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Effectiveness of Stabilized Hypochlorous Acid in Acute Peritonitis Treatment: A Murine Surgical Study

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EFFECTIVENESS OF STABILIZED HYPOCHLOROUS ACID IN ACUTE PERITONITIS TREATMENT: A MURINE SURGICAL STUDY

A Master's Thesis

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In Partial Fulfillment

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Master of Science, Cell and Molecular Biology

By

Benjamin Michael Pomeroy

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ABSTRACT

Peritonitis is an inflammatory condition affecting the mesothelial cells that line the peritoneal cavity and is commonly induced by bowel perforations. This medical emergency is treated through antibiotic therapy and surgical intervention followed by tissue irrigation (lavage). Acute treatments aim to remove the bacterial burden, however recurring peritoneal infections occur at high rates and contribute to patient morbidity. These recurring infections are likely due to the inability of lavage solutions to remove the entire massive intra-abdominal bacterial load due to intestinal perforation. Numerous antiseptic solutions and antibiotic additives have been evaluated in their ability to improve source control by abdominal lavage, without apparent benefit when compared to sterile saline washout. Saline is a common surgical lavage solution; however, it does not possess antimicrobial properties. Consequently, stabilized hypochlorous acid (HOCl) has been proposed as a safer and more effective irrigation solution with hopes of reducing recurrent infections and improving survival rates. In this study, we compared the efficacy of stabilized HOCl and common irrigation solutions of saline and chlorhexidine gluconate (CHX) on the acute treatment of induced peritonitis. Adult Sprague Dawley rats underwent laparotomy surgeries where peritonitis was induced through cecal ligation and puncture (CLP). Acute peritonitis was treated with resection of damaged tissue and peritoneal irrigation with either stabilized HOCl, saline, or CHX. Treatment efficacy was evaluated through the assessment of gross pathological investigation of peritoneal adhesions, histological analysis of pulmonary tissue, bacterial cultures of peritoneal fluid, analysis of pro-inflammatory TNFα levels, and survival rates. Stabilized HOCl, compared to saline, was found to provide no benefit for improving survival rates and decreasing pulmonary tissue injury; however, survival rates were improved when compared to CHX irrigation. Additionally, stabilized HOCl resulted in higher peritoneal bacterial contamination compared to saline and CHX, 24 hours post-irrigation. Stabilized HOCl also showed no detrimental peritoneal adhesion formation. Across all treatment groups, no serum TNFα was detected, 24 hours post-irrigation. This data suggests that normal saline remains the preferred lavage solution regarding peritonitis source control and further research is warranted to improve peritoneal lavage solutions.

KEYWORDS: peritonitis, sepsis, stabilized hypochlorous acid, chlorhexidine, saline

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INTRODUCTION

Bacterial peritonitis is defined as pathogen-induced inflammation of peritoneal cavity mesothelial cells. Most peritoneal infections are caused by perforations of the gastrointestinal (GI) tract due to tissue ulceration or trauma, resulting in bacterial contamination of the peritoneal cavity [1]. Peritonitis treatment relies on source control which is defined by any means taken to eliminate the infectious source, reduce bacterial contamination, and restore physiologic function. Source control requires laparotomy (abdominal surgical exploration), abdominal lavage (extensive irrigation of the peritoneal cavity for washout), and broad-spectrum intravenous antibiotics [1]. These source control measures are well established; however, post-operative infections and inflammation are common and negatively affect patient prognosis [1-3]. Sustained bacterial contamination within the peritoneal cavity often results in progression to a systemic inflammatory event known as sepsis. In a recent study by Riché et al., frequency of sepsis following peritonitis was 45%, with an overall mortality of 19% [4]. This prognosis may be due to lack of adequate source control to eliminate bacterial presence resulting in extensive intra-abdominal tissue injury [1]. Initial indication of peritonitis-induced sepsis is the onset of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) due to the fragile nature of pulmonary tissues and might be an indicator of peritonitis treatment efficacy. One approach to improving post-operative prognosis may be the improvement of lavage solutions. Sterile normal saline has long been preferred as the standard of care for abdominal lavage of peritonitis. Saline, however, does not possess any antiseptic properties and merely serves to wash away bacterial contamination. The addition of numerous antibiotics and antiseptics to abdominal lavage has shown some benefit for improving source control vs normal saline [5]. However, increased

antibiotic resistance and its effect on impairing wound healing is concerning [5]. Chlorhexidine gluconate (CHX), an antiseptic currently used for wound irrigation, has been researched for its use in the peritoneal cavity. However, its reported cytotoxic nature indicates that a safer alternative is warranted. Alternatively, hypochlorous acid (HOCl), an antimicrobial biomolecule produced naturally by mammalian immune cells in response to bacterial infection, may be the answer to improving source control. While evidence supports its use as a dermal wound cleanser, limited evidence supports its use as an intra-abdominal lavage which warrants further study. One previous drawback to using HOCl has been its rapid destabilization following synthesis, rendering it ineffective shortly after production. The development of a stabilized hypochlorous acid solution resolves this problem and may provide the key to improving source control efficacy over currently used lavage solutions like saline and CHX.

LITERATURE REVIEW

Peritonitis

Depending on the underlying cause, bacterial peritonitis is categorized either as primary, secondary, or tertiary [1]. Primary bacterial peritonitis results from spontaneous infection arising without a known underlying intra-abdominal pathology and is less common than secondary or tertiary. This condition often occurs during infancy/early childhood, in cirrhotic patients with ascites who receive peritoneal dialysis, and immunocompromised patients [1,6,7]. Secondary bacterial peritonitis, also referred to as general peritonitis, is the most common form that occurs following gastrointestinal (GI) tract trauma, perforation, bowel necrosis, or surgical intervention [1,8]. Perforations are predominant sources of general peritonitis, often due to ruptured colonic diverticula or perforated peptic ulcer disease causing bacterial peritoneal contamination [1,7,8]. Tertiary bacterial peritonitis is characterized by persistent or recurrent infections by organisms of low intrinsic virulence or patients who are predisposed to immunosuppression. This usually follows operative attempts to treat secondary peritonitis and is almost exclusively associated with a systemic inflammatory response [1,8]. These 3 forms of peritonitis are sub-classified as diffuse or local, based on infection location within the abdomen. Local infection is described as walled-off or contained by adjacent organs. Diffuse infection is characterized as having spread to the entire peritoneal cavity and is often synonymous with generalized peritonitis [1]. Without medical intervention, this inflammation progresses to sepsis, a life-threatening condition resulting in multi-organ dysregulation and eventual death.

Given that secondary peritonitis is the predominant form of peritonitis, it will become the focus of this paper and is referred to as bacterial peritonitis. Bacterial peritonitis diagnosis

follows several symptoms and clinical signs: abdominal pain, nausea, vomiting, decreased intestinal sounds, abdominal rigidity, fever, and shock. In many cases, urgent laparotomy is planned without further workup. In equivocal cases, where time and patient stability allow, abdominal imaging may be rendered via x-ray, ultrasound, and computerized tomography (CT) with the latter being preferred by clinicians as CT is more comprehensive and increases diagnostic accuracy [1]. Following identification of infectious source, several treatments are administered; in addition to abdominal lavage for source control, intravenous antibiotics are administered urgently [1]. Bacterial cultures of infection sites were once deemed necessary to build a suitable antibiotic treatment to effectively target potential pathogens. Intra-operative bacterial cultures obtained from secondary peritoneal infections indicate *Escherichia coli* (*E. coli*) and *Bacteroides fragilis* (*B. fragilis*) as predominant pathogens [1,8,9]. Antibiotic additives to lavage solutions have minimal efficacy against intra-abdominal bacterial contamination; however, intravenous broad spectrum antibiotic therapies have shown to be an effective measure of source control. Antibiotic therapy is restricted to less than 7 days for generalized secondary peritonitis to minimize bacterial desensitization and resistance [1]. If source control is inadequate, chronic fever and leukocytosis suggests further medical intervention via an additional surgical laparotomy [1].

Sepsis

Sepsis is a life-threatening medical emergency, and if left untreated, can progress to septic shock and eventual organ failure and death. Sepsis occurs when the body's inflammatory response to infection leads to systemic organ injury and dysregulation [10]. Following a bowel perforation, pathogen-associated molecular patterns (PAMPs) found on bacterial membranes

bind to toll-like receptors (TLRs) on the surface of mesothelial cells, which line the abdominal cavity and organs [11]. Damaged host tissues also release damage associated molecular patterns (DAMPs), which are molecules released in response to cell injury and death; these molecules also capable of binding TLR's [11]. These signals activate intracellular secondary messaging cascades such as the NF-kB pathway (Figure 1), resulting in gene transcription and extracellular release of pro-inflammatory cytokines such as Tumor Necrosis factor alpha (TNFα), Interleukin-1 (IL-1), and Interleukin 6 (IL-6) [11,12]. Once produced, these proteins are delivered via exocytosis to the extracellular environment where they bind to neighboring mesothelial cells or innate monocytes to initiate the innate immune response [10].

These pro-inflammatory cytokines initiate the proliferation of monocytes, macrophage migration, and activation of neutrophils [10]. After bacterial recognition and endocytosis, neutrophils engulf bacteria to through the process of phagocytosis and kill bacteria utilizing a process called respiratory burst (oxidative burst) in which nicotinamide adenine dinucleotide phosphate (NADPH) produces superoxide anions (O_2) [13]. These superoxide anions can be utilized to create hydrogen peroxide (H_2O_2). H_2O_2 can be combined with available intracellular halides, such as chloride, to form reactive oxygen species which are key mediators of bacterial damage and death [13]. During the oxidative burst, intracellular myeloperoxidase (MPO) converts most phagosome H_2O_2 into HOCl (Figure 2), which exhibits potent antimicrobial properties [14]. HOCl acts through oxidation and chlorination as its primary innate defense against bacteria through interaction with microbial proteins and lipids [13-15]. Protein fragmentation, DNA synthesis inhibition, and membrane dysregulation from HOCl action are key events for the removal of bacteria [13-15]. Should the innate immune system fail to eliminate the bacterial burden, proinflammatory mediators in the local tissue migrate to the

circulatory system causing a systemic inflammatory response otherwise known as sepsis [1]. Sepsis is the result of elevated levels of circulating proinflammatory mediators such as TNFα and their widespread physiological effect on all organs. These cytokines have a profound effect on tissues, one of which being increased vascular permeability thus leading to widespread tissue edema [16]. This edema leads to infiltration of proinflammatory mediators such as TNFα into tissues and onset of inflammation distant from the original source of infection. Untreated, this inflammation results in widespread tissue dysregulation and eventual tissue death.

Pulmonary Tissue and Sepsis-Induced Acute Lung Injury

Pulmonary tissue is where gas exchange occurs between the atmosphere and the blood capillary network within the inter-alveolar septa. The terminal structure of the conducting airway is comprised of respiratory bronchioles and alveolar sacs, separated by inter-alveolar septa. These alveoli are lined with a continuous barrier of type-I and type-II pneumocytes, as well as dust cells (alveolar macrophages) [16]. Additionally, there is a layer of capillary endothelium and connective tissue within the inter-alveolar septa [16]. Type-I pneumocytes are cells that comprise 95% of the alveolar sac that permit gas exchange [16]. Type-II pneumocytes are secretory cells which produce surfactant, a thin and continuous film lining the alveoli, which serves to support alveolar integrity and optimize gas exchange [16]. Pulmonary dust cells are resident macrophages which act to clear contaminants from the alveolar sac [16]. Together, these cells provide an environment in which gas exchange may occur to provide homeostasis to bodily tissues.

The fragile nature of the respiratory membrane and its exposure to inflammatory mediators make acute lung injury one of the most common complications to sepsis. Acute lung

injury (ALI) is defined as an acute inflammatory event which disrupts the integrity of the pulmonary endothelial and epithelial barriers, with more severe, life-threatening forms referred to as acute respiratory distress syndrome (ARDS) [17,18]. Sepsis is the most common cause of acute lung injury, and 60% of patients recovering from laparotomy for abdominal sepsis will develop ALI or ARDS [18]. ALI and ARDS occur when a source of infection, distant to the lungs, creates a systemic inflammatory response, leading to lung tissue damage. Following the innate immune response to peritonitis, pro-inflammatory cytokine levels increase within the cardiovascular system, resulting in a systemic inflammatory response [1,7]. Pro-inflammatory cytokines, IL-1, IL-6, and TNFα bind to receptors on the cellular surfaces of pulmonary vascular endothelium [7]. This interaction, results in activation of intracellular pathways, such as NF-kB (Figure 1), resulting in increased gene transcription and subsequent release of additional IL-1, IL-6, and TNF cytokines [7,12]. Additionally, these activated pathways result in production and release of chemokines which are proteins expressed on cell surface membranes that mediate cellular interactions [19]. These molecules are responsible for the localization and transmigration of granulated immune cells known as polymorphonuclear leukocytes (PMNL's) from the circulatory system into surrounding tissues [19]. These PMNL's, also known as neutrophils, are essential to the innate immune response due to their bacterial phagocytic properties [20].

Another characteristic of sepsis-induced lung injury is the alteration of pulmonary tissue structure and function [21,22]. Circulating pro-inflammatory cytokines during sepsis increases the permeability of capillary endothelial cells [21]. These cytokines signal the endocytosis of cell adhesion molecules (CAM's) which decrease endothelial interaction, thus permitting increased fluid loss from vasculature into surrounding tissues, resulting in edema [21,22]. This infiltration of blood, plasma, cytokines, and white blood cells collect within the inter-alveolar septa,

decreasing perfusion rates of oxygen and carbon dioxide [11,22]. This event also creates increased interstitial pressure on the alveolar walls, ultimately decreasing the alveolar structural integrity [16,22]. A similar event occurs within the alveolar sac pneumocytes. Pro-inflammatory cytokines bind to receptors on pneumocytes, resulting in decreased pneumocyte CAM's [16,22]. This weakening of the alveolar sac integrity, coupled with the increase of interalveolar septa, results in destruction of the alveolar walls and subsequent pulmonary edema. Pulmonary edema results in the infiltration of plasma and red blood cells (RBC's) into the alveolar space further disrupting perfusion and ventilation needed for gas exchange [16]. This decrease in ventilation and perfusion disrupts CO_2 and O_2 exchange, thus propagating systemic tissue anoxia and dysregulation [16,22].

Immediately following acute lung injury, a large influx of PMNL's within the pulmonary tissue is commonly seen [16,22]. A key role of neutrophil activity is their ability to fight pathogens; however, neutrophils are also capable of recruiting additional neutrophils to sites of inflammation [23]. As a result, large accumulations of PMNL's within tissues is a well-known characteristic of sepsis-induced lung injury [24]. While neutrophil activity is a key component to fighting bacterial infections, an overabundance of neutrophil activity can be damaging to host tissues [24]. In a study by Jimenez et. al, circulating neutrophil activity was evaluated in patients suffering from systemic inflammatory response syndrome (SIRS), an excessive host response to infection [24]. This finding, coupled with the work of Sookhai et al. suggest that given the cytotoxic nature of neutrophils, an overabundance of HOCl produced by excess neutrophil presence may contribute to excessive tissue damage in patients suffering from sepsis [24,25].

Abdominal Adhesions

One complication following abdominal surgery is an increased susceptibility to intra-abdominal adhesions. Abdominal adhesion formation occurs when two damaged peritoneal surfaces are opposed [1,26]. Surgical intervention obliterates existing networks of vasculature, resulting in reduction or elimination of tissue blood supply [26]. This leads to persistent production of fibrin matrix in compromised tissue and the formation of intra-abdominal adhesions [26,27]. The fibrin matrix is eventually replaced by tissue containing polymorphic leukocytes, fibrin, fibroblasts, macrophages, and formation of new blood vessels [26]. Affected peritoneal tissues become re-epithelialized with mesothelial cells within 5 to 6 days, with dermal restructuring occurring for subsequent months [26]. Tissue remodeling ultimately leads to fibrous adhesion formation covered by mesothelium. Inflammation brought on by peritonitis and surgical intervention drives increased fibroblast activity from mesothelial tissue to promote scar tissue formation [26]. The overabundance of scar tissue results in adhesions that can anchor organs to other organs or walls of the peritoneum [26]. Abdominal adhesions can cause acute small bowel obstructions as well as restrict blood supply to GI tissues, resulting in ischemia [26]. Although 95% of patients develop postoperative adhesions, only 35% of these patients present with symptoms due to adhesions [27]. Small bowel obstructions typically present with nausea, vomiting, abdominal pain, abdominal distention, and intolerance to oral intake. Treatment includes nasogastric decompression, withholding food and fluids orally, and often requires laparotomy for lysis of adhesions [26]. Intra-abdominal adhesion treatment is largely preventative by minimizing tissue damage, sterilizing surgical sites, or applying biodegradable films that cover wounds to prevent the joining of apposed tissues [27]. While no known data

points to saline as a cause of adhesion formation, it is imperative that any proposed lavage solution not result in detrimental adhesion formation.

