BearWorks

MSU Graduate Theses

Fall 2022

Effects of Natural Products on Inflammation

Riley Ann Nadler
Missouri State University, Nadler0205@live.missouristate.edu

As with any intellectual project, the content and views expressed in this thesis may be considered objectionable by some readers. However, this student-scholar's work has been judged to have academic value by the student's thesis committee members trained in the discipline. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

Follow this and additional works at: https://bearworks.missouristate.edu/theses

Part of the Immunology and Infectious Disease Commons

Recommended Citation

Nadler, Riley Ann, "Effects of Natural Products on Inflammation" (2022). *MSU Graduate Theses*. 3814.
https://bearworks.missouristate.edu/theses/3814

This article or document was made available through BearWorks, the institutional repository of Missouri State University. The work contained in it may be protected by copyright and require permission of the copyright holder for reuse or redistribution.
For more information, please contact bearworks@missouristate.edu.

EFFECTS OF NATURAL PRODUCTS ON INFLAMMATION

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Riley Ann Nadler

December 2022

Copyright 2022 by Riley Ann Nadler

EFFECTS OF NATURAL PRODUCTS ON INFLAMMATION

Biology

Missouri State University, December 2022

Master of Science

Riley Ann Nadler

ABSTRACT

Chronic inflammation is characterized by infiltration of inflammatory cells such as macrophages and lymphocytes into the tissue where they produce inflammatory cytokines that contribute to tissue damage. Worldwide, 3 out of 5 people die due to chronic inflammatory diseases like cardiovascular diseases, obesity, diabetes, and cancer. Since it is well-documented that diet and metabolism are key mediators of inflammation, I investigated the effects of dietary lectins on inflammatory cytokine production and the ability of sodium pyruvate, a metabolite, to decrease inflammation. In chapter 1, I examined the effect that lectins from either *Triticum vulgaris* (common wheat) or *Phaseolus vulgaris* (common bean) had on bone marrow derived macrophages infected with LPS + ATP or IAV. During infection, neither lectin significantly affected the levels of inflammatory cytokines IL-1β or IL-6. However, when the cells were uninfected but treated with the bean lectin, a significant amount of background inflammation was observed. While the presence of the lectins may not exacerbate an infection, they could contribute to a pre-existing inflammatory condition. In chapter 2, I collaborated with a company (Emphycorp) and investigated the effects of sodium pyruvate nasal spray on the symptoms of lung diseases like pulmonary fibrosis (PF), COVID-19 and long-COVID. All of these respiratory diseases result from excessive acute or chronic inflammation and can exacerbate each other (i.e. PF patients have more severe COVID-19, and COVID-19 can result in PF). Three separate clinical trials were conducted in COVID-19 infected patients, long-COVID patients, and pulmonary fibrosis patients to determine the efficacy of N115, a sodium pyruvate nasal spray. During an active COVID-19 infection, N115 decreased viral titers and improved some patient symptoms. However, it was more effective in chronic diseases (long-COVID and PF patients), where N115 significantly increased SaO_2 levels, improved lung function, headache, coughing/sneezing and breathing. Overall, my research demonstrates that dietary constituents and metabolic products can have harmful or beneficial effects on inflammation.

KEYWORDS: lectin, inflammation, metabolite, cytokine, influenza A virus, LPS, COVID-19, infectious disease, chronic inflammation, pyruvate

EFFECTS OF NATURAL PRODUCTS ON INFLAMMATION

By

Riley Ann Nadler

A Master's Thesis
Submitted to the Graduate College
Of Missouri State University
In Partial Fulfillment of the Requirements
For the Degree of Master of Science, Biology

December 2022

Approved:

Christopher R. Lupfer, Ph.D., Thesis Committee Chair

Babur S. Mirza, Ph.D., Committee Member

Kyoungtae Kim, Ph.D., Committee Member

Julie Masterson, Ph.D., Dean of the Graduate College

In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. Lupfer for his patience, kindness, and guidance during my journey through graduate school. He never gave up on me, even when I accidentally used TEMED instead of TMB. He always told me that as long as I learn something from my mistake, then that mistake was worth it. I can't express enough how grateful I am to have had such a genuinely good human as my advisor and mentor. I would also like to thank Dr. Mirza and Dr. Kim for their kindness and guidance as my committee members.

None of this would have been possible without the help and love of my lab mates, especially Devyn Worthley, Cathy Rippe, and Nayeon Son. They were always there to lend a helpful hand or to offer me advice, guidance, or a shoulder to cry on as I worked my way through this project. I wouldn't want to go through this without anyone else by my side.

I am so grateful for all of the opportunities I've had through Dr. Lupfer's lab, especially getting to present my poster on the long-COVID trial at the Immunology 2022 conference in Portland, OR alongside my friends.

I dedicate this thesis to my loved ones for the constant support and love they give me every day.

TABLE OF CONTENTS

Overview
- Background — Page 1
- Inflammation — Page 2
- Dysregulated Inflammation — Page 9
- Dietary Molecules and Metabolites — Page 11
- Scientific Question and Hypothesis — Page 12

Effects of Dietary Lectins on Macrophage Inflammatory Cytokine Production
- Abstract — Page 13
- Introduction — Page 14
- Materials and Methods — Page 16
- Results — Page 19
- Discussion and Conclusions — Page 22
- References — Page 25

Inhalation of Sodium Pyruvate to Reduce the Symptoms and Severity of Respiratory Diseases Including COVID-19, Long COVID, and Pulmonary Fibrosis
- Abstract — Page 26
- Introduction — Page 27
- Materials and Methods — Page 29
- Results — Page 31
- Discussion — Page 40
- Acknowledgements — Page 44
- References — Page 45

Overall Conclusions — Page 47

References — Page 49

Appendices
- Appendix A — Page 53
- Appendix B — Page 68
- Appendix C — Page 100
- Appendix D — Page 107

LIST OF TABLES

Inhalation of Sodium Pyruvate to Reduce the Symptoms and
Severity of Respiratory Diseases Including COVID-19,
Long COVID, and Pulmonary Fibrosis

Table 1. Patient Demographics and Symptom Data Page 33

Table 2. Patient Demographics and Symptom Prevalence Page 36

LIST OF FIGURES

Overview

Figure 1. Inflammatory pathway-mediated production of IL-6 Page 6

Figure 2. Production of IL-1β via NLRP3 inflammasome activation Page 8

Effects of Dietary Lectins on Mouse Bone Marrow Derived Macrophage Inflammatory Cytokine Production

Figure 1. 12-well plate layout for infection scheme. Page 18

Figure 2. Pro-inflammatory cytokine levels following LPS + ATP and lectin treatment. Page 21

Figure 3. Pro-inflammatory cytokine levels following Influenza A virus (IAV) and lectin treatment. Page 21

Inhalation of Sodium Pyruvate to Reduce the Symptoms and Severity of Respiratory Diseases Including COVID-19, Long COVID, and Pulmonary Fibrosis

