiled garlic extract inhibited TNF release below baseline levels. Trypsinized garlic extract also significantly inhibited HMGB1 and LPS stimulated TNF release at concentrations ≥ 0.5 ug/mL of trypsinized garlic extract.

**IL-1β Release by RAW 264.7 Cells is Increased by Garlic Extract**

 IL-1β release was stimulated using HMGB1 and LPS. It was found that HMGB1 did not stimulate IL-1β (Data not shown). In previous works, LPS stimulated IL-1β release was inhibited by garlic extract (Keiss et al., 2003). In my study it was found that garlic extract does not inhibit IL-1β, but it increases significantly at concentrations ≥ 1.0 ug/mL of garlic extract (Figure 3).



**IL-1β Release by RAW 264.7 Cells is not Affected by Boiled Garlic Extract**

In this experiment IL-1β release was stimulated using HMGB1 and LPS. As in the previous experiment HMGB1 did not stimulate IL-1β release. It was found that boiled garlic extract had no effect on LPS stimulated IL-1β release at any concentrations of boiled garlic (Figure 4).

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**IL-6 Release by RAW 264.7 Cells is Increased by Garlic Extract and Boiled Garlic Extract**

 In previous studies, LPS stimulated IL-6 released was inhibited by garlic extract (Hodge et al., 2002). In my experiment HMGB1 and LPS were used to stimulate IL-6 release in RAW 264.7 cells. Like IL-1β, IL-6 was not stimulated by HMGB1, but when RAW 264.7 cells were stimulated with LPS and treated with garlic extract or boiled garlic extract IL-6 release increased (Figure 5).



It was discovered that garlic extract causes a significant increase in LPS stimulated IL-6 release at concentrations ≥ 0.5 ug/mL garlic extract. It was also found that boiled garlic extract causes a significant increase in LPS stimulated IL-6 release at 0.1 ug/mL boiled garlic extract and 1.0 ug/mL boiled garlic extract.

**IL-10 Release by RAW 264.7 Cells is Inhibited by Garlic Extract and Boiled Garlic Extract**

In previous experiments, LPS stimulated IL-10 levels were found to significantly decrease when BALB/c mice were treated with garlic extract (Ghazanfari et al., 2000). Furthermore, other studies showed that LPS stimulated IL-10 in peripheral blood mononuclear cells (PBMC’s) increased when they were treated with garlic extract (Hodge et al., 2002). In my experiment RAW 264.7 cells were stimulated with HMGB1 and LPS. It was found that treatment of the cells with garlic extract and boiled garlic extract inhibited both HMGB1 and LPS stimulated IL-10 release (Figure 6).



The inhibition of LPS stimulated IL-10 release for both garlic extract and boiled garlic extract was significant at ≥ 0.1 ug/mL. Moreover, inhibition of HMGB1 stimulated IL-10 release for garlic extract was significant at ≥ 0.1 ug/mL. Lastly, HMGB1 stimulated IL-10 release was significantly inhibited by boiled garlic extract at ≥ 0.5 ug/mL.

**Inhibition of NF-kB Activity in RAW 264.7 cells using Garlic Extract, Boiled Garlic Extract and Trypsinized Garlic Extract**

Previous studies have proven that NF-kb activity is inhibited by garlic extract (Ok Ban et al., 2009). NF-kB is a very important transcription factor in inflammatory diseases since; it regulates many pro-inflammatory cytokines including TNF, IL-1, IL-6 and IL-12 (Oeckinghaus et al., 2009). Inhibition of NF-kB would be very significant due to the fact, that various key cytokines would be inhibited. In my study RAW 264.7 cells were stimulated with HMGB1 and various types of garlic including garlic extract, boiled garlic extract, and trypsinized garlic extract. After stimulating, the nuclear content of the cells was extracted and tested for active NF-kB expression at nuclear localization sequences (NLS) p50. It was found that HMGB1 stimulated NF-kB activity was significantly inhibited by garlic extract, boiled garlic extract, and trypsinized garlic extract (Figure 7).



All types of garlic extract significantly inhibited NF-kB activity at 1.0 ug/mL. In addition, all types of garlic extract inhibited NF-kB activity about 50%; this inhibition is identical to TNF inhibition at 1.0 ug/mL of garlic extract. This correlation further proves that garlic extract inhibits cytokine release through a biological mechanism.

**Discussion**

The addition of garlic extract to RAW 264.7 cells reduced HMGB1 and LPS-induced TNF release and caused a variety of significant changes in cytokines and NF-kB levels, revealing a potential therapeutic use in sepsis.