Peritonitis Treatment

Peritonitis treatment involves source control, which is described as any physical measure to eliminate a source of infection. Source control is focused on the elimination of bacteria foci initiating inflammation through tissue lavage, debridement, resection or repair of the perforated viscus, and administration of antibiotics [1,6]. Lavage of the peritoneal cavity is necessary for washing away bacteria and cellular debris, with some solutions possessing antimicrobial properties, in attempts to reduce post-surgical infections [1]. Tissue debridement is the removal of any damaged or necrotic tissue to prevent further propagation of an inflammatory response. Following debridement, the subsequent goal is to repair and restore organ function [1]. Intravenous (IV) antibiotics are administered to complement other measures of source control with antibiotic selection being dependent on the prevalent bacterium present [1]. With the common source of peritonitis bacterium originating from the terminal ileum and colon, a broad-spectrum antibiotic is required as the bacterial flora from this region of the GI tract includes gram-negative and gram-positive bacteria [1].

The utilization of source control measures relies on 3 classifications: contamination, mild infection, or severe infection [28]. Intra-abdominal contamination occurs when there is a bacterial presence within the peritoneum but a lack of inflammation [28]. This is often seen following perforation of the bowel with soiling of the peritoneal tissue; however, no pathogenic tissue invasion has occurred [28]. Mild intra-abdominal infections typically occur from gangrenous appendicitis resulting in inflammation [28]. Left untreated, mild infections progress

to severe infections as a result of the diffuse infection throughout the peritoneal cavity [28]. Source control measures are important tools used to reduce bacterial presence to prevent the onset of severe infection. However, even after the infection is cleared, prolonged inflammation from the bacterial burden can result in the development of tertiary peritonitis [28]. Patients who develop tertiary peritonitis experience higher rates of multiple organ failure and mortality [28]. Peritonitis treatment, therefore, should focus on successfully clearing bacteria, while limiting host tissue damage to improve patient prognosis.

Lavage Solutions

Lavage is a standard of care for source control of peritonitis and involves the use of an irrigation solution to washout the peritoneal cavity in an attempt to reduce the bacterial burden. Effective lavage solutions should be nontoxic to host tissue, noncorrosive, and relatively inexpensive. Sterile normal 0.9% saline is currently the preferred solution used by surgeons as it is 100% safe for use, but given its lack of antimicrobial properties, further research into alternatives is warranted. Analysis by Thomas et al. has demonstrated the necessity of lavage showing that irrigation with saline vs no irrigation provides a positive benefit to patients in reducing post-operative infections [29]. Additionally, additives to lavage, such as antibiotics and antiseptics, were also analyzed and was reported to reduce infection rates over non-additive solutions [29]. However, antibiotic lavage has raised concerns about the development of antibiotic resistance and the potential for inducing allergic reactions in patients. Antiseptic lavage has worked well, historically with povidone iodine (PVI), as iodine is a known bactericidal molecule [30]. Treatment with PVI demonstrated reduced bacterial contamination compared to saline, without any concerns of cytotoxicity [30]. However, concerns about wound

healing impairment and systemic iodine absorption through the peritoneal cavity warrant a continued search for a better antiseptic lavage [30,31].

Chlorhexidine gluconate (CHX), a biguanide compound with potent antibacterial properties, is also currently used as an antiseptic lavage [32]. It is a positively charged molecule that interacts with negatively charged microbial cell surfaces, disrupting the cellular membrane integrity, resulting in cellular component leakage, reducing bacterial viability [32]. CHX and its effectiveness as a source control lavage has previously been shown by Bondar et al. to effectively reduce mortality rates and enduring bacterial counts in peritonitis murine models when used in low concentrations (0.05%) [33]. Higher concentrations (0.1% and 0.5%) resulted in significant decreased survival indicating increased cytotoxicity to host tissues [33]. In a study by Maleckas et al. which evaluated 0.05% CHX in peritonitis treatment, increased dense adhesion formation was reported, raising questions about the effectiveness of CHX lavage [34]. Additionally, Shams et al. has demonstrated that use of 0.05% CHX intra-abdominal lavage resulted in a significant increase in peritoneal fibrosis over saline lavage in mice peritonitis models [35]. Overall, this suggests that CHX is successful in reducing abdominal bacterial pathogens. However, it induces injury to healthy tissues, impairs wound healing, and prolongs infection [34,35]. Despite advancements in lavage therapies, continued research is warranted to improve source control measures. HOCl, a potent oxidizer and antiseptic, has been used with success in the treatment of superficial wounds [14,36-43]. However, limited evidence supports its use as an intra-abdominal lavage and may be the key to improving the outcome of peritonitis patients.

HOCl Synthesis

Hypochlorous acid is produced naturally in mammalian innate immunity cells and is produced in response to pathogenic invasion [15,34,39]. This biomolecule belongs to a family of molecules known as reactive oxygen species (ROS) [38,39]. These ROS molecules are unstable free radicals and will readily react with other cellular components to induce cellular component damage [38,39]. As ROS molecules accumulate within the cell, there is a significant rise in oxygen consumption, a process known as the oxidative burst (Figure 2) [15,39]. The primary enzyme responsible for ROS production is the oxidative burst enzyme, nicotinamide adenine dinucleotide phosphate (NADPH), a membrane-bound molecule which transfers electrons (e^-) to form superoxide anion (O_2-) [15,19]. Superoxide dismutase (SOD) then converts O_2^- to H_2O_2 through reaction with hydrogen ions (H^+) [39]. Myeloperoxidase (MPO) enzymes then utilize H_2O_2 to oxidize cytoplasmic halides such as Cl^- ions to form HOCl [15,39]. During bacterial engulfment by neutrophilic phagosomes, HOCl is produced to combat the invading pathogen (Figure 2) [38]. HOCl is an electrically neutral molecule, which can passively diffuse through bacterial membranes and react with nucleophilic structures, such as amino acids, purine and pyrimidine bases, and amines [13]. The resulting oxidation of these structures by HOCl leads to peptide bond cleavage and protein misfolding and aggregation leading to cellular signaling dysfunction [13]. The resulting damage to DNA, RNA, proteins, and lipids ultimately decreases bacteria survivability (Figure 3) [1,13]. The primary mechanism by which HOCl is produced is a superoxide anion(O_2^-), an oxygen radical, which is a precursor to other reactive oxygen species (ROS) [15,38,39]. The exogenous production of a HOCl solution (Figure 4) is synthesized by either electrolysis of a salt solution (eq 1A & 1B) or acidification of hypochlorite (eq 2) [39]. Equation 1A&B is carried out by electrolysis of a weak sodium chloride (NaCl) solution. At the

anode, water (H_2O) is transformed into O_2 and H^+, along with chlorine (Cl_2) from chlorite ions (Cl^-) [39]. Chlorine then reacts with water to form HOCl and hydrochloride (HCl) [39]. At the cathode, water is converted to hydrogen (H_2) and hydroxide ion (OH^-). These reactions result in a synthesized solution of HOCl [39]. One disadvantage of this method is the difficulty in controlling the final solution concentration, which effects solution efficacy. Solution pH plays a significant role in HOCl concentration as high [Cl^-] and low pH (<3) favor Cl_2 formation [39]. Low pH is problematic as Cl_2 will gas off, decreasing available chlorine to form HOCl. To produce a solution of predominant HOCl, a pH range of 3 to 6 is preferred [39]. Another problem in synthesizing HOCl solution from NaCl electrolysis is the short shelf life. Exposure to UV radiation, sunlight, contact with air, or temperatures above 25 ºC render the solution instable [39]. When exposed to, or sheltered from sunlight, chlorine reduction began on day 4 and day 14, respectively [39]. Consequently, to prolong HOCl solution shelf life, it should be stored in a sealed, dark, and cool environment [39]. The preferred method for HOCl production is acidification of hypochlorite as it is easily controllable and safe, comparatively [6,39,41].

Recent proprietary methods have created stabilized HOCl solutions, which extend shelf life to 2 years by maintaining a pH of 5.5-6.5 and stabilizing solution equilibrium. These solutions exhibit antimicrobial activity against a broad spectrum of microorganisms at varying concentrations but it has been minimally studied in the treatment of peritonitis. Treatment against *E. coli,* the predominant peritonitis pathogen, results in >99.99% bacterial death within the first two minutes of HOCl exposure [39]. HOCl is currently used in superficial wound treatments and is being evaluated for its effectiveness in abdominal infection treatments. Studies conducted by Kubota et al, have reported increased survival rates in murine model peritonitis and decreased infectious rates in humans following perforated appendicitis following use of electrolyzed strong

acid water (ESAW) as a source of HOCl disinfection [36,42]. These findings further support its research as a peritoneal lavage solution. Stabilized HOCl has been minimally studied in regard to peritonitis and comparisons with chlorhexidine or saline lavage are lacking.

Peritonitis is a medical emergency that is common in human medicine; however, additional research is needed to improve post-operative recovery. Standard treatment involves surgical intervention, abdominal lavage, and parenteral antibiotic therapy depending on its categorization [1]. Despite these treatment options, peritonitis patients experience high rates of multiple organ failure and higher mortality rates [2,3,28]. A recent study by Ballus et al. reported that out of 343 patients with peritonitis, 185 (54%) progressed to tertiary peritonitis, from which 90 (48%) died [44]. This data clearly indicates that reduction of recurrent intra-abdominal infections is a key focus for improving patient prognosis and improving survival rates associated with peritonitis-induced sepsis. One way to address this is the improvement of intra-abdominal lavage solutions used during source control. While sterile normal saline is primarily used, it lacks antimicrobial properties, which may be the key to reducing bacterial contamination following peritonitis treatment. Previously used antiseptics, such as CHX have shown efficacy compared to saline but concerns regarding cytotoxicity warrant further research. One proposed lavage antiseptic is stabilized HOCl, as it has had success with superficial wound healing and reduction of abdominal bacterial contamination [42,43]. However, its use as an intra-abdominal lavage solution is minimally researched and warrants further experimentation.

Aims & Hypotheses

This project was designed to investigate the effectiveness of utilizing stabilized HOCl as an intra-abdominal lavage solution for treatment of acute peritonitis in an animal model. This

model evaluated the treatment of experimentally induced peritonitis using cecal ligation and puncture (CLP) in rats as described by Kubota et al [36]. Four specific aims were addressed. The first aim examined survival among rats acutely treated with saline, CHX, or stabilized HOCl intra-abdominal lavage solutions. It was hypothesized that rats treated with stabilized HOCl will have significantly higher survival rates compared to rats treated with saline or CHX.

The second aim compared lung tissue damage among rats treated with saline, CHX, or stabilized HOCl abdominal lavage. Failed source control of peritoneal infections leads to a system inflammatory response, and multiorgan dysregulation and damage [22]. Pulmonary tissue is acutely affected, following onset of sepsis, due to its fragile tissue structure and is easily observed by infiltration of PMNL's, thickening of the interalveolar septa, and hemorrhaging [22]. Using Hematoxylin and Eosin (H&E) staining, the base of the right lung was collected, stained, and viewed under confocal microscopy to determine pulmonary tissue damage. It was hypothesized that stabilized HOCl will successfully prevent peritonitis-induced sepsis compared to saline or CHX, resulting in little or no pulmonary tissue damage.

The third aim compared intra-abdominal adhesion formation between rats treated with saline, CHX, or stabilized HOCl intra-abdominal lavage solutions. Intra-abdominal adhesions are well-known occurrences following acute peritonitis treatment and effective lavage solutions should not produce detrimental adhesion formation [27,34]. No supporting data suggests saline contributes to adhesion formation however CHX concentrations higher than 0.05% have been shown to produce significantly higher peritoneal adhesion formation and fibrosis when compared to saline in murine models of peritonitis [34,35]. With the development of stabilized HOCl, the incidence of intra-abdominal adhesions following abdominal lavage is not well studied [36]. This aim was addressed by performing a gross anatomical evaluation of intra-abdominal tissues,

following euthanasia. Using the Majuzi adhesion scoring method it was hypothesized that rats treated with stabilized HOCl will present with similar adhesion scores to rats treated with saline and will compare favorably to CHX.

The fourth aim compared the enduring intra-abdominal bacterial growth following lavage with saline, CHX, or stabilized HOCl. Saline is the standard of care lavage solution in peritonitis treatment; however, saline provides no bactericidal or bacteriostatic properties and only serves to wash away cellular debris. A lavage solution that reduces bacterial presence through cytotoxic mechanisms while maintaining host tissue integrity is preferred. This aim was addressed by collecting peritoneal fluid from the retro-uterine and retro-vesicular sacs of animals 24 hours after irrigation of either saline, CHX, or stabilized HOCl. Peritoneal fluid was grown in bacterial cultures to determine the presence of remaining bacteria following irrigation. It was hypothesized that stabilized HOCl irrigation will result in less bacterial growth compared to animals treated with saline.

The fifth aim compared blood serum levels of TNFα in animals treated with saline, CHX, or stabilized HOCl intra-abdominal irrigation solutions. Ineffective irrigation solutions used in the treatment of peritonitis may lead to peritonitis-induced sepsis. Sepsis is categorized driven by elevated levels of pro-inflammatory cytokines in the bloodstream, having migrated from inflamed peritoneal tissues. Ultimately, this results in systemic inflammation and tissue dysregulation. Blood samples of animals treated with saline, CHX, or stabilized HOCl were collected via the saphenous vein of rats 24 hours after intra-abdominal irrigation. Samples were subjected to ELISA analysis for comparison of TNFα levels across animals treated with saline, CHX, or stabilized HOCl. It was hypothesized that animals treated with stabilized HOCl

irrigation will present with lower levels of TNFα within the bloodstream, compared to saline or CHX.

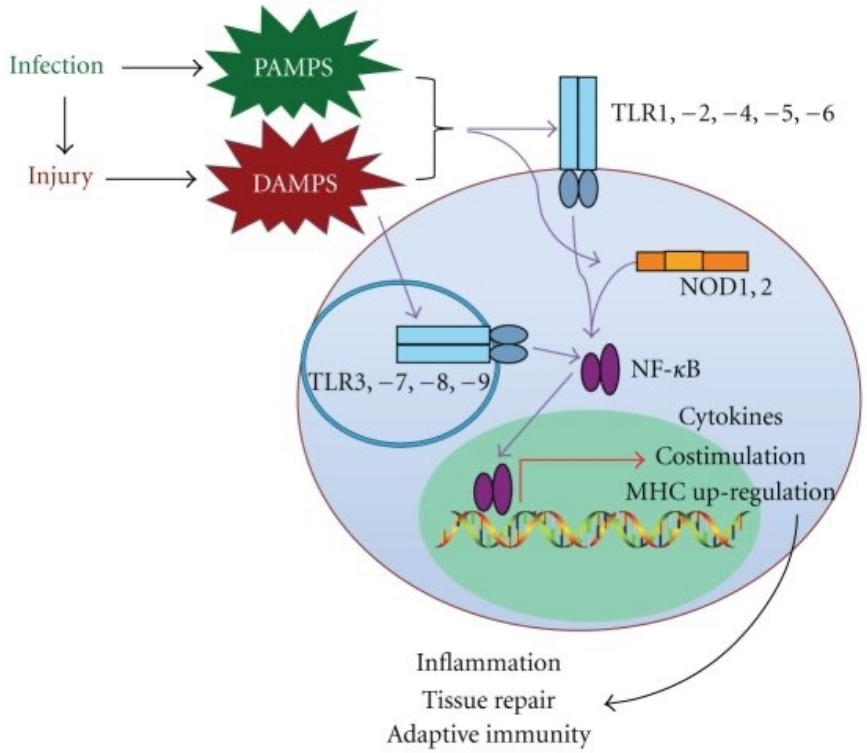

Figure 1. Inflammatory response via TLR binding to PAMPs/DAMPs due to cellular injury or pathogen invasion. Bacterial PAMPs and cellular DAMPs interact with extracellular TLR's to initiate intracellular NF-kB inflammatory pathway. Activation of NF-kB results in the up-regulation of pro-inflammatory cytokine production leading to inflammation [12].

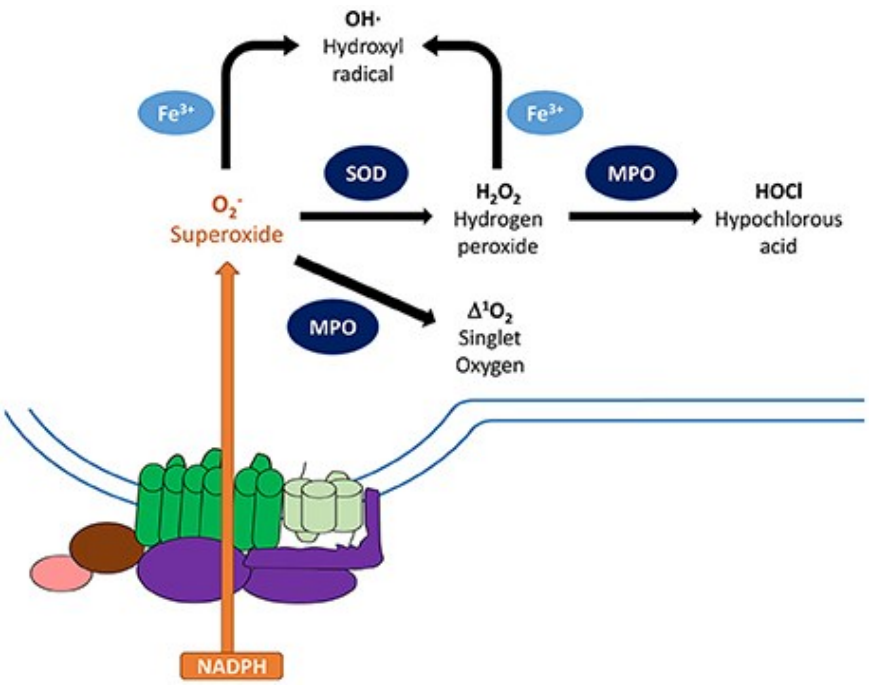

Figure 2. Metabolism of ROS. Illustrative image of endogenous HOCl production through respiratory burst mechanism. NADPH oxidase produces superoxide (O_2^-) which is then converted by Superoxide Dismutase (SOD) to Hydrogen Peroxide (H_2O_2). H_2O_2 is converted by Myeloperoxidase (MPO) to HOCl [14].