Figure 1. Effects of N115 treatment in mice infected with SARS Page 31

Figure 2. Effects of N115 treatment in Active COVID-19 Infecti Page 34

Figure 3. Results from N115 treatment of Long COVID Patients. Page 37

Figure 4. Sub-Chronic treatment of PF patients with N115. Page 38

Figure 5. Acute treatment of PF with N115. Page 39

OVERVIEW

Background

In order to survive and to thrive, a strong, properly functioning immune system is crucial. All cells require adequate nutrition to function optimally, including the immune cells. When the immune system is actively fighting an infection, there is an increased rate of metabolism, which requires more energy and substrates like vitamins, trace elements, amino acids, and fatty acids. Diet and metabolism have a huge effect on the immune response, since our diet is what primarily provides the needed nutrients for cellular functions. Diet not only directly affects the immune system, but "you are what eats what you eat", meaning the types of foods you consume can alter your gut microbiota and environment, which can alter your immune response too [1]. Nutrients derived from diet or endogenous pathways that produce and divert metabolites into other pathways regulate the initiation, duration, and termination of the inflammatory response [2]. When these nutrients are not provided, the immune system cannot function properly. However, over-nutrition and obesity also alter the immune system.

In our society, a Western diet characterized by an overconsumption of calorically rich foods, processed foods, refined sugars, and saturated fats combined with chronic overnutrition, and a sedentary lifestyle promotes a state of chronic metabolic inflammation [3]. Chronic low-grade inflammation in adipose tissue is a hallmark of obesity and metabolic disease and is characterized by an accumulation of T cells, macrophages, and other mediators such as inflammatory cytokines. Along with obesity, chronic inflammation has been linked to many other diseases such as cardiovascular disease, rheumatoid arthritis, autoimmune diseases, and other metabolic disorders [4]. With more than half of the American population being obese or

overweight and 3 of 5 people worldwide dying due to chronic inflammatory diseases, it is extremely important to study the effects of diet and metabolic processes on the immune system [5].

As of 5:06 PM CET, November 7th, 2022, there have been ~ 630 million confirmed cases of COVID-19, including ~ 6.6 million deaths reported to WHO [6]. While the initial chaos of the pandemic has calmed, the COVID-19 pandemic continues to affect people every day. The COVID-19 pandemic caused a lot of people to start wondering how best they could support and strengthen their immune system, whether that be through dietary changes or consumption of supplements. However, it is important to realize how diet and inflammatory disorders prior to infection with COVID-19 could significantly impact the disease outcome. Since chronic inflammatory diseases cause delay and dysfunction of the immune response to pathogens, conditions such as obesity, metabolic syndrome, type 2 diabetes, cardiovascular disease, and hypertension are risk factors for increased severity of COVID-19 [7]. Thus, understanding the connection between diet, metabolism, and inflammation is important for infectious diseases as well as chronic inflammatory conditions like diabetes or arthritis.

Inflammation

Role of Inflammation During Infectious Disease. Inflammation is a critical aspect of how the immune system responds to harmful stimuli, such as cell damage, irradiation, pathogens, or toxic compounds. It works to eliminate these stimuli and initiate the healing process [8]. Inflammation is characterized by redness, swelling, heat and pain. There are critical events that occur during inflammation including alterations in vascular permeability, leukocyte recruitment and inflammatory mediator release, such as cytokines and chemokines. Cytokines are small,

secreted proteins that facilitate communication between immune cells and assist in the resolution of infectious diseases [9]. Chemokines are small, secreted proteins that are able to stimulate the migration of leukocytes. They play a critical role in the development and homeostasis of the immune system and are involved in all protective and destructive immune/inflammatory responses [10]. This is accomplished by attracting leukocytes to tissues during inflammation and response to infection [11]. While the inflammatory response depends on the kind of initial stimulus and location in the body, all inflammatory responses are mechanistically similar. First, cell surface pattern receptors recognize the stimuli. Second, these receptors activate inflammatory pathways. Third, inflammatory markers/mediators are released. Last, inflammatory cells are recruited to the area to fight the infection [8].

Inflammatory Pathways. Inflammatory pathways affect the pathogenesis of many chronic diseases and often involve multiple regulatory pathways and inflammatory mediators. Innate immune cells express pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs), which are microbial components like lipopolysaccharide (LPS), or damage-associated molecular patterns (DAMPs), which are molecules that are released by necrotic cells and damaged tissue (i.e. ATP). Relevant to my research, LPS interacts with toll-like receptors (TLRs) and ATP is detected by Nod-like receptors (NLRs). Viral RNA in the cytoplasm stimulates three different inflammatory immune pathways: retinoic acid-induced gene-I (RIG-I), TLRs and NLRs. These pathways are responsible for the immune response to viral infection, specifically RNA viruses [9]. Regardless of the pathway, receptor activation triggers intracellular signaling pathways such as the nuclear factor kappa-B (NF-κB) pathway or activation of the inflammasome.

Toll-like Receptors. TLRs were the first PRRs identified and are receptors of the innate immune system that detect PAMPs and DAMPs to initiate immune responses. These receptors are classified into six major families, TLR1, TLR3, TLR4, TLR5, TLR7 and TLR11. The TLR1 family consists of TLR1, TLR2, TLR6 and TLR10. These TLRs are plasma membrane receptors and recognize components of microbial cell walls and membranes [12]. TLR4 is also a plasma membrane receptor and recognizes bacterial lipopolysaccharide (LPS). Members of the TLR3, TLR7, and TLR11 families are intracellular and expressed in endosomes and lysosomes. The TLRs that are members of the TLR3 and TLR7 families recognize double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA) respectively. During a viral infection, activation of TLR signaling in endosomes can lead to interferon (IFN) or inflammatory cytokine production. However, cell surface TLR signaling (with the exception of TLR4) only results in inflammatory responses, not IFN expression [12]. Proper TLR functioning, like most immunological responses, requires adequate amounts of micronutrients and is significantly affected by diet [13]. For example, TLR2 and TLR4 are involved in inflammation due to high-fat diet (HFD)-induced obesity in rats. It was found that HFD decreased TLR2 and TLR4 expression on CD14 monocytes and altered their function by increasing levels of IL-1β, IL-6 and TNF-α [14]. HFD also induced macrophage activation with a significant increase in NF-κB and IL-6 levels [15]. There are also various non-microbial stimulants that affect the functioning of TLRs such as plant polyphenols, polyunsaturated fatty acids, saturated fatty acids, glucans, and pectins [13]. TLR4 is particularly relevant for this study, as it is the receptor that recognizes LPS and induces the secretion of pro-inflammatory cytokine IL-6 through NF-κB signaling (Figure 1).