 Various concentrations of boiled garlic extract and garlic extract increased LPS stimulated IL-6 and IL-1β levels in RAW 264.7 cells. The IL-6 results suggest that IL-6 is working through classic signaling, giving it anti-inflammatory properties, during this experiment. The difference between the two types of signaling is that classical signaling leads to an anti-inflammatory response; trans-signaling leads to a pro-inflammatory immune response (Rose-John, 2012). It is found that IL-10 down regulates IL-6 and IL-1β (Platzer et al., 1994).These relationships were confirmed in my results since, as IL-10 decreases IL-6 and IL-1β levels increase.

 Additionally, multiple concentrations of boiled garlic extract and garlic extract decreased HMGB1 and LPS stimulated IL-10 levels. It has been proven that TNF production up-regulates IL-10 levels (Platzer et al., 1994). This previously described relationship was confirmed in my results since; as TNF levels decrease IL-10 levels also decrease.

 NF-kB is the key pro-inflammatory transcription factor since; it creates TNF, IL-1, IL-6 and IL-12. NF-kB attaches to various nuclear localization sequences (NLS). The most common NLS for NF-kB is p50 (Oeckinghaus et al., 2009). For this reason, NF-kB levels were measured at the NLS p50. It was found that garlic extract, boiled garlic extract, and trypsinized garlic extract inhibited NF-kB activity at NLS p50 by 50%. This inhibition is equal to garlic extract’s inhibition of TNF levels, which was also 50%.

 From the results that were gathered I determined that the inhibitory molecule/molecules are heat-stable and not a protein. The anti-inflammatory molecule/molecules in the garlic extract were proven to be heat stable, since the garlic extract did not lose its effect when boiled. In fact, the effect of the garlic extract was amplified when it was boiled. This increased effect can be seen in the TNF release, since boiled garlic extract at 1.0 ug/mL return TNF levels to below the baseline; compared to the 50% inhibition seen with normal garlic extract. This is an extremely significant increase in inhibition. Moreover, it can be seen that the anti-inflammatory molecule/molecules in garlic extract are not proteins. The reason for this is that trypsin, a protease, rendered the proteins in the garlic extract inactive. In addition, proteins denature at high temperatures, so the boiled garlic extract results show that the anti-inflammatory molecule/molecules were not protein since the boiled garlic extract retained its anti-inflammatory affect. It could be concluded that the anti-inflammatory molecule/molecules in garlic extract could be an antagonist of Toll-like receptor 4 (TLR4). The reasoning behind this is that it is known that TLR4 binds with both HMGB1 and LPS (Wang, 1999); subsequently TLR4 also activates NF-kB (Hoshino et al., 1999). These functions and mechanisms of TLR4 make a likely candidate of inhibition for the anti-inflammatory molecule/molecules in garlic extract.

**Conclusion**

 Garlic extract was proven to act as an effective inhibitor of HMGB1. Garlic extract, boiled garlic extract, and trypsinized garlic extract were able to reduce HMGB1 and LPS stimulated cytokine release (TNF release, IL-10 release) and NF-kB activity (as measured by p50 expression). Also, garlic extract and boiled garlic extract increased IL-1β and IL-6 release. Additionally, my findings showed that the anti-inflammatory molecule/molecules in the garlic extract were heat stable and not proteins. This is evident since the boiled and trypsinized garlic extract both maintained their anti-inflammatory properties.

 These results fulfill the aim of finding a better treatment for sepsis. The overall results are encouraging and prove that garlic extract has potent anti-HMGB1 effects, which have been proven successful in previous studies (Wang et al., 2004). Moreover, garlic extract was also proven to be non-toxic using a general protein assay (results not show). This lack of toxicity and garlic extract’s potent effects provide hope that garlic extract could be used as an effective inhibitor of HMGB1 in animal models and it would lack the adverse side effects of a synthesized drug. If taken with antibiotics, garlic extract could have therapeutic potential for the treatment of sepsis and my ultimate aim is to save the lives of people like Rory.

**Future Research**

The results of this project created many new directions for further research. First, the effects of dithiothreitol (DTT) on garlic extract could be tested. This would reveal whether or not the anti-inflammatory molecules in garlic could be reduced. Next, the garlic extract could be broken down into different molecules using chromatography, and then each individual molecule’s anti-inflammatory effect would be tested. This would reveal which molecules are responsible for the anti-inflammatory effect of garlic extract. Lastly, the effect of garlic extract on the survival rate of septic mice could be tested. This would show if the garlic extract could be metabolized by an organism’s body and be effective in preventing experimental induced sepsis in mice.

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