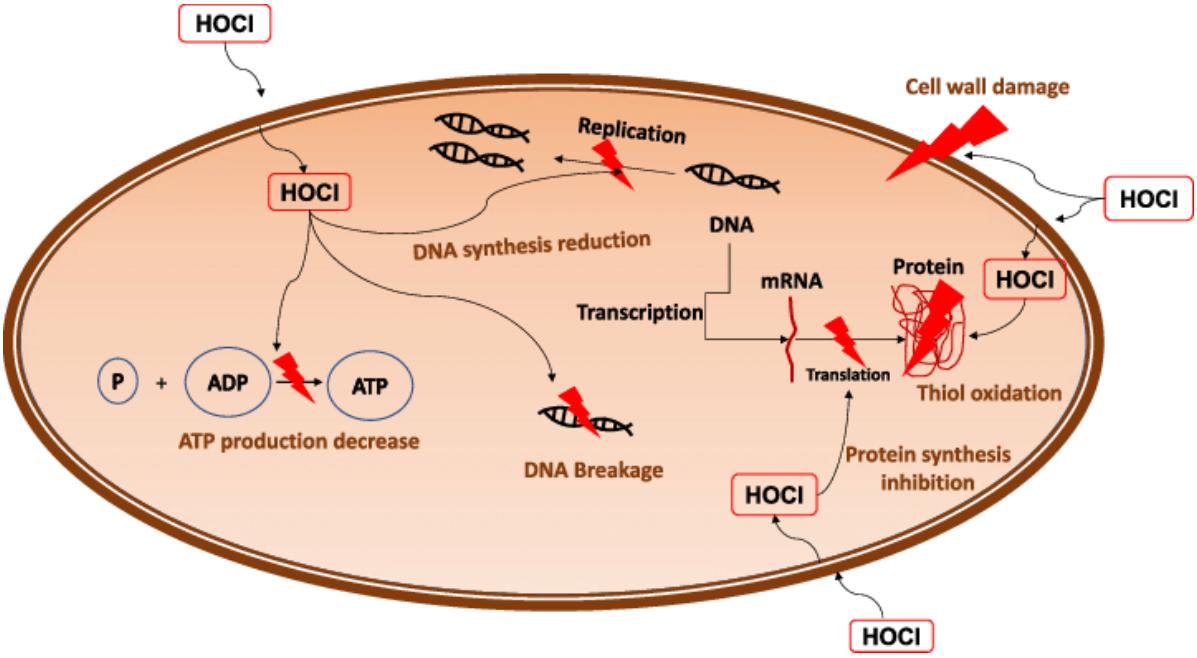

Figure 3. Proposed mechanism of action for HOCl. Exogenous HOCl, which is permeable to cellular membranes, is capable of oxidizing cellular proteins thus interrupting ATP production, DNA replication, and damages genomic DNA. Additionally, exogenous HOCl can oxidize membrane phospholipids, disrupt mRNA translation, as well as protein folding resulting in cellular damage [40].

$$2Cl^- + 2e^- \longrightarrow Cl_2 \qquad (1A)$$

$$CL_2 + H_2O \rightleftharpoons HOCl + H^+ + Cl^- \qquad (1B)$$

$$OCl^- + H^+ \rightleftharpoons HOCl \qquad (2)$$

Figure 4. Synthesis of HOCl. HOCl is produced by two methods: salt solution electrolysis and acidification of hypochlorite [39].

MATERIALS & METHODS

Animal Subjects

This study was approved by the Institutional Review Board/Institutional Animal Care and Use Committee/Biosafety Committee on 03/1/2021 and received approval for protocol #2020-14 (see Appendix A). No IRB approval, no biosafety approval, or radioactive approval was required, and all CITI training requirements for animal research were met (see Appendices B-E). Sprague-Dawley rats, 5-7 weeks old, weighing 180–250 g, were used for this study. Forty-two rats, females and males (21 of each) were randomly divided into three groups (7 female and 7 males in each group). One animal was lost during surgery and one during recovery. Both were replaced for the study. Animals were housed in a 12-hour light/dark cycle and fed with Labdiet 5001 chow ad libitum.

Cecal Ligation and Puncture

General anesthetic was induced by inhalation of isoflurane via an induction box, and maintenance of general anesthetic was accomplished by inhalation of a 1:1.5 (2 lpm/3 lpm) mixture of oxygen/isoflurane mixture via nose cone. Subjects were then weighed to determine proper analgesic medication and fluid resuscitation. Saline (0.9%) was warmed in a water-bath (37.7 °C) and administered in the dorsal subcutaneous tissue, in no less than 3 locations, at a dosage of 80 mL/kg prior to all laparotomies. Buprenorphine was administered subcutaneously in the femoral triangle prior to laparotomy at a dosage of 0.24 mL/kg. Abdominal hair was trimmed with clippers and skin was disinfected with povidone iodine. The animal was placed supine on a tabletop warmer at 37-38 °C to minimize hypothermia during and after surgery.

Using sterile surgical instruments and aseptic techniques, a midline abdominal incision of 2-cm was made through cutaneous and fascial layers, taking care not to puncture the underlying GI tract. An abdominal retractor was placed, and the cecum was retrieved from the upper left quadrant of the abdomen. The cecum was ligated 2-cm from the apex with a silk tie, maintaining adequate ileocecal junction communication, and punctured twice with an 18-gauge needle (Figure 5; [45,46]). The cecum was returned to the abdomen, and the abdomen was closed with a running 3-0 monofilament suture through the fascial and cutaneous layers. Following completion of the laparotomy and withdrawal of anesthetic, the animal remained on tabletop warmer until the animal could ambulate. The subject was placed in a clean cage and returned to the animal holding room and placed on a warming pad, set at 102 °C to minimize the risk of post-operative hypothermia.

Irrigation Solution Preparation and Assignment

Preparation of 50 mL of 0.09% saline, 50 mL of 0.05% chlorhexidine gluconate (Irrisept), and 50 mL of 100 ppm Pure&Clean© stabilized HOCl was performed each week. The solutions were all clear, colorless, and identical in appearance and without identifying odor. Solutions were placed in identical sealed glass vials and delivered to an unbiased vivarium staff member who then assigned each solution an identification number and random order of use. All solutions were stored in a cool environment, with no exposure to light, to prevent chemical instability. Following use of each solution, the animal ID and fluid identification was shared with and recorded by the vivarium staff. A total of 14 solutions of saline, 15 solutions of stabilized HOCl, and 13 solutions of CHX were randomly delivered to subjects. 7 males and 7 females were treated with saline, 8 males and 7 females were treated with stabilized HOCl, and 6 males

and 7 females were treated with CHX for a total of 42 irrigation procedures. The study was unblinded at the end of all data collection (see Appendix F).

Irrigation and Suction

Three hours following CLP, a secondary laparotomy was performed for source control. Isoflurane induction, maintenance of general anesthetic, and abdominal preparation were performed as during the initial procedure. The laparotomy was performed, ligated cecum recovered, and resected just distal to the ligation. The peritoneal cavity was irrigated with a warmed solution (37.7 °C) of blinded, randomized solution. A total of 50 mL was administered in increments of 10 mL with a solution contact time of 1 minute before suction of administered solution. Suction was performed with a vacuum pump attached to a modified microcentrifuge tube and catheter (Figure 6). Irrigation was performed in each quadrant with special attention at the site of cecal resection to retrieve maximum amounts of fluid. Following the final round of irrigation, suctioning was performed until there was no visible remaining solution in the peritoneal cavity. The abdominal incision was closed in 2 layers with a 3-0 monofilament running suture in the fascial layer and cutaneous layer. Animals recovered from anesthesia in the same fashion as the initial procedure.

Blood Serum and Peritoneal Fluid Collection

Twenty-four hours following the secondary laparotomy, animals were prepared for a third laparotomy. Isoflurane induction, maintenance of general anesthetic, and abdominal preparation were performed as during the initial procedure. Laparotomy was performed and using a P20 micropipette, 10 μL of peritoneal fluid was collected from the cul-de-sac of the

pelvis and was stored at -80 °C. Additionally, 200 µL of blood was collected from the great saphenous vein via venous puncture with an 18 ga needle. Blood samples were centrifuged for 10 minutes at room temperature for a duration of 10 minutes. Blood serum was allocated and stored at -80 °C. Blood serum was subjected to ELISA testing for TNFα detection.

Animal Health Evaluation and Euthanasia

Animal welfare checks were provided at a minimum of twice daily, once in the morning and another approximately 12 hours later in the evening. Animals in apparent discomfort (arched backs, head pressing) were administered subcutaneous buprenorphine at a dosage of 0.24 mL/kg pro re nata for analgesia. The vivarium veterinarian was consulted with any animals presenting with labored breathing or hastened respiratory rate, diminished/no reaction to stimuli, and any recommendations for euthanasia were adhered to. Moribund animals were euthanized per IACUC (Institutional Animal Care and Use Committee) protocol 2020-14 via administration of CO_2 at a rate of 7.9 lpm until unconscious. Once animal was unconscious, CO_2 was adjusted to 10 lpm and delivered for a minimum of 5 minute to ensure death. Any subjects surviving to the end of the planned observation of 10 days were also euthanized by the same procedure. Secondary physical method to confirm death was a thoracotomy prior to tissue collection and gross evaluation. A midline incision was made and both lungs and heart were collected. The esophagus above the stomach at the diaphragm was ligated, along with the terminal end of the large intestine and the GI tract was removed after ligation of mesenteric arteries. The liver, left kidney, and a 2 cm x 2 cm area of peritoneal abdominal wall was collected for tissue preservation. Animal carcasses were subsequently stored in vivarium freezer at -18 °C.

Tissue Fixation and Paraffin Embedding

Abdominal and thoracic tissues collected from the Sprague-Dawley rats following death, were washed with 25 mM PBS, and fixed with 4% paraformaldehyde. The following tissues were collected for analysis; base of right lung, cecal tissue at ligature site, terminal ileum, and intra-abdominal wall opposing the cecum. Collected tissues were embedded with paraffin utilizing a LEICA ASP300S tissue processor for a duration of 10 hours and transferred to base molds for tissue sectioning. Tissue samples were labeled with animal ID, tissue type, and date of processing. Embedded tissues were stored at 2 °C until sectioning was performed.

Tissue Sectioning and Mounting

Processed tissues embedded in paraffin were sectioned using a rotary microtome at a thickness of 3 μm and were then placed in a warm water bath and subsequently transferred to adhesive microscope slides (Fisher Scientific cat. 23-888-114) and dried overnight at 60 °C. Slides were then transferred to a slide case stored at room temperature.

Hematoxylin and Eosin Lung Tissue Staining

Rehydration and Hematoxylin Staining. The tissue was deparaffinized and rehydrated with 3 washes for 3 minutes each in Xylene (Fisher Scientific cat. X3P-1GAL). Subsequent ethanol solutions were made from 100% absolute ethanol (Fisher Chemical A406F-1GAL). Tissues were then washed 2 times for 3 minutes each with 100% ethanol, 1 time for 3 minutes in 95% ethanol, 1 time for 3 minutes in 70% ethanol, and finally 1 wash for 5 minutes in deionized (DI) H_2O. Slides were transferred to hematoxylin stain (Fisher Scientific 220-109) and were dipped 6 times, followed by a DI H_2O rinse. Slides were washed 1 time for 5 minutes in tap

water to allow stain development. Slides were quickly dipped 12 times in acid ethanol (1 mL of HCl and 400 mL of 70% EtOH) for differentiation, followed by 2 washes for 1 minute each in tap water and finally 1 wash for 2 minutes in DI H_2O.

Eosin Staining and Dehydration. Slides were transferred to eosin-y stain (Fisher Scientific 220-104) and washed for 20 seconds in concentrated eosin, followed by 1 wash for 5 minutes in 95% ethanol, 1 time for 5 minutes in 100% ethanol, and finally 2 times for 5 minutes each in Xylene. Permount was placed on tissue slides with a P1000 pipette and coverslips were placed over tissue; slides were dried overnight under hood at room temperature.

Lung Tissue Scoring

Lung tissue collected (as described in Tissue Fixation and Embedding) from subjects were subjected to H&E staining and viewed under confocal microscopy. Pulmonary tissues were evaluated to determine tissue morphology due to systemic infection, brought on by peritonitis. Tissues were analyzed by a blinded researcher. Researchers analyzed 4 random locations of the tissues and scored tissues on a scale of 1-4 according to morphological changes (Table 1).

Abdominal Adhesion Scoring

Animals were monitored until death, moribund and requiring euthanasia, or surviving to the maximum timeline of ten days. Adhesion scoring was then performed on necropsy using the Mazuji adhesion scale (Table 2) with no adhesions present receiving a score of 0. A small, irregular adhesion received a score of 1. An easily separable medium intensity adhesion received a score of 2. An intense, not easily separable regular adhesion received a score of 3. A very intense, not easily separable adhesion received a score of 4.

Bacterial Culture and ELISA Analysis

Bacterial cultures of peritoneal fluid and ELISA analysis of blood serum TNFα levels were carried out by Dr. Christopher Lupfer's Missouri State University biology team.

Statistical Analysis

GraphPad Prism was utilized for data analysis of survival rates using the Kaplan-Meier Survival Curve. Lung tissue analysis was performed using a two-tailed student t-test. Assessment of significance between early adhesion scores among stabilized HOCl, CHX, and saline were performed using a one-way ANOVA. Late adhesion analysis between stabilized HOCl and saline were performed using a two-tailed student t-test. Bacterial counts were analyzed using a one-way ANOVA across all treatment groups. Assessment of TNFα was performed using an ELISA assay kit. A p value ≤ 0.05 was set for statistical significance within all analysis group.

Table 1. Pulmonary tissue scoring criteria.

Pulmonary Morphology Description	Score
Thickening of inter-alveolar septa, PMNL and RBC infiltration in septa and alveolar sac, and changes in alveolar integrity in less than 25% of microscopic field	1
Thickening of inter-alveolar septa, PMNL and RBC infiltration in septa and alveolar sac, and changes in alveolar integrity in more than 25% but less than 50% of microscopic field	2
Thickening of inter-alveolar septa, PMNL and RBC infiltration in septa and alveolar sac, and changes in alveolar integrity in more than 50% but less than 75% of microscopic field	3
Thickening of inter-alveolar septa, PMNL and RBC infiltration in septa and alveolar sac, and changes in alveolar integrity in more than 75% of microscopic field	4

Table 2. Majuzi adhesion scoring.

Adhesion Description	Score
No adhesions present	0
Small irregular, easily separable adhesion	1
Medium intensity, easily separable adhesion	2
Intense, not easily separable adhesion	3
Very intense, not easily separable adhesion	4

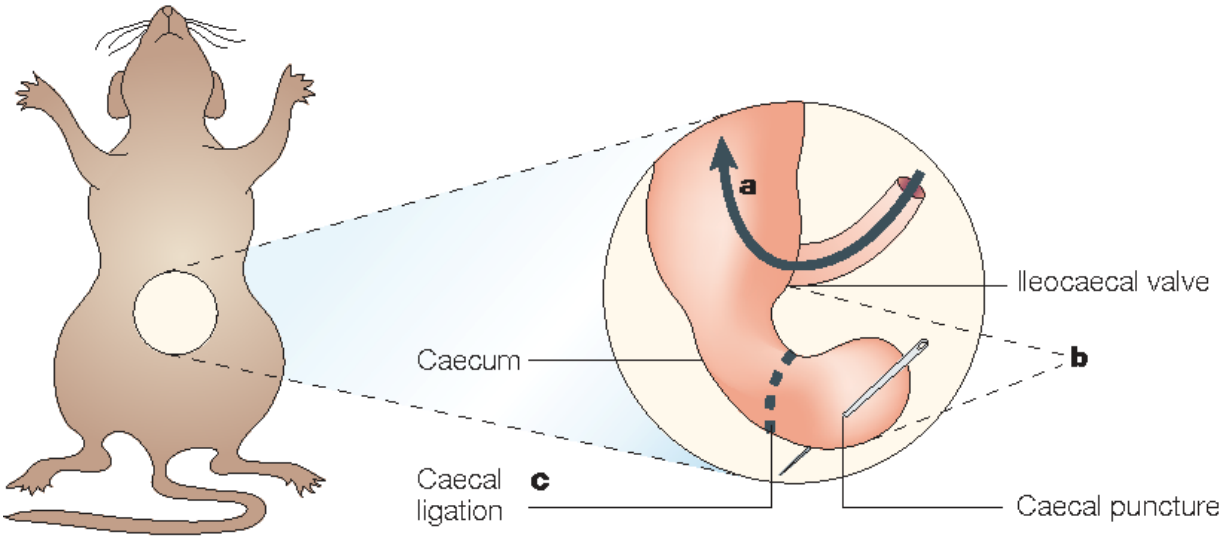

Figure 5. Cecal ligation and puncture. Animals under isoflurane anesthesia were subjected to laparotomy with cecal ligation and puncture. A midline incision was performed, and the cecum was retrieved from the peritoneal cavity. The apex of the cecum was ligated with a 3-0 silk tie while maintaining ileocecal junction communication and punctured twice with an 18 ga needle. The cecum was returned to the abdomen prior to closure [45].