RIG-I-like Receptors. Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are located in the cytosol and play an important role in antiviral host responses by mediating the

production of type I interferons upon detection of RNA [16]. Type I interferons are a type of cytokine that are involved in antiviral defense. RLRs can be activated by viral or host RNAs detected in the cytosol. Also, RLR activation is seen in several autoimmune and autoinflammatory diseases and in cancer. This occurs either from mutations in the absence of a viral infection or due to errors in RNA processing that may result in the detection of endogenous RNAs [16]. RIG-I deficiency was found to promote obesity and insulin resistance induced by a HFD, indicating a regulatory role of RIG-I in metabolic stress, obesity, and insulin resistance. It is speculated that this is due to decreased type I IFN production, as they typically play a protective role against metabolic stress [17]. In this research RLRs recognized the double-stranded RNA present in the cytoplasm during infection with influenza A virus, which results in the secretion of pro-inflammatory cytokines like IL-6 via the NF-κB pathway (Figure 1).

NFκB Pathway. NF-κB plays important roles in inflammation, immune responses, and apoptosis and is induced by many different stimuli. This pathway regulates pro-inflammatory cytokine production and inflammatory cell recruitment. RLRs, TLRs, and NLRs can all activate the NF-κB pathway, which leads to the transcription of pro-inflammatory cytokines, chemokines, and other inflammatory mediators that can directly and indirectly mediate the inflammatory response [18]. This pathway is relevant to this study because it is involved in the production of pro-inflammatory cytokines like IL-6 (Figure 1). NF-κB is a central inflammatory mediator and its deregulation is involved in a variety of inflammatory diseases, such as obesity [19]. The NF-κB pathway links metabolic signals with inflammation-driven cellular responses in physiology and disease, suggesting that diet and metabolism can significantly affect this pathway [20]. Therefore, it is an important pathway to investigate when studying dietary and metabolic effects on inflammation.

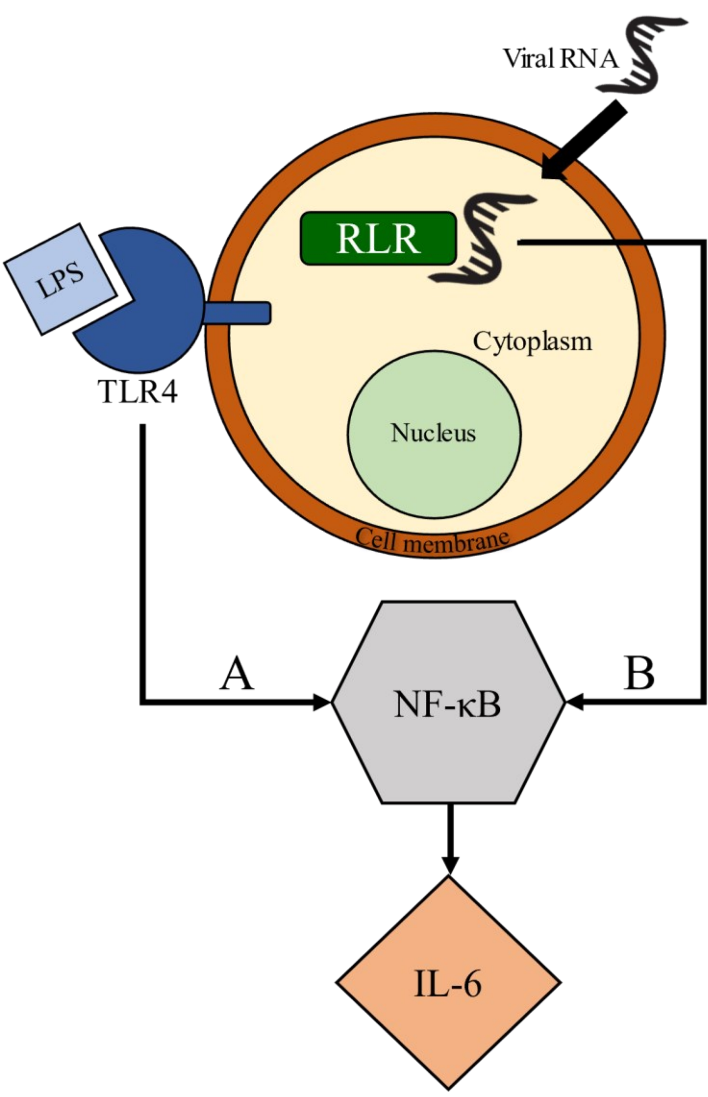


Figure 1. Inflammatory pathway-mediated production of IL-6. A, Toll-like receptor (TLR) 4 in combination with the adaptor protein MD-2 recognizes bacterial endotoxin lipopolysaccharide (LPS). Once activated by LPS, TLR4 signals through MyD88 and TRIF-dependent pathways to initiate the translocation of transcription factor NF-κB into the nucleus. Activation of NF-κB induces the production of interleukin-6 (IL-6). B, Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) present in the cytoplasm detect viral RNAs. Detection of viral RNAs results in the activation of NF-κB, which then stimulates the production of pro-inflammatory cytokines like IL-6.

NOD-like Receptors. Nucleotide oligomerization domain (NOD)-like receptors (NLRs) are intracellular cytosolic receptor proteins that are activated by cytoplasmic PAMPs [21]. Some of the NLRs activate inflammasomes, while others stimulate the innate immune system by

activating NF-κB, mitogen-activated protein kinases (MAPKs) and interferon (IFN) regulatory factor (IRF) pathways. Dysfunction in NOD-1 and NOD-2, members of the NLR family, is associated with chronic inflammatory and metabolic diseases, such as inflammatory bowel disease (IBD), asthma, arthritis, and periodontitis [22]. Recently, NOD-1 and NOD-2 have been implicated as mediators of metabolic disease, with increased expression seen in metabolic diseases such as obesity, diabetes, non-alcoholic fatty liver disease, and metabolic syndrome [23].

Inflammasomes. The inflammasomes are innate immune complexes triggered by PAMPs and DAMPs that recruit and activate the inflammatory protease caspase-1, which is a required molecule for the processing and maturation of inflammatory cytokines IL-1β and IL-18 [24]. NLRP3, a member of the NOD-like receptor family, is the most widely studied inflammasome activator. It is able to detect a wide range of PAMPs, including LPS, bacterial and viral RNA, double-stranded RNA analog polyinosinic-polycytidylic acid (polyI:C), and nonmicrobial DAMPs like uric acid, ATP and asbestos [21]. The NLRP3 inflammasome has been implicated in various metabolic diseases, such as obesity, insulin resistance, atherosclerosis and Alzheimer's disease [24]. Upon activation of the inflammasome by a PAMP or DAMP, pro-caspase 1 is cleaved into its active form, caspase-1, which then converts the inactive pro-IL-1β into its active form IL-1β (Figure 2).