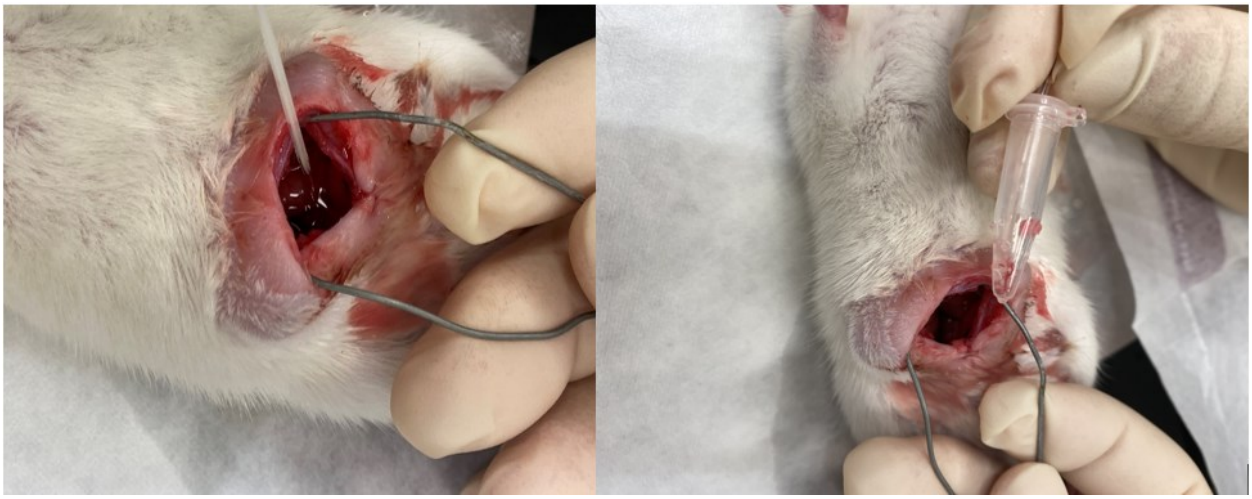

Figure 6. Peritoneal irrigation and suction. Peritoneal cavities were irrigated with a catheter attached to a 30 mL syringe. Suction was performed with a modified microcentrifuge tube and catheter attached to a vacuum pump.

RESULTS

Survival Rates

Animals were monitored until death, moribund and requiring euthanasia, or surviving to the maximum timeline of ten days post-irrigation with timeline of survival beginning at time of the secondary laparotomy with abdominal irrigation. Survival rates were assigned in increments of 24 hours and animal welfare checks were provided every 12 hours to best estimate time of death. Thirteen rats treated with CHX intra-abdominal irrigation were monitored after the second laparotomy and survival rates were recorded (Table 3). Survival rates in this group were averaged with an overall survival of 1.9 days. Fifteen rats treated with stabilized HOCl intra-abdominal irrigation were monitored after the second laparotomy and survival rates were recorded (Table 4). Survival rates in this group were averaged with an overall survival of 5.9 days. Fourteen rats treated with saline intra-abdominal irrigation were monitored after the second laparotomy and survival rates were recorded (Table 5). Survival rates in this group were averaged with an overall survival of 8.7 days. One animal died during initial laparotomy and another during postoperative recovery immediately following cecal ligation and puncture. These animals were not included in the data.

Survival rates of all three treatment groups were analyzed by a Kaplan Meier survival analysis and displayed in Figure 7. This data indicates a significant difference in survival rates ($p < 0.0001$). Pairwise comparisons between groups showed a trend towards decreased survival with stabilized HOCl compared to saline, however, it did not reach statistical significance ($p = 0.0634$). Pairwise comparisons between stabilized HOCl and CHX were statistically significant

($p = 0.0052$). Additionally, pairwise comparisons between saline and CHX were statistically significantly ($p < 0.0001$).

Pulmonary Tissue Morphology

Pulmonary tissues were analyzed by means of qualitative analysis by a blinded researcher using bright field microscopy. Following the death of each animal, a thoracotomy was performed, and lung tissue was collected and washed in 25 mM PBS and stored in 4% paraformaldehyde at 4 °C for up to 6 months. The base of the right lung was collected and embedded with parafilm and tissue sections, cut at 3 μm, were mounted to charged glass slides. Tissue samples were subjected to H&E staining for analysis of morphological changes in pulmonary tissues. Tissues of random 10-day survivors were analyzed from the saline (n = 4) and stabilized HOCl (n = 4) groups. Tissues in each group were analyzed by a blinded researcher, with each tissue being observed in 4 different locations and scores were assigned per Table 1 and with examples of tissue scoring in Figure 8 and Figure 9. Recorded scores for each tissue location were averaged and assigned to treatment groups (Table 6).

Averaged tissue scores were analyzed by a two-tailed student t-test to determine any statistical differences between pulmonary tissue changes across treatment groups 10-days after irrigation treatments. Analysis (Figure 10) of tissue scores indicates no significant difference between pulmonary morphological changes between saline and stabilized HOCl treatment groups ($p = 0.0732$).

Peritoneal Adhesions

Adhesion formation within the peritoneal cavity was determined by visual and physical evaluation following animal death. During necropsy, the midline incision was opened, and peritoneal cavity explored for adhesion formation. Adhesions were graded per the Majuzi adhesion scoring method (Table 2) and the best representative images of each adhesion score is illustrated in Figures 11–15. Adhesion separability was determined by using a probe and lifting on each adhesion.

Thirteen animals (6 male, 7 female) were evaluated for adhesion formation following treatment with CHX intra-abdominal irrigation (Table 7). Adhesions were recorded and animals presented with an overall average adhesion score of 1.2. Fourteen animals (8 male, 6 female) were evaluated for adhesion formation following treatment with stabilized HOCl intra-abdominal irrigation (Table 8). Subject 6.13 was omitted from data analysis due to a lack of adhesion evaluation. Animals in this group presented with an overall average adhesion score of 1.1. Thirteen animals (7 male, 7 female) were evaluated for adhesion formation following treatment with saline intra-abdominal irrigation (Table 9). Subject 6.24 was omitted from data analysis due to a lack of adhesion evaluation. Animals in this group presented with an overall average adhesion score of 0.9. Data for all treatment groups were recorded for statistical analysis (Table 10).

Adhesion formation was evaluated in the early stages of post-operative recovery and the late stages of post-operative recovery. Early adhesion formation was assessed within the first 4 days after irrigation across all groups using a one-way ANOVA analysis (Figure 16). Upon analysis, adhesion formation was found to have no statistical significance across all groups (p = 0.7501). Late adhesion formation was assessed beginning at day 5 and ending at day 10 after

irrigation using a two-tailed student t-test analysis (Figure 17). It was determined that there was no statistical difference in adhesion formation between animals treated with saline and stabilized HOCl ($p = 0.2826$).

Peritoneal Fluid Bacterial Counts

Effectiveness of irrigation solution to remove bacterial contamination from the peritoneal cavity was investigated through the collection of peritoneal fluid, followed by bacterial culture and calculation of colony forming units (CFU). A third laparotomy was performed 24 hours after intra-abdominal irrigation, and 10 μL of peritoneal fluid was collected via P20 pipette from the retro-uterine or retro-vesicular sac. The peritoneal fluid was placed in a microcentrifuge tube and stored at -4 °C and moved to -80 °C storage. Peritoneal fluid was subjected to bacterial cultures by Dr. Christopher Lupfer's Missouri State University biology team and CFU counts were calculated for each subject (Table 11).

A one-way ANOVA was utilized to determine any statistical difference between bacterial CFUs presence among the treatment groups (Figure 18). Upon analysis, there was a statistically significant difference in bacteria found within the abdomen within the three treatment groups, 24 hours following irrigation procedures ($p = 0.0303$). A pairwise comparison between stabilized HOCl and CHX treatment was performed, and a significant increase of bacterial presence was observed in the abdomen of stabilized HOCl treated subjects ($p = 0.0064$). A pairwise comparison between stabilized HOCl and saline treatment was performed and there was a significantly lower count of bacteria within the abdomen of saline treated subjects than stabilized HOCl treated subjects ($p = 0.0299$). The mean of bacterial counts for stabilized HOCl presented

with 2.55648 Log10 CFU/mL while saline and CHX presented with a mean of 1.14777 Log10 CFU/mL and 0.69231 Log10 CFU/mL, respectively.

Blood Serum TNFα Levels

The effectiveness of stabilized HOCl to prevent a systemic inflammatory response was assessed through TNFα profiling in blood serum samples. Twenty-four hours after intra-abdominal irrigation, blood was collected from the great saphenous vein of anesthetized subjects. Blood was centrifuged for 10 minutes at room temperature and serum was allocated, stored at 4 °C and finally stored at -80 °C. Serum was subjected to ELISA analysis for TNFα profiling by Dr. Christopher Lupfer's Missouri State University biology team, with Emma Loder receiving special recognition. Following ELISA analysis, no detectable levels of TNFα were observed in any subject in any treatment group.

Table 3. Survival rate of rats treated with CHX.

Chlorhexidine (CHX) Group Survival (n = 13)		
Animal ID	Sex (M/F)	Survival (Days)
7.16	M	0
8.12	M	0
6.26	M	1
7.13	M	1
7.15	M	1
5.5	M	2
7.32	F	0
7.35	F	0
8.49	F	0
6.9	F	1
5.33	F	2
6.8	F	2
7.38	F	2

Table 4. Survival rate of rats treated with stabilized HOCl.

Stabilized Hypochlorous Acid (HOCl) Group Survival (n = 15)		
Animal ID	Sex (M/F)	Survival (Days)
5.51	M	1
6.23	M	1
8.14	M	1
5.53	M	4
7.12	M	6
6.29	M	10
7.11	M	10
8.13	M	10
7.34	F	1
6.12	F	2
6.7	F	3
6.13	F	10
7.4	F	10
7.36	F	10
7.39	F	10

Table 5. Survival rate of rats treated with saline.

Saline 0.9% Group Survival (n = 14)		
Animal ID	Sex (M/F)	Survival (Days)
5.52	M	7
6.24	M	10
6.27	M	10
6.28	M	10
7.17	M	10
7.14	M	10
8.15	M	10
6.11	F	1
8.48	F	4
5.32	F	10
6.1	F	10
7.33	F	10
7.41	F	10
7.37	F	10

Table 6. Qualitative scores of pulmonary tissues of 10-day survivors in saline and stabilized HOCl treatment groups.

Animal ID	Treatment Group	Scores of Tissues	Average Scores
7.11	HOCl	3, 4, 3, 4	3.50
7.40	HOCl	4, 4, 4, 4	4.00
7.36	HOCl	1, 2, 2, 2	1.75
8.13	HOCl	3, 2, 3, 3	2.75
8.15	Saline	2, 3, 3, 2	2.50
7.41	Saline	1, 1, 1, 1	1.00
7.17	Saline	2, 1, 2, 2	1.75
7.14	Saline	4, 3, 4, 4	3.75

*Qualitative scores were determined by a blinded researcher at 4 different locations of the tissue sample and listed under “Scores of Tissues” column. Scores were averaged.

Table 7. Adhesion scores of rats treated with CHX irrigation.

CHX Group Adhesion Scores (n = 13)			
Animal ID	Sex (M/F)	Adhesion Score	Survival (Days)
7.16	M	0	0
8.12	M	0	0
6.26	M	2	1
7.13	M	1	1
7.15	M	4	1
5.5	M	1	2
7.32	F	0	0
7.35	F	0	0
8.49	F	0	0
6.9	F	1	1
5.33	F	1	2
6.8	F	2	2
7.38	F	4	2

Table 8. Adhesion scores of rats treated with stabilized HOCl irrigation.

HOCl Group Adhesion Scores (n = 15)			
Animal ID	Sex (M/F)	Adhesion Scores	Survival (Days)
5.51	M	1	1
6.23	M	0	1
8.14	M	1	1
5.53	M	2	4
7.12	M	1	6
6.29	M	1	10
7.11	M	2	10
8.13	M	2	10
7.34	F	0	1
6.12	F	1	2
6.7	F	1	3
6.13	F	OMIT	10
7.4	F	2	10
7.36	F	2	10
7.39	F	0	10

* Subject 6.13 was omitted due to no adhesion analysis following death.

Table 9. Adhesion scores of rats treated with saline irrigation.

Saline Group Adhesion Scores (n = 14)		
Sex (M/F)	Adhesion Scores	Survival (Days)
M	0	7
M	OMIT	10
M	1	10
M	2	10
M	0	10
M	1	10
M	3	10
F	0	1
F	1	4
F	0	10
F	0	10
F	1	10
F	1	10
F	2	10

*Subject 6.24 was omitted due to no adhesion analysis following death.

Table 10. Adhesion scoring of all treatment groups.

Adhesion Score	Total Number of Rats (n = 40)	CHX (n = 13)	Stabilized HOCl (n = 14)	Saline (n = 13)
0	13	5	3	5
1	16	5	6	5
2	8	1	5	2
3	1	0	0	1
4	2	2	0	0

Table 11. Intra-abdominal bacterial counts of treatment groups 24 hours post-irrigation (Log10 CFU/mL).

Treatment Groups		
HOCl (n = 15)	Saline (n = 14)	CHX (n = 13)
3.397940	5.574031	5.60206
3.397940	3.397940	3.39794
3.698970	3.698970	0.00000
4.352183	3.397940	0.00000
4.477121	0.000000	0.00000
3.397940	0.000000	0.00000
3.397940	0.000000	0.00000
3.698970	0.000000	0.00000
3.875061	0.000000	0.00000
4.653213	0.000000	0.00000
0.000000	0.000000	0.00000
0.000000	0.000000	0.00000
0.000000	0.000000	0.00000
0.000000	0.000000	-
0.000000	-	-

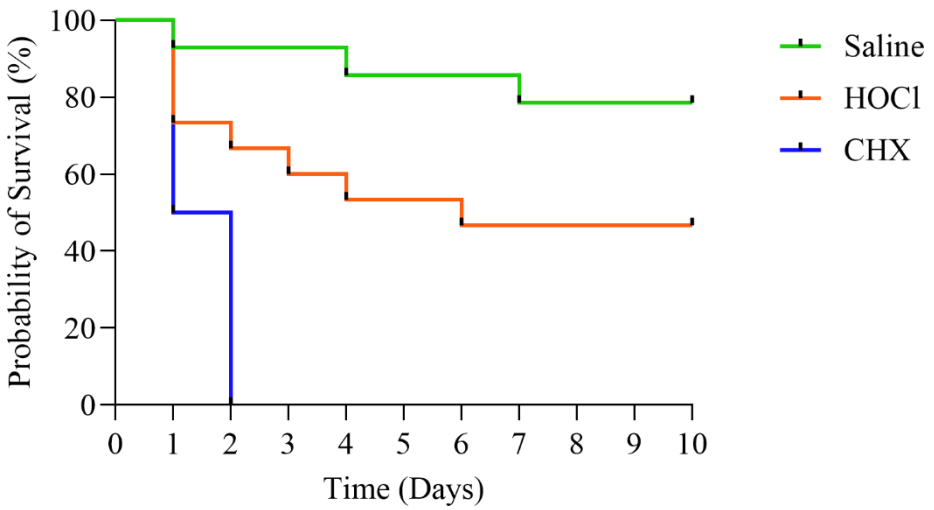

Figure 7. Survival rates of rats treated with CHX, stabilized HOCl, and saline. Each line represents the survival rate observed among 3 different treatments: saline (n = 14), stabilized HOCl (n = 15), and CHX (n = 13). Values on the x-axis indicate a maximum timeline of 10 days, while the y-axis indicates the percentage of animal survival. One-way ANOVA showed a statistical significance among the three groups. ($p < 0.05$). A two-tailed student t-test of HOCl and saline showed no statistical significance ($p > 0.05$). A two-tailed student t-test of HOCl vs CHX showed statistical significance ($p < 0.05$).

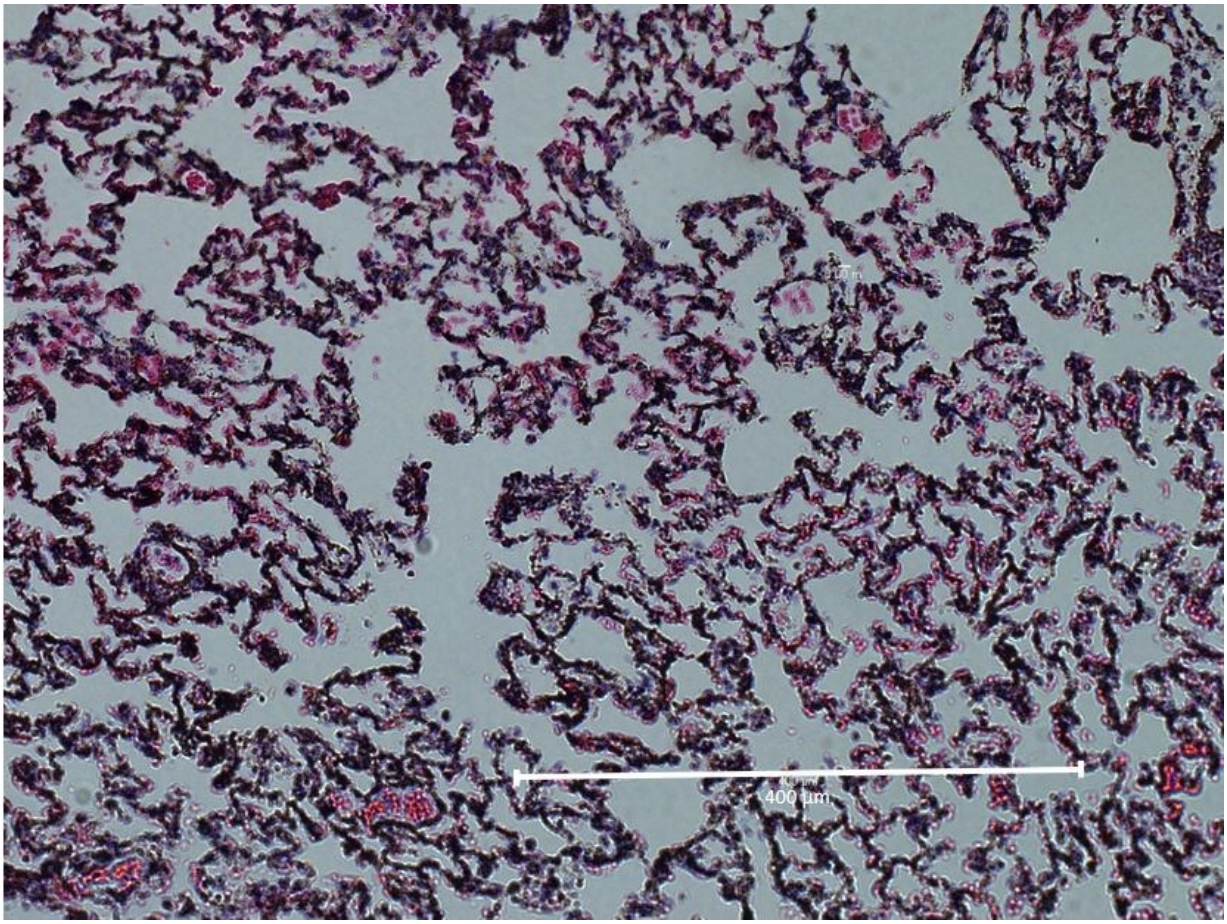

Figure 8. Pulmonary tissue morphology score 1. Best representative images of 10-day surviving Sprague-Dawley pulmonary tissues subjected to H&E staining for tissue morphological analysis in stabilized HOCl and saline treatment groups. Tissues with alveolar morphological changes and RBC and WBC infiltration in less than 25% of the microscopic field received a score of 1. Tissues sectioned at 3 µm.

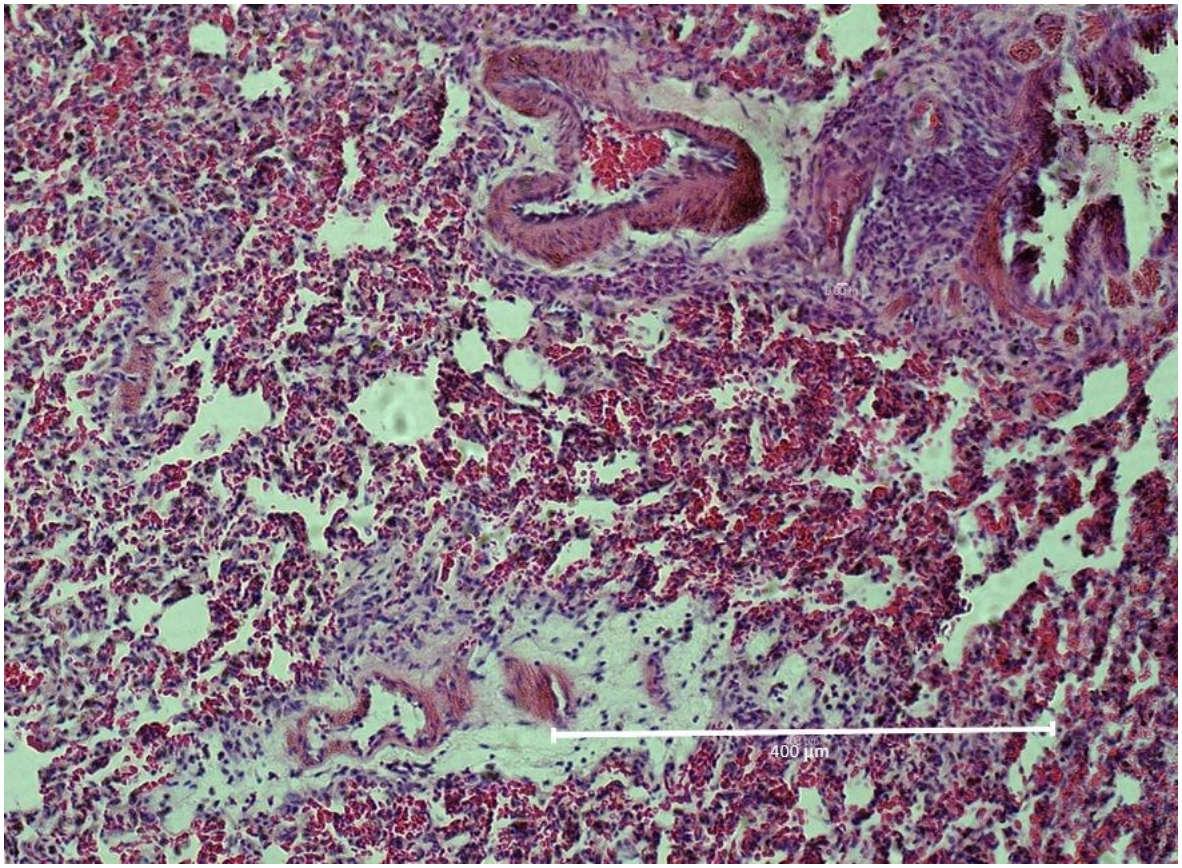

Figure 9. Pulmonary tissue morphology score 4. Best representative images of 10-day surviving Sprague-Dawley pulmonary tissues subjected to H&E staining for tissue morphological analysis in stabilized HOCl and saline treatment groups. Tissues with alveolar morphological changes and RBC and WBC infiltration over 75% of the microscopic field received a score of 4. Tissues sectioned at 3 µm.

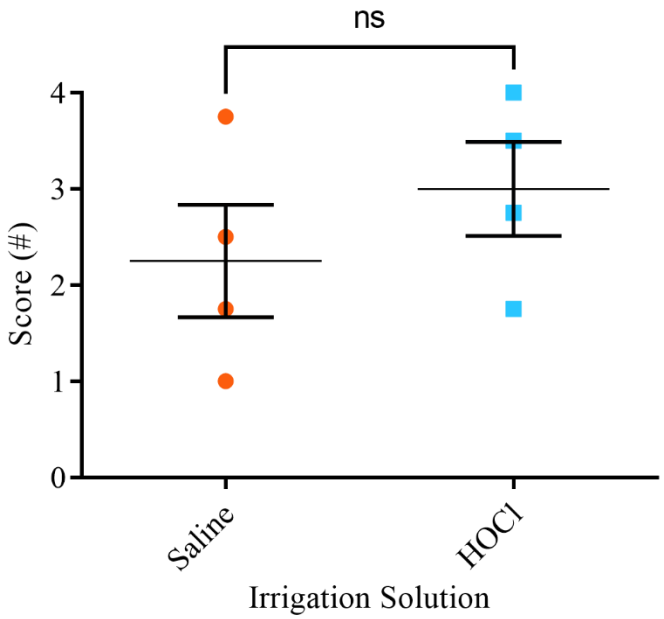

Figure 10. Pulmonary tissue scores of saline and stabilized HOCl 10-day survivors. Sprague-Dawley rat pulmonary tissue of animals surviving 10 days following intra-abdominal irrigation of Saline or Stabilized HOCl were collected and subjected to H&E staining for tissue morphological analysis. A blind researcher viewed 4 different regions of the same tissue and scored according to changes in morphology. The four scores of each tissue were averaged and assigned to the subject from which the tissue was collected. Tissues were scored on a 1-4 scale with a score of 1 indicating alveolar septa thickening, hemorrhaging, and alveolar sac morphology in less than 25% of the microscope field view. Score 2 was received for morphological changes in 25-50% of the field view. Score 3 was given for morphological changes up to 75% of the field view. Score 4 was given for morphological changes in at least 75% of the field view. Saline (n = 4), HOCl (n = 4). A two-tailed student t-test between saline and HOCl groups indicates no statistical significance ($p > 0.05$)

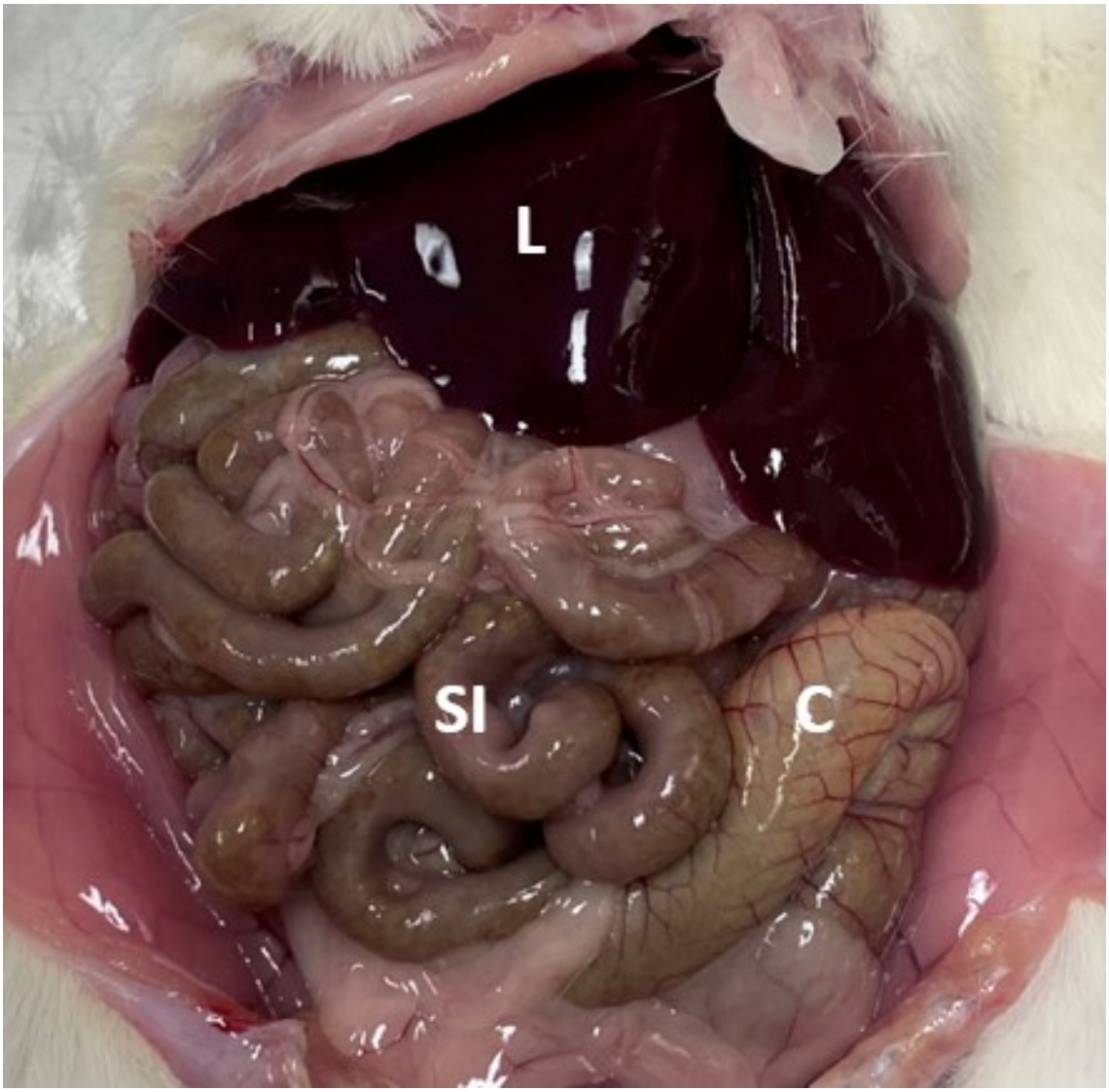

Figure 11. Adhesion score grade 0. Best representative image of healthy peritoneal cavity with no adhesions present. Small Intestine (SI), Colon (C), Liver (L).

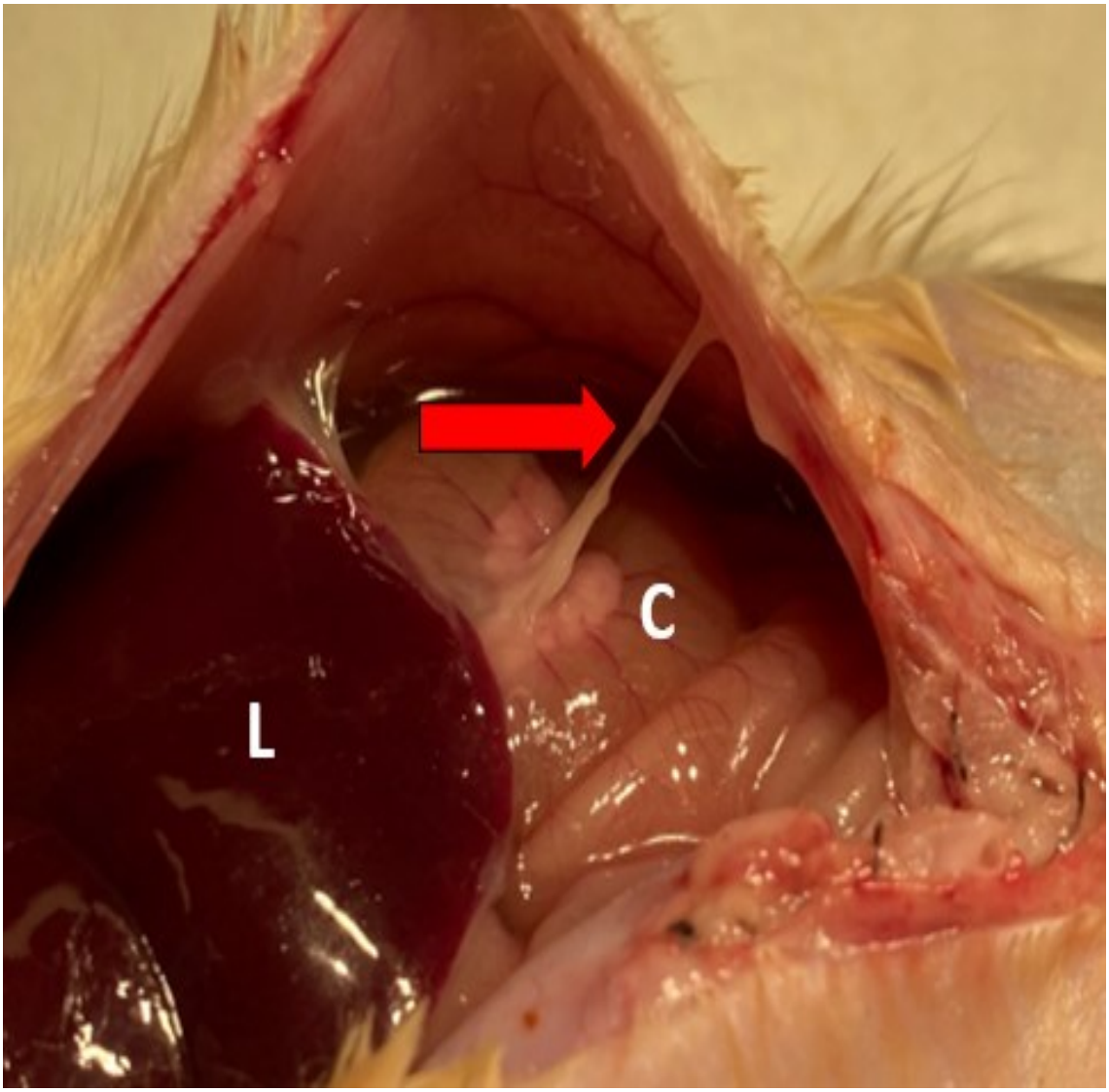

Figure 12. Adhesion score grade 1. Best representative image of peritoneal cavity with one small adhesion, indicated by the arrow, that is easily separable. Colon (C), Liver (L).