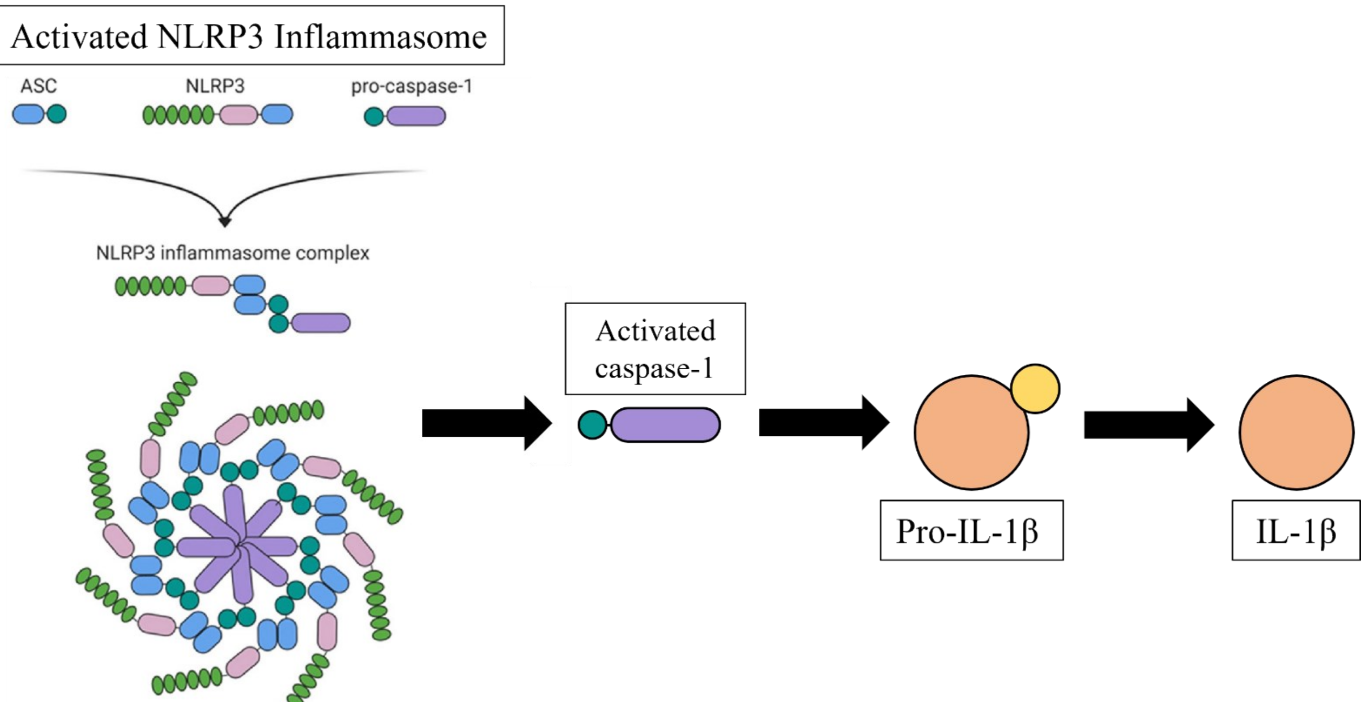


Figure 2. Production of IL-1β via NLRP3 inflammasome activation. It is believed that NLRP3 inflammasome activation requires two signaling steps: priming and activation. The first signal is an inflammatory stimulus like TLR4 agonists that induce NF-κB-mediated NLRP3 and pro-IL-1β expression. The second signal is from PAMPs and DAMPs. Once both signals have been provided, the NLRP3 inflammasome is activated and pro-caspase-1 is cleaved into its active form caspase-1. Caspase-1 then cleaves the cytokine pro-interleukin-1β (pro-IL-1β) into the biologically active form IL-1β.

Inflammatory Cytokines. Inflammation relies on inflammatory cytokines to recruit leukocytes to the area and trigger physiological changes in blood vessels and metabolism to induce fever. They are also important to begin the healing process. Cytokines can be either pro- or anti-inflammatory. They are primarily released by immune cells such as macrophages, lymphocytes and monocytes and are critical in the recruitment of other leukocytes to the location of infection or injury. They are also involved in modulating the immune response to prevent excessive inflammation and tissue damage. In this study, I specifically looked at the levels of two pro-inflammatory cytokines, IL-6 and IL-1β. IL-6 is a pro-inflammatory cytokine produced by macrophages and other innate immune cells and plays a critical role in inflammatory

responses, viral infections and autoimmune diseases. IL-1β is a pro-inflammatory cytokine that is produced when Caspase-1 is cleaved during inflammasome activation. It plays a role in homeostasis and acute and chronic inflammatory and autoimmune disorders [25].

Inflammatory Cells. While there are many types of cells involved in the inflammatory response, this study focuses on bone marrow-derived macrophages. Macrophages are innate immune cells that are present in all tissues [26]. During inflammation, macrophages present antigens, perform phagocytosis and regulate the immune response via cytokine and growth factor production [8]. Once activated by PAMPs or DAMPs, macrophages differentiate into different states, the classically activated (M1) and alternatively activated (M2) macrophages. M1 macrophages produce pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, and they also promote the differentiation of inflammatory T cells into Th1 and Th17 cells which mediate inflammation. M2 macrophages produce anti-inflammatory cytokines, such as IL-10 and IL-13. They are important in the resolution of inflammation and in the wound healing process [12]. Measuring these cytokines can be used to determine the strength of the immune response and help in diagnosing disease.

Dysregulated Inflammation

Cytokine Storms. Sometimes, the immune system malfunctions or is over-stimulated and makes too many cytokines which leads to a cytokine storm. A cytokine storm is described as excessive production of pro-inflammatory cytokines leading to aggressive pro-inflammatory responses and insufficient control by anti-inflammatory responses [9]. Influenza A virus infections can result in severe disease. While it may seem that the viral load is associated with the severity of the disease, the host's inflammatory response to the viral infection also

contributes to disease severity. If the pro-inflammatory cytokines that are released to combat the infection are produced excessively and the anti-inflammatory response is insufficient, a cytokine storm is produced, which can cause organ damage, systemic inflammation and even death [9]. COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Once the virus is inside the cell, a cytokine storm is triggered, largely caused by IL-6 and the NLRP3 inflammasome [27]. It is thought that the pathological, clinical and sometimes deadly symptoms of COVID-19 are more due to the cytokine storm produced by the immune system rather than the viral load [28]. Finally, sepsis is an inflammatory disease mediated by the immune system in response to systemic infection and is the cause of approximately 20% of deaths worldwide [29]. There are many challenges to diagnosis sepsis in the early stages, so the need for better biomarkers is critical. Initially, sepsis is similar to typical infections, where receptors respond to PAMPs and DAMPs via signaling pathways [30]. However, as the disease progresses and more and more leukocytes respond to the stimuli, a cytokine storm ensues. This can lead to disseminated intravascular coagulation (DIC), multi-organ dysfunction syndrome (MODS), inflammation-coagulation due to platelet activation, and peripheral vasodilation leading to low blood pressure [30]. A common cause of sepsis is infection with Gram-negative bacteria, where the immune system overreacts to the presence of the endotoxin LPS.