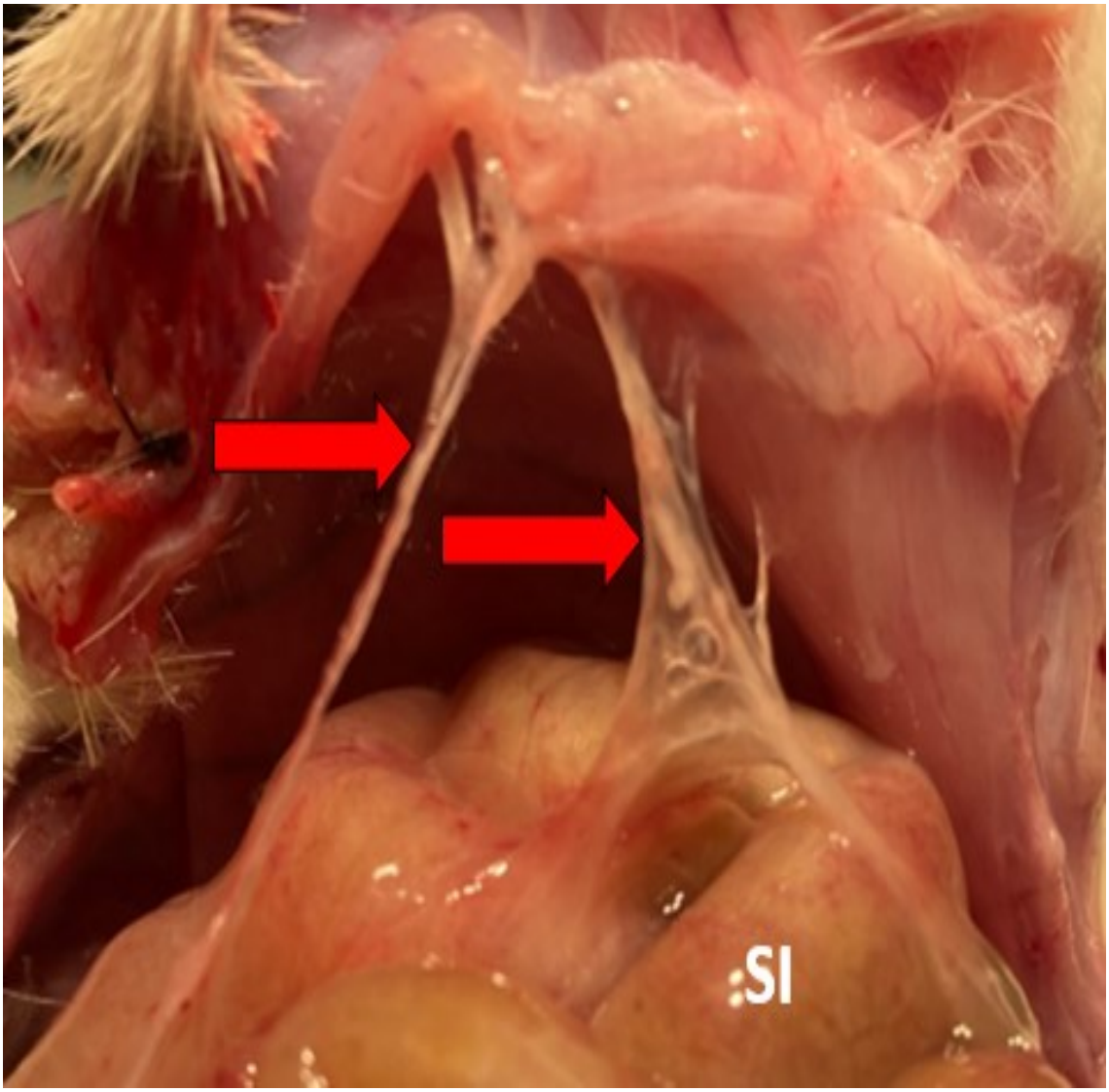

Figure 13. Adhesion score grade 2. Best representative image of peritoneal cavity with multiple adhesions that are easily separable as indicated by arrows. Small Intestine (SI).

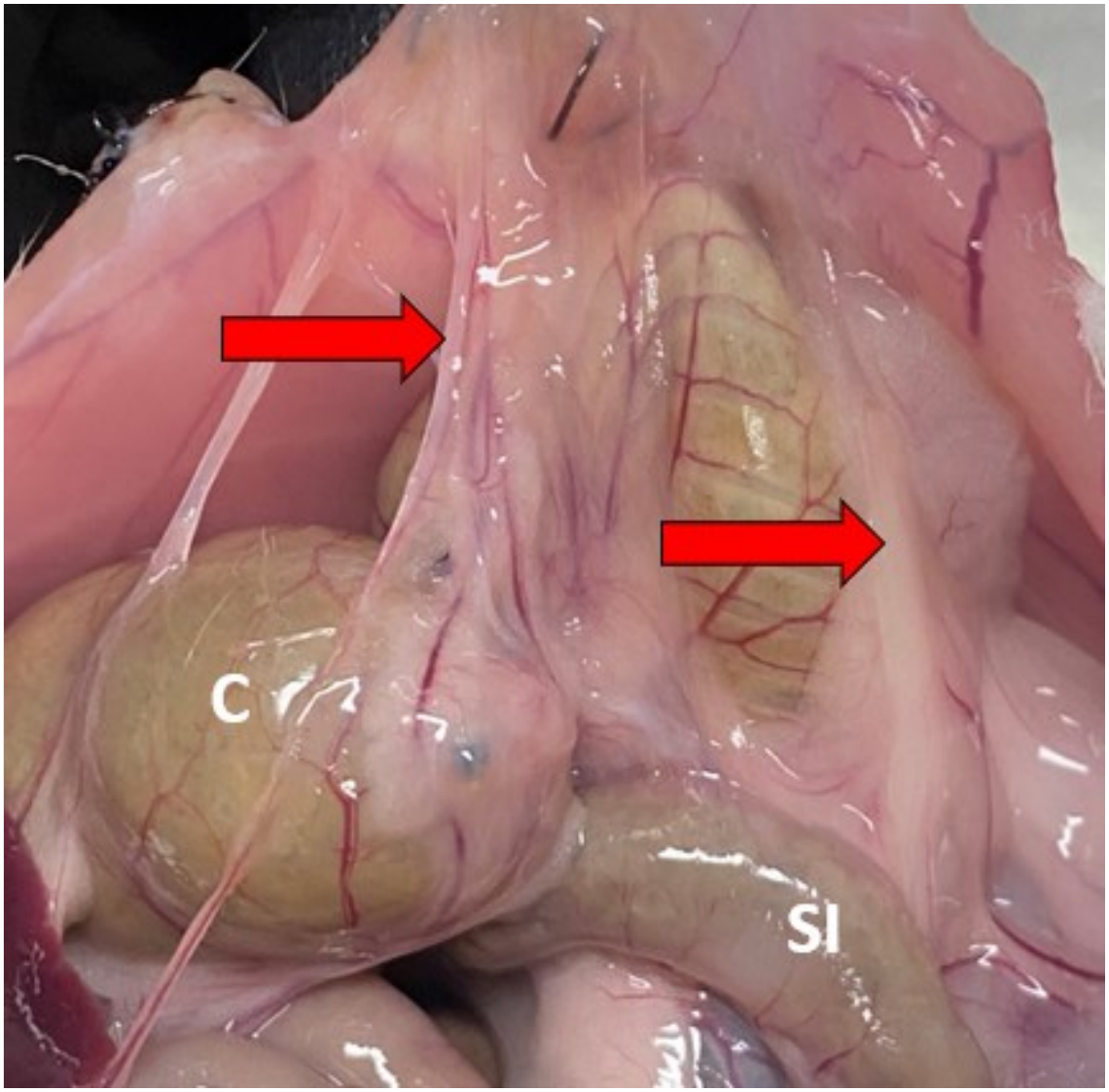

Figure 14. Adhesion score grade 3. Best representative image of peritoneal cavity with adhesion formation on the small intestine, colon, and abdominal wall with mild intensity as indicated by arrows. Small Intestine (SI), Colon (C).

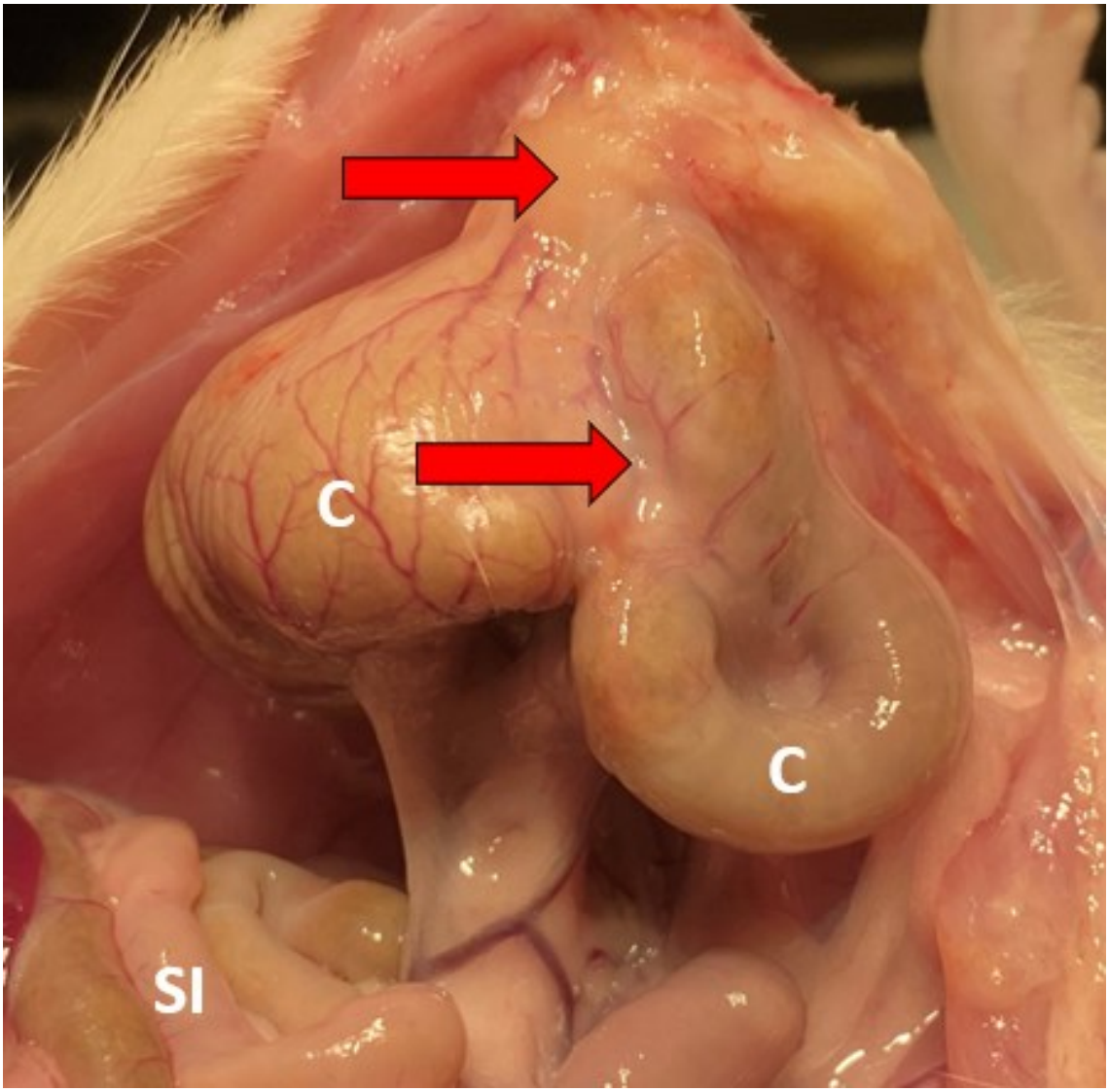

Figure 15. Adhesion score grade 4. Best representative image of peritoneal cavity with very intense adhesions that are not easily separable as indicated by the arrows. Small Intestine (SI), Colon (C).

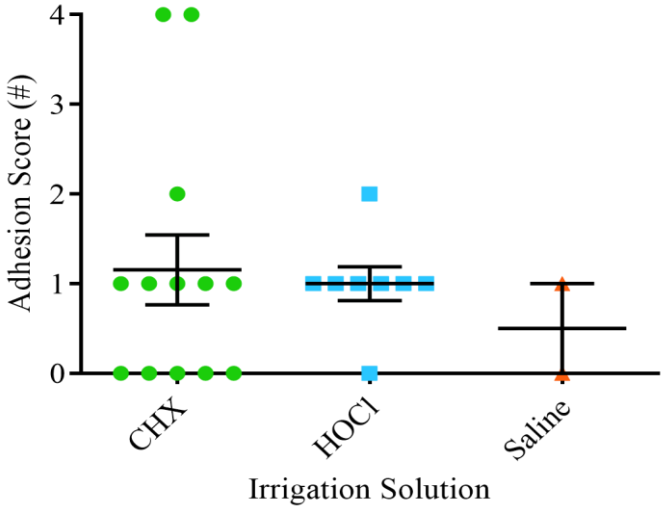

Figure 16. Early adhesion formation. Adhesion formation 1-4 days post irrigation across three treatment groups. CHX (n = 13), HOCl (n = 8), and Saline (n = 2). ($p > 0.05$)

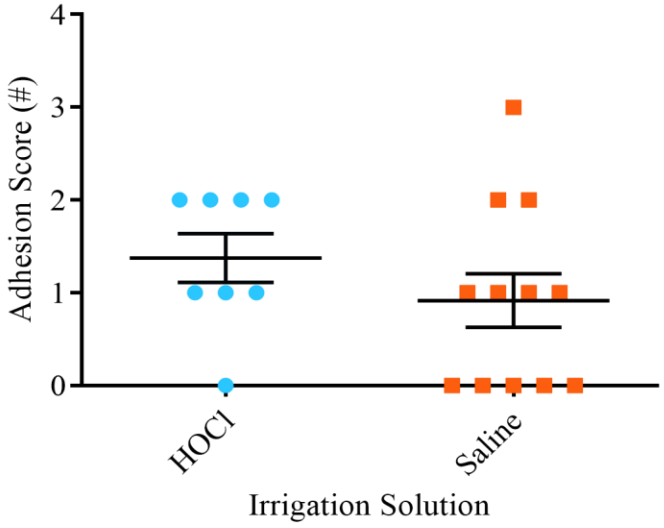

Figure 17. Late adhesion formation. Adhesion formation 5-10 days post irrigation. Stabilized HOCl (n = 7), Saline (n = 12). ($p > 0.05$)

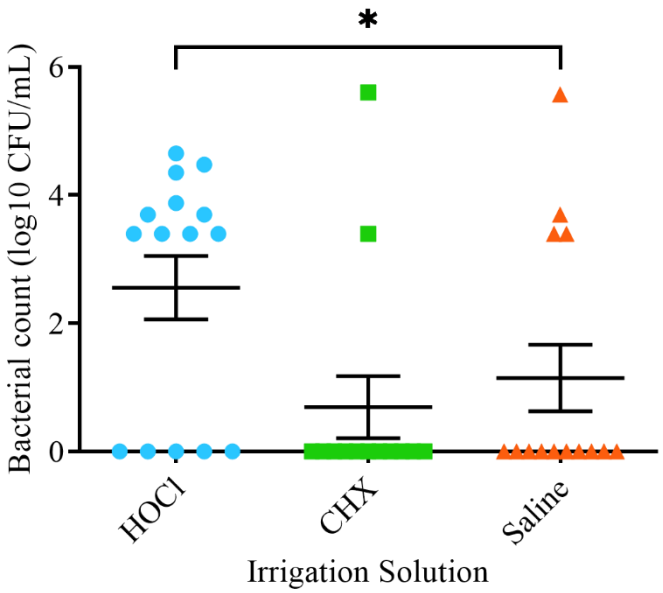

Figure 18. Bacterial counts of peritoneal fluid 24 hours post-irrigation. Peritoneal fluid of animals 24 hours post-irrigation subjected to bacterial cultures and displayed as log10 CFU/mL). Stabilized HOCl (n = 13), CHX (n = 15), and saline (n = 14). One-way ANOVA indicates no statistical significance among the three treatment groups ($p < 0.05$).