Chronic Inflammation. While inflammation primarily occurs to protect and heal our bodies, inflammation can become extremely detrimental if left unchecked. Typically, inflammation is resolved by the dilution of chemokine gradients over time. This halts the recruitment of circulating leukocytes. However, malfunctions in this process can result in chronic inflammation, which is characterized by slow, long-term inflammation, lasting several months to years. The World Health Organization (WHO) ranks chronic diseases as the greatest threat to

human health [5]. Worldwide, 60% of deaths are attributed to chronic inflammatory diseases such as stroke, heart disorders, cancer, obesity, diabetes and chronic respiratory diseases [5]. Arthritis and other inflammatory joint diseases affect approximately 350 million people worldwide, and allergies affect more than 50 million people in the United States alone [31]. Although COVID-19 can result in a cytokine storm, a chronic inflammatory condition known as Long-COVID has impacted the lives of millions of COVID-19 survivors. The symptoms of Long-COVID vary, but the most common are fatigue, trouble breathing, fever, cough/sneezing, low SaO_2, and loss of taste and/or smell.

Dietary Molecules and Metabolites

Lectins. As diet and metabolism are known to contribute to inflammation, I wanted to examine the role for specific dietary molecular and metabolic compounds. Lectins are proteins found in plants that bind to carbohydrates present on cell membranes. Dietary lectins can be found in various foods such as vegetables, fruits, grains, and nuts [32]. While many common foods contain lectins, raw legumes, like beans, and whole grains, like wheat, contain the highest amounts of lectins. These proteins protect plants by resisting digestion and retaining stability in acidic environments, basically acting as a toxin so that the plant does not get eaten. When eaten, lectins can elicit negative side effects such as nausea, vomiting, diarrhea, bloating and gas. Also, previous studies have shown that lectins can interfere with nutrient absorption and gut microbiota by binding to epithelial cells of the gastrointestinal tract. The presence and buildup of lectins in the body can elicit an immune response and may be linked to inflammatory conditions such as rheumatoid arthritis and type 1 diabetes [33].

Sodium Pyruvate. Pyruvate is an antioxidant and is a key metabolite in energy metabolism and cellular respiration. It enters into the mitochondria where it is utilized as an energy molecule to produce ATP in the tricarboxylic acid cycle (TCA), bypassing many glycolysis-controlled metabolic regulatory pathways. Pyruvate is also involved in amino acid production and its reduction is used to replenish nicotinamide adenine dinucleotide (NAD^+). Pyruvate can be found in various forms such as sodium pyruvate (NaPyr), ethyl pyruvate, and pyruvic acid. It is well tolerated with little to no toxicity in the body. Pyruvate has been shown to elicit a wide range of anti-inflammatory and protective effects across many body systems and cell types. [34]. Traditional steroids down-regulate nasal nitric oxide synthesis, but NaPyr is able to reduce nasal inflammation while up-regulating nasal nitric oxide synthesis. This is important as nitric oxide can be used in the lungs to fight infections and increase lung function [35]. Since NaPyr has been shown to have therapeutic properties in other inflammatory diseases, especially related to lung function, I wanted to examine the effect of inhalation of NaPyr on the symptoms of an active COVID-19 infection and in long-COVID patients with decreased SaO_2, coughing/sneezing, fever, fatigue, and trouble breathing.

Scientific Question and Hypothesis

Based on the previously cited scientific literature, I wanted to know how dietary and metabolic products affected inflammation. I hypothesized that treating macrophages with dietary lectins during an infection would result in increased inflammatory cytokine production. Alternatively, based on its antioxidant and anti-inflammatory properties, I hypothesized that sodium pyruvate inhalation would result in improvement of physiological symptoms experienced by long-COVID patients.

EFFECTS OF DIETARY LECTINS ON MACROPHAGE INFLAMMATORY CYTOKINE PRODUCTION

Abstract

Background. Inflammation is a critical component of the immune system resulting from the release of inflammatory cytokines, like IL-1β and IL-6. Typically, once the stimulus is cleared, homeostasis is restored. Sometimes though, the inflammatory response can become chronic and contribute to various diseases. The source of inflammatory responses is not always clear. Therefore, the effect of diet on inflammation is a crucial topic to be investigated.

Design. Bone marrow derived macrophages (BMDMs) were treated with LPS + ATP, LPS + ATP + lectin, influenza A virus (IAV), or IAV + lectin. Cell culture supernatants collected from control and infected BMDM were analyzed for IL-1β and IL-6 to determine if dietary lectins could affect inflammatory responses of macrophages.

Findings. Macrophage pro-inflammatory cytokine secretion was not affected when treated with lectins. Neither IL-1β nor IL-6 levels were statistically different when treated with LPS + ATP compared to LPS + ATP + lectin. The same was true for samples treated with IAV compared to IAV + lectin. However, some lectins were able to stimulate IL-6 production in the absence of infection.

Conclusions. IL-1β and IL-6 inflammatory cytokine levels produced by infected BMDMs did not show statistically significant differences from the control levels when treated with two different dietary lectins in cell culture, but further experiments are needed to determine if other lectins, cells, or treatment during other infections can alter inflammatory cytokine release.

Introduction

Inflammation is a critical part of the innate immune system responding to a stimulus, such as injury or pathogens, through pattern recognition receptors (PRRs). PRRs recognize pathogen-associated molecular patterns (PAMPs), like viral RNA or LPS as well as damage-associated molecular patterns (DAMPs), like extracellular release of nuclear contents or ATP [1]. Activation of some PRRs results in their association with ASC and procaspase-1 to form a protein complex called an inflammasome. There are multiple types of inflammasomes, but the NLRP3 inflammasome is able to respond to the widest variety of PAMPs and DAMPs. While the exact mechanism of activation is not fully understood, it is known that the NLRP3 inflammasome is responsible for the activation of pro-inflammatory cytokines, such as IL-1β [2]. IL-1β plays a role in increasing transport of neutrophils and T cells to infection sites and is involved in pain, inflammation and autoimmune conditions [3]. It also induces epithelial and endothelial cells to produce other cytokines like IL-6 [4]. IL-6 is a pro-inflammatory cytokine produced by macrophages, mast cells and other innate immune cells that has a critical role in inflammatory responses, viral infections and autoimmune diseases. It also plays a role in pathophysiological events like fever, liver acute-phase response, and in the transition from acute to chronic inflammation [5]. During a primary infection with influenza A virus, IL-6 plays a protective role by clearing the virus and promoting the innate phase of the immune response [6]. Although IL-6 in necessary for the resolution of an influenza A virus infection, excessive levels of IL-6 have been linked to poor prognosis of influenza A virus infected patients [4].

Typically, once the immune system is no longer recognizing the stimulus, the immune response is resolved, and homeostasis is restored. However, when a stimulus lingers or the immune cells are continuously activated, the inflammatory response can become chronic.

Chronic inflammation, usually associated with elevated levels of pro-inflammatory cytokines, has been associated with various mental and physical disorders and diseases such as depression, schizophrenia, cancer, autoimmune diseases, cardiovascular disease, gastrointestinal disorders and obesity [7]. While inflammatory sources are clear for some diseases, in other diseases, the source is unclear. Thus, it is important to investigate the effects of our diets on chronic inflammation.