DISCUSSION

Assessment of Survival Rates of Animals Treated With Intra-Abdominal Lavage Solutions Saline, CHX, or Stabilized HOCl Suffering From CLP-Induced Peritonitis

The first aim of this project was to compare survival rates of rats affected by CLP-induced peritonitis and their subsequent treatment with different intra-abdominal lavage solutions. In a study by Riché et al. 41% of 180 patients suffering from general peritonitis progressed to septic shock with an overall survival of 19% [4]. Peritonitis generally results from bowel perforation which allows the gut flora to contact the mesothelial cells lining the intra-abdominal cavity, thus inducing an inflammatory response. Many works of literature have shown CLP in murine models to be an effective means of inducing peritonitis [36,38]. This study further reiterates the success of the CLP methodology when studying peritonitis. Of the established source control measures, improvements of intra-abdominal irrigation solutions may be a step towards improving patient prognosis and reducing mortality rates. Saline is primarily used as source control for intra-abdominal lavage; however, it lacks antimicrobial properties. CHX is another common irrigation solution but unlike saline, it possesses antimicrobial properties [33]. However, CHX's cytotoxicity has been shown to exhibit excessive damage to healthy host tissues, in the absence of follow-up irrigation with saline [34]. This may lead to excessive inflammation, further driving peritonitis-induced sepsis. Analysis of survival rates may be reflective of the effectiveness of irrigation solutions used to treat peritonitis. It was hypothesized that rats treated with stabilized HOCl lavage would present with higher survival rates than rats treated with saline or CHX; suggesting that stabilized HOCl effectively prevents peritonitis-induced sepsis.

Analysis of the survival rates allowed for the comparison of effectiveness across irrigation treatment group of stabilized HOCl, CHX, and saline. The median survival of animals treated with CHX, without the manufacturer recommendation of follow-up irrigation with saline, shows a severely poor survival rate of 1.9 days. Use of stabilized HOCl shows a significant increase of survivability compared to CHX but surprisingly no significant difference in survival when compared to saline (Figure 7). This data suggests that treatment with stabilized HOCl as an intra-abdominal lavage solution provides no benefit or detriment to survival rates when compared to treatment with saline lavage in CLP-induced peritonitis. Stabilized HOCl has been a successful treatment for superficial skin wounds when compared to saline [47] but may not provide a benefit over commonly used saline irrigation within the abdomen. While there is no statistical significance between saline and stabilized HOCl, the trend of survival rates of each group suggests that additional test subjects in each group may provide a significant difference. One potential explanation is the physiological differences between dermal, epidermal, and the mesothelial cells that line the peritoneal cavity. The skin maintains a pH of 5 while the peritoneal fluid that lines the cavity maintains a pH of 7.5 - 8.0 [48]. Stabilized HOCl maintains a pH of approximately 5.5-6.5 and may disrupt tissue homeostasis into an acidic environment, thus creating tissue dysregulation, further driving the inflammatory response. Furthermore, work by Davies et al. and Klebanoff have shown that excess HOCl results in cytotoxicity to healthy host tissues, further driving inflammation and tissue damage [49,50]. While 0.9% saline, which also maintains a pH of 5.5, may disrupt the intraperitoneal environment, it does not possess the same cytotoxic mechanisms as stabilized HOCl. Therefore, saline may induce less damage to healthy peritoneal tissue.

Another challenge facing the use of HOCl as an intra-abdominal lavage is the host cellular mechanism in response to excess reactive oxygen species present in tissues. Mammalian tissues inactivate excess ROS, such as HOCl, through anti-oxidative systems in an attempt to maintain homeostasis and improve cell viability. These antioxidant systems in peritoneal tissues may contribute to the observed lack of lavage efficacy. Information regarding antioxidant systems within the peritoneal cavity tissues and their effects on exogenous HOCl is lacking and further research is warranted to determine any effect they may have on solution efficacy. Given the recent advances in HOCl detection fluorescence probing, future experimentation could be performed to observe the degree of exogenous HOCl inactivation in peritoneal tissues.

Assessment of Acute Lung Injury via H&E Staining

The second aim of this project was to assess the potentiality of stabilized HOCl to prevent peritonitis-induced acute lung injury. Following poor acute peritonitis treatment, local infection leads to systemic inflammation and multi-organ dysregulation. During the initial stages of sepsis, systemic inflammation results in widespread cellular and organ dysfunction [10,11]. Notably, the lungs are impacted swiftly during the onset of sepsis and can be visualized through morphological changes in tissue structure [22,51]. Elevated circulating inflammatory cytokines cause increased vascular permeability and tissue infiltration of polymorphonuclear leukocytes, pro-inflammatory cytokines, and fluid [7,21,22]. Given that pulmonary function and survival is acutely affected by changes in inter-alveolar septa, alveolar integrity, and cellular infiltrate, pulmonary tissue analysis is an indicator of peritonitis-induced sepsis. It was predicted that animals treated with stabilized HOCl would prevent the onset of sepsis, thus preventing pulmonary tissue dysregulation caused by systemic inflammation.

Pulmonary tissues in the saline group presented an overall mean score of 2.25, while the stabilized HOCl group presented an overall median score of 3. This data (Figure 10) suggests that 10-day survivors treated with saline developed no significant change in pulmonary morphological changes compared to 10-day survivors treated with stabilized HOCl. Pulmonary injury observed as a result of ALI occurs in 3 phases; exudative, proliferative, and remodeling [51]. In humans, the acute exudative phase is seen during the first week following injury which include tissue edema and hemorrhage [51]. During this phase, an influx of PMNL's and RBC's are observed, initiating tissue dysregulation and decreased pulmonary function [51]. At the end of this phase, fibrin deposition and pneumocyte hyperplasia is observed and continues into the proliferative phase [51]. This phase is characterized by alveolar septa thickening, diminished PMNL infiltration, and continued fibrin deposition [51]. Some cases of ALI resolve without permanent pulmonary damage following the proliferative phase; however fibrotic activity within the lungs is likely to result in pulmonary remodeling [51]. This remodeling drastically changes pulmonary function and is viewed histologically as diminished alveolar air space and excess collagen deposition within interalveolar septa [51]. Patients who experience significant pulmonary remodeling suffer from diminished pulmonary function and higher mortality rates.

Surprisingly, this data suggests that intra-abdominal lavage with stabilized HOCl results in pulmonary injury and tissue reorganization similar to that of animals treated with saline lavage 10-days following acute peritonitis treatment. The tissues analyzed at day ten appear to be within the proliferative phase, given the minimal presence of PMNL's and the increased observation of cell proliferation. This could be from the inability of stabilized HOCl to successfully clear the bacteria foci from the peritoneal cavity, thus allowing for an elevated and sustained systemic inflammatory response from the host. Additionally, exogenous administration of HOCl to

mesothelial tissues may induce excess damage to healthy mesothelial cells, thus driving systemic inflammation leading to acute lung injury. Alternatively, another mechanism by which stabilized HOCl affects organs distant to the peritoneum may be possible through the movement of stabilized HOCl into the lymphatic system and creating a systemic inflammatory response. Due to the experimental design, pulmonary tissues were analyzed for 10-day survivor which excluded any evaluation of the CHX treatment group. Further experimentation would likely benefit from the collection and analysis of pulmonary tissues at differing timelines following peritonitis treatment. Evaluation of surviving animal tissues gain insight into the proliferative/remodeling phase which is important assessment into the possible long-term impact of pulmonary function; however, acute analysis of tissues within the early phase of ALI/ARDS may provide valuable information. Designing an experiment in which pulmonary tissues are analyzed acutely, within the first 2 days following irrigation across all treatment groups, would provide larger sample sizes, increase statistical power, and provide valuable data on the exudative timeline of ALI/ARDS in a murine model, which is lacking in literature.

Assessment of Peritoneal Adhesion Scoring

The third aim of this study was to determine if use of stabilized HOCl as a lavage solution resulted in detrimental adhesion formation compared to saline or CHX. This was addressed by performing a gross anatomical inspection of the intra-abdominal cavity following animal death. Adhesion formation is a common occurrence following abdominal surgeries and an irrigation solution which does not produce detrimental adhesions is warranted [27,34]. Adhesions were examined to determine their resistance to separation from tissues and compared to the Majuzi adhesion scoring method (Table 2). It was hypothesized that stabilized HOCl

lavage in acute peritonitis would result in less adhesion formation compared to CHX which has previously been reported to produce severe adhesions and similar adhesion formation seen in the saline treatment group. Early adhesion formation from days 1-4 were analyzed across all three groups using a one-way ANOVA. Thirteen animals treated with CHX, five treated with stabilized HOCl, and two animals treated with Saline survived a maximum of 4 days. Although CHX has been reported to result in severe adhesion formation, early adhesion scores across the three treatment groups were not significantly different (Figure 16). This may have been due in part to the low survival rates seen in animals treated with CHX. Late adhesion formation analyzed from days 5-10 post-irrigation in saline and stabilized HOCl groups were subjected to a two-tailed student t-test. This data indicates that treatment with stabilized HOCl results in no significant adhesion formation up to ten days post-irrigation when compared to rats treated with intra-abdominal lavage with saline (Figure 17). This would suggest that stabilized HOCl lavage would be a safe alternative to saline in that it does not result in detrimental adhesion formation within the abdomen. Surgical procedures within the abdomen often result in transient paralytic ileus that often resolve during post-operative recovery. It was seen that animals treated with CHX presented with severe abdominal distention compared to animals treated with saline or stabilized HOCl (see Appendix G). Rats treated with CHX, without subsequent irrigation with saline, may result in prolonged paralytic ileus, resulting in abdominal distention.

Assessment of Bacterial Contamination of Peritoneal Fluid 24 Hours Post-Irrigation

The fourth aim of this study was to determine the effectiveness of stabilized HOCl and by assessing the level of bacteria within the peritoneal fluid 24 hours post-irrigation. Poor source control results in sustained bacteria foci within the abdomen, resulting in chronic peritonitis and

the likely progression to sepsis. The lack of saline's antimicrobial characteristic is a hinderance to its effectiveness in clearing bacterial contamination. A lavage solution that possesses antimicrobial properties could provide an advantage over saline. It was hypothesized that animals treated with stabilized HOCl would present with decreased peritoneal bacterial presence 24 hours after irrigation compared to animals treated with saline. Bacteria cultures were grown and analyzed by Dr. Christopher Lupfer's Missouri State University biology team member Emma Loder. This data suggests use of stabilized HOCl as a peritoneal lavage does not effectively decrease bacterial foci within the abdomen when compared to CHX or saline. CHX is known for its aggressive cytotoxic properties [34] so it was expected that bacterial counts would be lower in the CHX treatment group (Table 11 and Figure 18). Bacterial counts of peritoneal fluid in animals treated with stabilized HOCl presented with a higher bacterial presence compared to CHX and saline 24 hours after irrigation. Despite HOCl's antimicrobial properties, administration to the peritoneal cavity appears to have neither the anticipated antibacterial effect nor improved subject survival. It should be noted that while animals were fed the same diet, stool consistency during CLP procedure was observed to be different among subjects. This would suggest that initial bacterial contamination from CLP may vary, thus inducing a varied degree of inflammatory response. Thus, subjects treated with saline may have had less initial bacterial contamination from CLP than subjects treated with stabilized HOCl.

Assessment of Blood Serum TNFα Levels

The fifth aim for this study was to assess blood serum TNFα levels of rats 24 hours after intra-abdominal irrigation with stabilized HOCl, CHX, or saline to determine a solutions effect on systemic inflammation. Poor peritonitis treatment results in sustained bacterial presence

within the peritoneal cavity. This leads to chronic peritonitis and eventual progression to sepsis. As inflammatory mediators from the source of infection migrate to the circulatory system, systemic levels of pro-inflammatory cytokines, such as TNFα, increase and further drive inflammation [10,52]. An effective lavage solution for treating peritonitis should remove peritoneal bacteria thus reducing the inflammatory response induced by pro-inflammatory cytokines. This reduction in pro-inflammatory cytokines produced in local peritoneal tissues lessens the systemic inflammatory response, thus resulting in decreased serum levels of TNFα. It was hypothesized that stabilized HOCl would decrease systemic elevated pro-inflammatory TNFα levels compared to saline or CHX. Surprisingly, ELISA analysis reported undetectable levels of TNFα within the blood serum of all subjects. This was unexpected as systemic inflammation results in the upregulation of TNFα during early phases of inflammation and sepsis. Findings by Aliomrani et al. reported detectable serum TNFα levels 24 hours following CLP in Wistar rats in the absence of abdominal irrigation [52]. Consequently, it was unexpected to observe subjects dying of peritonitis-induced sepsis and presenting no serum TNFα after infection. It was anticipated that subjects that died by day 2 or 3 from sepsis would have detectable elevated levels of TNFα. Complete absence of ELISA results may indicate poor blood serum preparation or inadequate storage. Blood serum stored long term at incorrect temperatures can negatively impact sample quality and result in a poor analysis. Additionally, poor or old reagents provided with the ELISA kit can affect the outcome of ELISA testing and result in a complete lack of results. Due to time constraints of this project, ELISA troubleshooting could not be performed but it is likely that improper sample storage prior to ELISA is the cause of the lack of results for all animal subjects.

CONCLUSIONS AND FUTURE DIRECTIONS

Peritonitis, an intra-abdominal infection, is a medical emergency that is common in human medicine. Most occurrences of peritonitis are sourced from bowel perforations due to gastrointestinal ulcers or trauma. Treatment relies on categorization of peritonitis resulting from contamination, infection, or sepsis, with surgical intervention, peritoneal lavage, and antibiotic therapy being standard treatment [1]. A common post-operative complication is recurring intra-abdominal infections that place patients at a high risk for developing sepsis, contributing to high mortality rates [1,6]. Consequently, the focus for increasing survival rates associated with peritonitis-induced sepsis is to reduce recurring peritoneal infections. Peritoneal lavage with saline is routinely performed as a measure of source control, and while saline is sufficient for removing cellular debris, it lacks antimicrobial properties. Lavage solutions that kill pathogens but maintain healthy host tissue is warranted to prevent recurring infections, thus increasing survival rates. HOCl, which has antimicrobial properties, has been used in previous studies with successful elimination of bacteria but HOCl production and solution stability is transient and not suitable for clinical applications [23,36,47]. However, recently developed stabilized HOCl has shown to be beneficial in superficial wound healing and may be suitable for intra-abdominal irrigation [42,43]. Research about its use for peritoneal irrigation is insufficient, which calls for further research.

HOCl is produced naturally by mature neutrophils within the body in response to pathogenic invasion. This is synthesized during the respiratory burst event leading to an abundant production of ROS within neutrophil phagosomes, which destroy microbes [29]. These ROS are formed from available intracellular halides and H_2O_2 and act as potent oxidants that lead

to the disruption of bacterial protein synthesis and bacterial death [14,37,37]. This study, in part, aimed to research the effectiveness of stabilized HOCl as a lavage solution in the treatment of acute peritonitis. This study demonstrated that bacteria were found within the peritoneal fluid of more animals treated with stabilized HOCl than that of animals treated with saline or CHX (Figure 18). This study suggests that stabilized HOCl is not an effective intra-abdominal lavage given the enduring bacterial counts in the abdomen following lavage and trend of low survivability. This might be due, in part, to antioxidant systems of peritoneal tissues, diminishing the effectiveness of stabilized HOCl against bacterial contamination. Regardless of a standardized diet, it was noted that animals within the study had different stool consistencies during the CLP procedure. These inconsistencies may have resulted in higher bacterial contamination of the peritoneal cavity, following the cecal ligation and puncture procedure.

As a result of an overabundance of bacterial contamination within the peritoneal cavity, peritonitis can progress to systemic inflammation, or sepsis. During sepsis, pro-inflammatory cytokine production is elevated and is detectable in blood serum. Cytokines such as TNFα induce systemic inflammation and act as a positive feedback mechanism in uncontrolled infection [10,11]. It was expected that stabilized HOCl would eliminate excess bacteria found within the abdomen following CLP and prevent a large systemic inflammatory response. Thus, animals treated with stabilized HOCl intra-abdominal lavage would result in lower levels of TNFα within the blood serum, 24 hours after irrigation, indicating diminished systemic inflammation. The results of this project suggest that regardless of the lavage solution used, no TNFα is detected within the blood serum, 24 hours after irrigation for acute peritonitis treatment. This does not support the previously reported characteristics of inflammation and inflammatory mediators during sepsis in murine peritonitis models [10,52]. This result is confounding, however given the

amount of time in which the samples were stored, storing the serum at -80 ˚C as opposed to -20 ˚C would likely prevent sample degradation. This hypothesis could be addressed in future experimentation for pro-inflammatory cytokine analysis following abdominal irrigation. Alternatively, analysis of other proinflammatory mediators such as interleukin 1 and interleukin 6 may be a better indication of inflammation as they are present much later during the infectious timeline, as opposed to the transient elevation of TNFα.