Lectins are a group of proteins that were first discovered in plants but were later found in other species, from microbes to humans. They specifically and reversibly bind to carbohydrates present on cell membranes, which allows them to participate in many biological processes, such as cell development, cell recognition, tumor metastasis, host defense, and inflammation [2]. Plant lectins are found in many different kinds of foods such as vegetables, fruits, grains, legumes and nuts and are considered anti-nutrients. These lectins can increase intestinal permeability, which allows for increased translocation of dietary and microbial antigens into the body [2]. Once in the periphery, the lectins can provoke IgG and IgM antibody production, and they can bind to cell surface glycoproteins, such as epidermal growth factor receptor and insulin receptor, which disrupts their normal functioning. It is thought that lectins may exacerbate the pathogenesis of food intolerance, food allergy and other inflammatory diseases, such as type 1 diabetes, rheumatoid arthritis, and inflammatory bowel disease [2]. In a study done by Gong et al., they found that plant lectins acted as a DAMP, activating the NLRP3 inflammasome. Specifically, a plant lectin called wheat germ agglutinin (WGA) has been suggested to increase intestinal permeability. In individuals with celiac disease, they found significantly higher antibody levels to WGA, suggesting that WGA may be involved in the pathogenesis of the disease [2]. Increased intestinal permeability has been associated with autoimmune diseases, such as type 1 diabetes,

rheumatoid arthritis and multiple sclerosis. Surprisingly, increased intestinal permeability has also been associated with other diseases related to chronic inflammation, such as inflammatory bowel disease, asthma, and depression [7]. WGA has been shown to stimulate histamine secretion from non-stimulated peritoneal mast cells, induce NADPH-oxidase activity in human neutrophils, and stimulate the release of cytokines IL-4 and IL-13 from human basophils. Phytohaemagglutinin (PHA) is a lectin found in red kidney beans. It is known to be mitogenic, inflammatory, and causes aggregation of erythrocytes and leukocytes. In a previous study, it was shown that PHA treatment resulted in increased expression of IL-2, IL-2R, IL-6, IL-10, TNF-α, and IFN-γ in human peripheral blood mononuclear cells [8].

In this research, I examined the effects of two dietary lectins on cytokine release from macrophages treated with LPS and ATP or infected with influenza A virus.

Materials and Methods

Animal Welfare. In the Temple Hall Vivarium at Missouri State University, WT C57BL/6J mice were bred, raised, and then euthanized via CO_2 asphyxiation and cervical dislocation. The bone marrow was collected for differentiation into macrophages. All breeding and experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines (protocols 19.019, Appendix A and 19.005, Appendix C), NIH regulations, the AVMA Guidelines on Euthanasia, and the U.S. Animal Welfare Act of 1966.

Reagents. Lectin from Phaseolus vulgaris (red kidney bean) was purchased from Sigma Aldrich (L8754). Lectin from Triticum vulgaris (wheat) was purchased from Sigma Aldrich (L9640). Adenosine 5`-triphosphate (ATP) disodium salt hydrate was purchased from Sigma

Aldrich (A1852-1VL). Influenza A/PR/8/34 H1N1 virus was purchased from ATCC and grown in 10-day old embryonated hen's eggs. LPS was purchased from Sigma Aldrich (LPS25).

Production of Bone Marrow Derived Macrophages. To collect bone marrow from the femur and tibia of each hindlimb, C57BL/6J mice that were 7-14 weeks old were euthanized. Bone Marrow Derived Macrophages (BMDMs) were produced by growing the bone marrow cells in bone marrow differentiation media (BMDM media) for 5 days. This media contains Dulbecco's Modified Eagle Medium (DMEM) + 10% FBS + 1% Pen/Strep + 1% non-essential amino acids (NEAA) and supplemented with L929 cell media. The L929 medium contains macrophage colony-stimulating factor (M-CSF), which was produced by growing L929 cells in DMEM + 10% FBS + 1% Pen/Strep for 10 days, followed by filtering the media with a 0.2 μm filter. After allowing the BMDMs to grow for 5 days, cells were scraped and re-plated into 12-well plates containing 1 mL BMDM media at $1x10^6$ cells/well. After incubating overnight to allow the cells to adhere to the plate, the macrophages were collected and used for subsequent experiments.

Infection Schemes and Treatment. For LPS + ATP treatment, BMDMs were washed twice with phosphate buffered saline (PBS) and 500 μL of DMEM + 10% FBS was added to each well of two 12-well plates. LPS was added to 6 wells of each 12-well plate at 1μg/ml final concentration. Bean lectin was added to 6 of the 12 wells of one 12-well plate, and wheat lectin was added to 6 wells of the other 12-well plate, both at 1 mg/mL final concentration (Fig. 1a-b). The plate was incubated for 3.5 hours, and then ATP was added to each of the 6 wells containing LPS at 5 mM final concentration. After 30 more minutes, 200 μL of the media was collected from each well and placed into a 96-well plate to be used later for Enzyme-Linked Immunosorbent Assay (ELISA), as described later.

For influenza A virus infection, BMDMs were washed twice with PBS and 200 µL of RPMI 1640 media was added to each well of two 12-well plates. Influenza A virus was added to 6 wells of each 12-well plate at a concentration of 10,000,000 PFU/well (10MOI). The plates were incubated for one hour, shaking them every 15 minutes. After one hour, 250 µL of RPMI + 20% fetal bovine serum was added to each well. At this time, bean lectin was added to 6 wells of one of the 12-well plates, and wheat lectin was added to 6 wells of the other 12-well plate both to a final concentration of 1 mg/mL, (Fig. 1c-d). The plates were incubated for 24 hours, and then 200 µL of the media was transferred to a 96-well plate to be used later for ELISA.

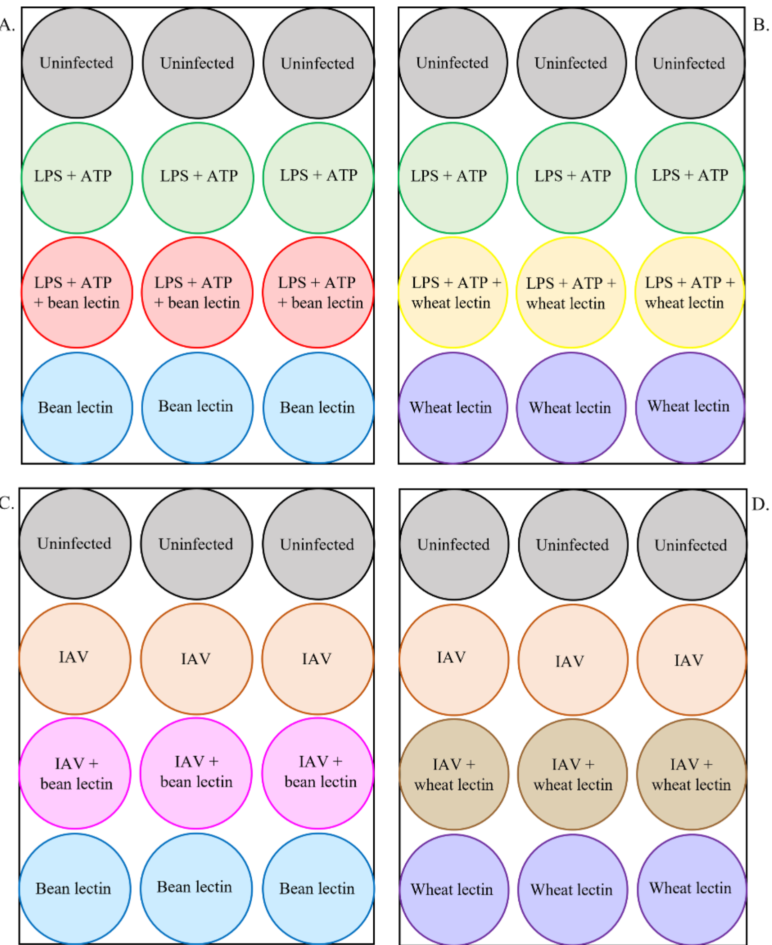


Figure 1. 12-well plate layout for infection scheme. A, infection scheme for LPS + ATP and bean lectin. B, infection scheme for LPS + ATP and wheat lectin. C, infection scheme for IAV and bean lectin. D, infection scheme for IAV and wheat lectin.

Enzyme-Linked Immunosorbent Assay (ELISA). Supernatants from infected, treated, infected/treated, and uninfected/untreated cell cultures were analyzed for IL-1β and IL-6. Mouse IL-1β and mouse IL-6 uncoated ELISA kits were purchased from Thermo Fisher Scientific (88-7013-88, 88-7064-88), and assays were performed following the manufacturer's recommendations. Then, plates were read using a BioTek ELx808 microplate reader at 450 nm.

Statistical Analysis. GraphPad PRISM9 was used to perform statistical analysis. Comparison of the treatment groups was performed using one-way ANOVA along with Tukey's post-hoc test. A p-value <0.05 was considered statistically significant.

Results

IL-1β Cytokine Response. I initially examined the effects of bean or wheat lectin on the cytokine response of BMDM treated with LPS + ATP by quantifying the levels of IL-1β produced via ELISA (Figure 2a). Treatment with LPS + ATP resulted in a mean of 25,151 pg/ml ±10,026. Treatment with LPS + ATP + wheat lectin resulted in a mean of 60,909 pg/mL ±54,494. Treatment with LPS + ATP + bean lectin resulted in a mean of 32,924 pg/mL ±43,895. Although higher IL-1β levels were seen in the LPS + ATP + wheat lectin group, this was not statistically different from the control group. In the absence of a PAMP or DAMP like LPS and ATP, respectively, treatment with wheat lectin alone resulted in a mean of 2,712 pg/ml ±4,090. Treatment with bean lectin alone resulted in a mean of 2,748 pg/mL ±4,388. Finally, untreated cells had a mean of 1,671 pg/mL ±1,816. Overall, there were no significant differences in IL-1β produced in the LPS + ATP experiment (Figure 2a).

I also examined the effects of bean or wheat lectin on the IL-1β cytokine response of BMDM infected with influenza A virus (IAV) (Figure 3a). Treatment with IAV resulted in a

mean of 123 pg/mL ±12.67. Treatment with IAV + wheat lectin resulted in a mean of 143 pg/mL ±100.2 and treatment with IAV + bean lectin resulted in a mean of 109 pg/mL ±28.67. Treatment with wheat lectin alone resulted in a mean of 120 pg/mL ±5.437 and treatment with bean lectin alone resulted in a mean of 134 pg/mL ±22.5. Finally, untreated cells resulted in a mean of 129 pg/mL ±32.27. I observed that IAV infection did not induce significant amounts of IL-1β and that lectins also had no effect on IL-1β levels (Figure 3a).

IL-6 Cytokine Response. I also examined the effects of bean or wheat lectin on the IL-6 cytokine response of BMDM treated with LPS + ATP (Figure 2b). Similar to IL-1β, I did not observe any effect of lectin treatment on IL-6 levels in LPS+ATP treated cells. However, I did notice that treatment of cells with bean lectin alone stimulated a significant increase in IL-6. Treatment with bean lectin resulted in a mean of 1,019 pg/mL ±983.1. However, treatment with wheat lectin resulted in a mean of 377 pg/mL ±269.9, and untreated cells had a mean of 254 pg/mL ±174.6. Similar results were observed during IAV infection. IL-6 levels were not affected by lectin treatment during IAV infection (Figure 3b). However, treatment with bean lectin resulted in a mean of 7,455 pg/mL ±5,808 compared to treatment with wheat lectin 805 pg/mL ±193.7 or untreated cells 449 pg/mL ±375.4.

Since I hypothesized that the treatment with lectins would cause an increase in the levels of inflammatory cytokines, it was expected that the groups treated with either LPS + ATP or IAV and either bean or wheat lectin would show higher levels of cytokines than any other treatment groups. However, this was not the case. Instead, I observed that there was an increase in IL-6 levels when comparing the cells treated with just bean lectin versus the uninfected cells (Figure 2b) (p-value = 0.0138) and in the amount of IL-6 produced by cells treated with bean lectin versus cells treated with wheat lectin (p-value = 0.0076) (Figure 3b).

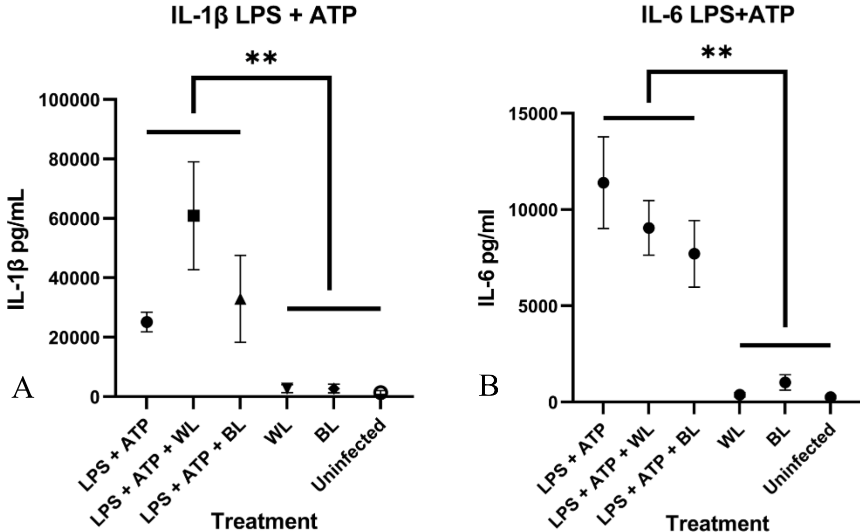


Figure 2. Pro-inflammatory cytokine levels following LPS + ATP and lectin treatment. BMDMs were mock infected, infected with 1µg/mL LPS for 4 hours + 5mM ATP for the last 30 minutes, wheat lectin for 4 hours, bean lectin for 4 hours, or LPS + ATP + lectin. Cell supernatants were collected after 4 hours of treatment and examined for IL-6 (Fig. 2a) or IL-1β (Fig. 2b) expression by ELISA. Cytokine concentration was determined by standard curve generation using spectrophotometry. Statistical significance was determined using one-way ANOVA with Tukey post-hoc for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001.