Sepsis is characterized by multiple organ failure due to tissue dysregulation brought on my chronic elevations in systemic pro-inflammatory mediators. These mediators result in tissue edema due to increased vascular permeability and a large influx of PMNL's, RBC's, and fluid within the interstitial space of organs [16]. This event leads to an impairment of tissue function and morphological changes of tissue structure. Pulmonary tissues are quickly altered during sepsis and the morphological changes in survivors may lend insight to the severity of sepsis. A goal of this project was to evaluate the effectiveness of stabilized HOCl in preventing the onset of peritonitis-induced sepsis. Qualitative analysis of H&E stained pulmonary tissues of 10-day survivors treated with saline and stabilized HOCl suggest that tissue reorganization was not significantly different (Figure 10). This is suggestive that stabilized HOCl provides no benefit over saline in preventing or reducing acute-lung injury due to peritonitis-induced sepsis and does not significantly prevent pulmonary dysfunction.

Patients who undergo peritonitis treatment with surgical intervention often present with post-operative adhesions or fibrosis, which contribute to poor patient prognosis. Adhesions are caused by excessive tissue damage within the peritoneal cavity and can be minimized by reducing the amount of healthy tissue damage [27,34]. Saline has not yet previously been reported to induce adhesion formation but its inability to kill bacteria may limit its effectiveness

as a lavage solution. A lavage solution that possesses antimicrobial properties but also does not induce detrimental adhesion formation might prove beneficial over saline. Our findings indicate that the use of stabilized HOCl as an intra-abdominal lavage solution produces no detrimental adhesion formation in comparison to saline or 0.05% CHX (Figures 16-17), which is reported to induce significant adhesion formation and fibrosis within the abdomen [34,35].

The main goal of this study was to evaluate the effectiveness of stabilized HOCl as an intra-abdominal lavage treatment for acute peritonitis and its effect on survival rates. Following peritonitis treatment, patients are at risk of developing recurring peritoneal infections which may lead to sepsis. One study found that out of 108 patients suffering from peritonitis, 41% progressed to sepsis with an overall survival rate of 19% [4]. Another study by Ballus et al. reported that out of 343 patients suffering from peritonitis, 54% progressed to tertiary peritonitis and of those patients, 48% died [44]. This data emphasis the need for improved source control measures in peritonitis patients. The standard treatment of acute peritonitis, following a bowel perforation, includes tissue debridement followed by cavity lavage. While saline is currently the preferred intra-abdominal lavage solution, it lacks antimicrobial properties, which may limit its overall efficacy. Given the efficacy of stabilized HOCl as a superficial wound lavage solution, it has been proposed to be an effective intra-abdominal lavage solution. Our data suggests that animals who were treated with an intra-abdominal irrigation of stabilized HOCl had no significant change in survival rates compared to animals treated with saline irrigation (Figure 7). Overall, the data collected in this project does not support the use of stabilized HOCl as an intra-abdominal lavage but supports the continued use of saline as the preferred irrigation for peritonitis treatment.

Given the trend of survival rates between stabilized HOCl and saline, further experimentation could include the addition of a few subjects for each group to determine if statistical significance would be achieved. Pulmonary tissue morphology was scored by one blinded researcher due to time constraints; however, it would be beneficial to have the tissues analyzed by additional blinded researchers. There are also more 10-day survivors for saline and stabilized HOCl which can be analyzed histologically, therefore increasing statistical power. Experimental design which analyzes lung tissue acutely following irrigation would also provide a strong statistical analysis as the timeline for comparison becomes standardized across all treatment groups, and not solely for surviving animals. Future projects utilizing this model for peritonitis could utilize different means of euthanasia, such as transcardial perfusion prior to lung tissue analysis to differentiate acute lung injury as a result from systemic inflammation or pulmonary edema following CO_2 euthanasia. Carbon dioxide induces pulmonary hemorrhaging and thus alters morphological observations [53], which would be undesirable when comparing pulmonary tissue samples acutely after treatment.

It would be feasible to accrue additional data to further investigate the potential effects of stabilized HOCl intra-abdominal irrigation and the development of acute and chronic lung injury. The effectiveness of intra-abdominal lavage solutions may also be assessed by altering the volume of solutions utilized and contact time within the abdomen. Initial irrigation with an antimicrobial solution followed by a washout with saline to remove cellular debris may provide additional insight into advancements in source control for peritonitis. Experimental design to analyze any detrimental cytotoxic effects of stabilized HOCl to the mesothelium and peritoneal tissues could also be assessed by the omission of the CLP procedure. Abdominal irrigation with these solutions, in the absence of pathogenic invasion, would provide valuable data to the effects

of stabilized HOCl to healthy peritoneal tissues. Additionally, treatment and analysis of cell cultures of dermal epithelium and mesothelial cells with stabilized HOCl and saline would provide insight on the differences of solution effectiveness observed in this study. Repeated experimentation with serum cytokine profiles of additional subjects may provide insight on the lack TNFα detection following peritonitis; one could assess ELISA kit reagents for solution quality. Analysis of serum IL-1 and IL-6 may prove to be better indicators of systemic inflammation in a murine model. There may also be a benefit to collecting peritoneal fluid during tissue resection and prior to irrigation to establish baseline for the severity of bacterial contamination following CLP. These cultures, when compared to peritoneal fluid collected after irrigation may provide insight on a lavage solutions ability to remove contamination within the peritoneal cavity. Blood samples could also be drawn at different times post-CLP to analyze changes with serum proinflammatory mediators to assess systemic inflammation. Additional histology analysis, such as immunohistology, of previously harvested ileum, cecal, and abdominal wall tissues could also be performed to analyze lavage cytotoxicity of host tissues.

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APPENDICES

Appendix A: IACUC Approval Notice

March 1, 2021

RE: IACUC Protocol 2020-14 amendment

Hello Patrick,

The amendment for IACUC protocol 2020-14, "Treatment of Peritonitis with Hypochlorous acid" has been approved.

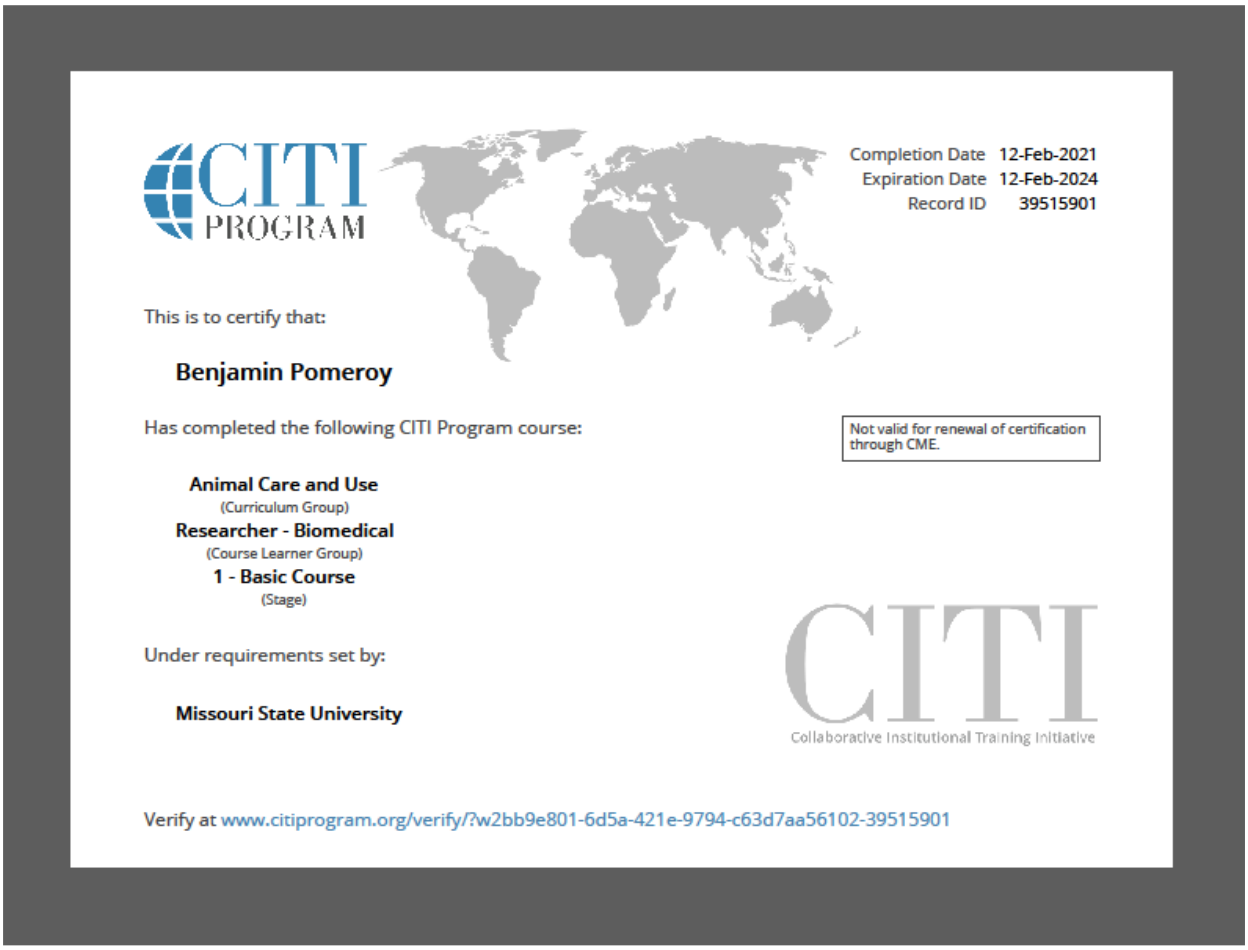
Thank you and if you need anything in the future regarding this protocol please contact me either via email (janeneproctor@missouristate.edu) or at 417-836-8419.

Sincerely,

Janene Proctor

OFFICE OF RESEARCH ADMINISTRATION
901 South National Avenue, Springfield, MO 65897 • Phone: 417-836-5972
www.missouristate.edu

Appendix B: Animal Care and Use Certification



CITI PROGRAM

Completion Date 12-Feb-2021
Expiration Date 12-Feb-2024
Record ID 39515901

This is to certify that:

Benjamin Pomeroy

Has completed the following CITI Program course:

Not valid for renewal of certification through CME.

Animal Care and Use
(Curriculum Group)
Researcher - Biomedical
(Course Learner Group)
1 - Basic Course
(Stage)

Under requirements set by:

Missouri State University

CITI
Collaborative Institutional Training Initiative

Verify at www.citiprogram.org/verify/?w2bb9e801-6d5a-421e-9794-c63d7aa56102-39515901

Appendix C: Reducing Pain and Distress in Laboratory Mice and Rats Certification

CITI PROGRAM

Completion Date 12-Feb-2021
Expiration Date N/A
Record ID 40908926

This is to certify that:

Benjamin Pomeroy

Has completed the following CITI Program course:

Not valid for renewal of certification through CME.

Reducing Pain and Distress in Laboratory Mice and Rats
(Curriculum Group)
Reducing Pain and Distress in Laboratory Mice and Rats
(Course Learner Group)
1 - Basic Course
(Stage)

Under requirements set by:

Missouri State University

CITI
Collaborative Institutional Training Initiative

Verify at www.citiprogram.org/verify/?we2b04814-3ab6-4f13-b101-e48ba832b422-40908926

Appendix D: Responsible Conduct of Research Certification

CITI PROGRAM

Completion Date 20-Feb-2021
Expiration Date N/A
Record ID 41075442

This is to certify that:

Benjamin Pomeroy

Has completed the following CITI Program course:

Not valid for renewal of certification through CME.

Responsible Conduct of Research (RCR)
(Curriculum Group)
Responsible Conduct of Research (RCR)
(Course Learner Group)
1 - Basic Course
(Stage)

Under requirements set by:

Missouri State University

CITI
Collaborative Institutional Training Initiative

Verify at www.citiprogram.org/verify/?w789311f9-ba51-46b2-9fc2-a439f1d4e4b1-41075442

Appendix E: Working With Rats in Research Certification

CITI PROGRAM

Completion Date 19-Feb-2021
Expiration Date N/A
Record ID 40908927

This is to certify that:

Benjamin Pomeroy

Has completed the following CITI Program course:

Not valid for renewal of certification through CME.

Working with Rats in Research Settings
(Curriculum Group)
Working with Rats
(Course Learner Group)
1 - Basic Course
(Stage)

Under requirements set by:

Missouri State University

CITI
Collaborative Institutional Training Initiative

Verify at www.citiprogram.org/verify/?w6a2df3b0-04d8-43bb-b366-13d8513b3a4f-40908927

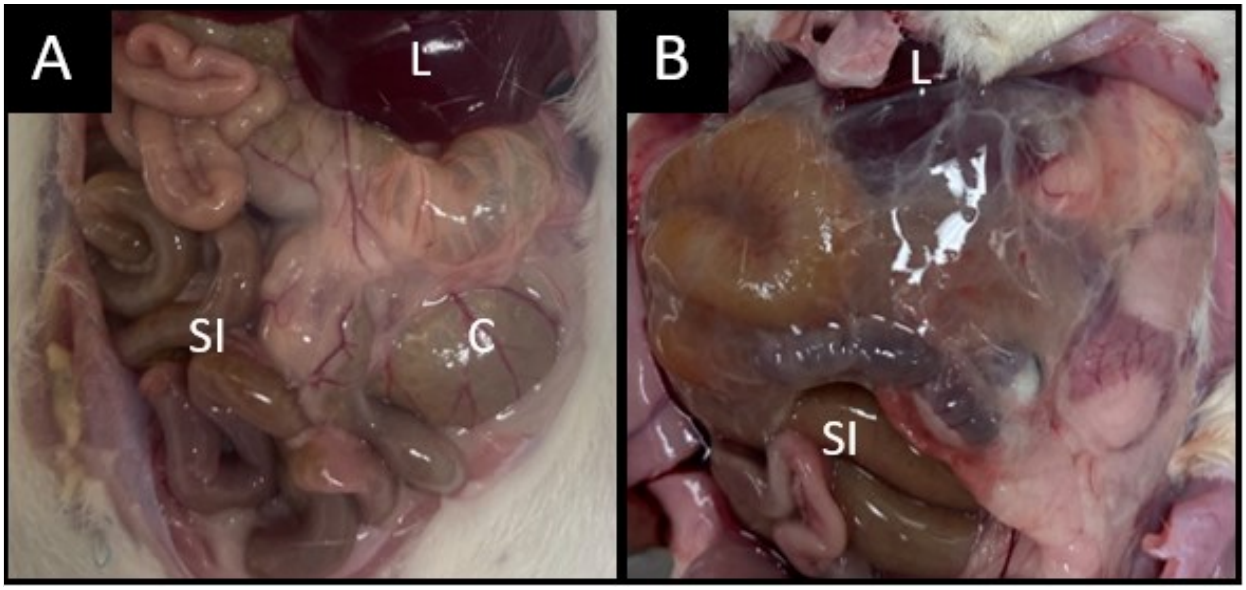
Appendix F: Assignment of Treatment Solution Groups to Subjects in Protocol 2020-14

Key:	Green	Pink	Blue
	Red/Orange	Yellow	Conical Tube

Protocol 2020-14 Irrigation ID List					
Female Cohort			Male Cohort		
Solution	ID	Notes	Solution	ID	Notes
HOCl #3	6.12		HOCl #2	5.51	conical tube
HOCl #1	6.13		HOCl #1	5.53	
HOCl #1	6.7		HOCl #1	6.23	
HOCl #2	7.40		HOCl #3	7.11	
HOCl #2	7.34		HOCl #1	6.29	
HOCl #4	7.39		HOCl #6	8.13	
HOCl #4	7.36		HOCl #5	8.14	
Saline #1	5.32		HOCl #2	7.12	Spilled male green #2 bottle (which was CHX), used female green #2 bottle instead (which was HOCl).
Saline #3	6.10		Saline #3	5.52	
Saline #1	6.11		Saline #2	6.24	
Saline #3	7.33		Saline #2	6.27	
Saline #2	7.41		Saline #1	6.28	
Saline #5	8.38		Saline #3	7.17	
Saline #5	7.37		Saline #4	7.14	
CHX #2	5.33		Saline #6	8.15	
CHX #3	6.8		Saline #5	Extra/Not Used	
CHX #2	6.9		CHX #1	5.50	conical tube
CHX #3	7.32		CHX #3	6.25	Died during surgery
CHX #1	7.35		CHX #3	6.26	
CHX #6	8.49		CHX #2	Spilled	Spilled male green #2 bottle (which was CHX), used female green #2 bottle instead (which was HOCl).
CHX #6	7.38		CHX #2	7.13	
			CHX #3	7.16	
			CHX #5	8.12	
			CHX #4	7.15	

Solutions were prepared and assigned unique identifications known only to blinded researcher (ex. Yellow Female #3). Once a solution had been utilized during irrigation, the solution ID was provided to blinded researcher who then assigned to the animal ID of which it had been used (ex. Yellow Female #3 – ID 6.12). Following all animal deaths and data analysis, the blinded researcher revealed the treatment group for which each solution identification belonged.

Appendix G: Comparison of Peritoneal Cavity and Organs of Subjects Treated With Saline vs CHX



A. Best representative image of healthy peritoneal cavity of rat treated with saline surviving 10 days. B. Best representative image of peritoneal cavity with abdominal distention 1 day after irrigation with CHX. Small Intestine (SI), Liver (L), Colon (C).