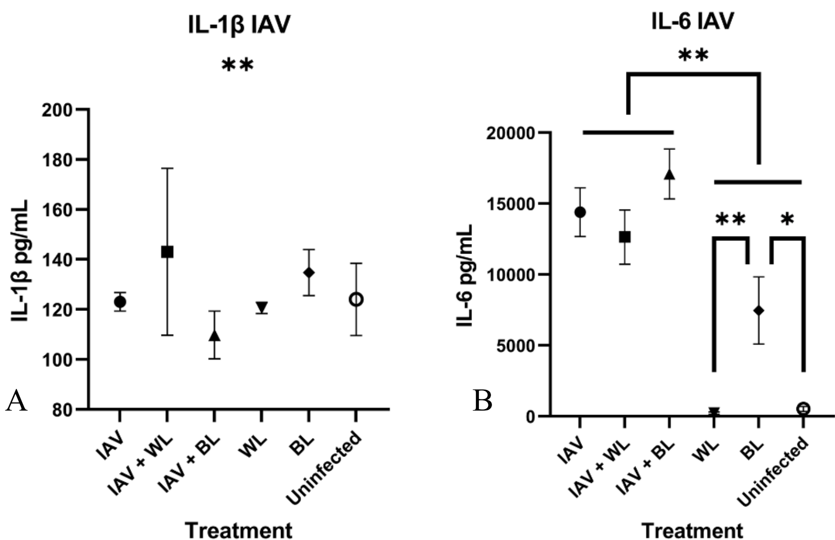


Figure 3. Pro-inflammatory cytokine levels following influenza A virus (IAV) and lectin treatment. BMDMs were mock infected, infected with 10 MOI influenza A virus (IAV), wheat lectin, bean lectin, or IAV + lectin and incubated for 24 hours. Cell supernatants were collected and examined for IL-6 expression by ELISA. Cytokine concentration was determined by standard curve generation using spectrophotometry. Statistical significance was determined using one-way ANOVA with Tukey post-hoc for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001.

Discussion and Conclusions

When faced with a stimulus, the immune system elicits a response tailored to that stimulus through PRRs that recognize PAMPs and DAMPs. Typically, inflammation is triggered, and inflammatory cytokines are released, leading to the recruitment of other leukocytes. Inflammatory cytokines can be used as inflammatory markers to determine the severity and type of immune response. IL-1β and IL-6 are common pro-inflammatory cytokines secreted by macrophages and other immune cells that are used as inflammatory markers. Once the immune system has cleared the body of the initial stimulus, the immune response terminates, and homeostasis is restored. Sometimes though, a stimulus can linger, or the immune system can malfunction, resulting in chronic inflammation. Chronic inflammation is associated with many mental and physical disorders, such as cancer, autoimmune diseases, depression, obesity, and gastrointestinal disorders [7]. With so many people suffering from chronic inflammatory disorders and other metabolic disorders, it is important to evaluate the effect of diet on the immune system, as metabolic processes can alter the immune response [9].

Lectins are proteins that bind to carbohydrates on cell membranes, making them important in various biological processes. They are also capable of triggering an immune response. Since plant lectins are a common component of what I eat daily, I wanted to investigate the possible inflammatory effect they have on our digestive and immune systems. Wheat germ agglutinin (WGA), a lectin found in wheat, is able to bind to N-glycolylneuraminic acid, the sialic acid found in humans, meaning that it can bind to cell surfaces such as the epithelial layer of the gut tissues [7]. Previous studies have shown that WGA can stimulate immune cells and increase intestinal permeability in mice by inducing structural changes that elicit functional changes of the cells [10]. This is important because increased permeability allows WGA to enter cells and

potentially stimulate a pro-inflammatory immune response. In a study using murine peritoneal macrophages, WGA induced the production of TNF-α, IL-1β, IL-12, and IFN-γ [11]. In another study using isolated human PBMCs, WGA stimulated the release of pro-inflammatory cytokines, and a significant increase in the intracellular concentration of IL-1β was found after treatment with WGA. These results showed that WGA is able to directly stimulate monocytes and macrophages when delivered *in vitro* [7]. It is possible that my data did not show a significant increase in the amount of IL-1β or IL-6 produced when BMDMs were treated with only wheat lectin due to the concentration of lectin used. As previous studies have shown, wheat lectin is capable of inducing macrophages to produce inflammatory cytokines *in vivo* and *in vitro*, so theoretically, I should have seen an increase in those cytokine levels [7]. However, the doses used by other researchers was much higher than the dose I used.

Phytohaemagglutinin (PHA) is a lectin present in red kidney beans. It causes leukocytes and erythrocytes to aggregate, and it can act as an exogenous pyrogen. Upon entrance into the blood, exogenous pyrogens interact with monocytes and macrophages, which results in the release of proinflammatory cytokines. Also, PHA is used as a mitogen in biological, immunological, and biochemical research. As a mitogen, PHA activates T cells through TLRs and induces proliferation and differentiation of lymphocytes. In one study, it was reported that PHA treatment increased the expression of IL-2, IL-2R, IL-6, IL-10, TNF-α, and IFN-γ in human peripheral blood mononuclear cells (PBMCs) [8]. This claim is also supported by data from my experiment, as I did see a statistically significant (p-value = 0.0138) increase in IL-6 levels when cells were treated with bean lectin versus the untreated cells (Figure 3b). However, it is interesting that this result was not consistent with the results in Figure 2b, where IL-6 levels produced in response to bean lectin treatment were not significantly different from the IL-6

levels of untreated cells. The difference between Figures 2 and 3 is the conditions of the 12-well plate that the cells were treated in. LPS + ATP treated cells and control cells in those experiments were maintained in DMEM + 10% FBS. For the groups in the 12-well plate of the IAV treated cells, RPMI 1640 and 20% FBS media was used as the medium. It is not clear whether this difference in medium explains the difference seen in the amount of IL-6 produced by the cells when treated with the bean lectin, but it is something worth noting. Also, it is possible that there would have been a more significant difference in my data had I used human THP-1 cells rather than mouse BMDMs, as previous studies have reported that PHA treatment of THP-1 cells resulted in the increased production of inflammatory cytokines like IL-6 [8]. When treated with bean lectin alone, there was an increase in the level of IL-6 produced by the cells but not IL-1β. This could be due to the bean lectin inducing the production of IL-6 through a mechanism that is independent of the inflammasome, as there would be an increase in the level of IL-1β as well if the inflammasome was involved.

For future studies, it would be interesting to analyze the effects of feeding mice a high-lectin diet by observing their physical condition and cytokine production *in vivo*. Dietary lectins may exert a much different effect when ingested rather exposing cells to them *in vitro*. Also, it would be interesting to look at other inflammatory markers to see if lectins more strongly induce the production of cytokines other than IL-1β and IL-6, such as anti-inflammatory cytokines, like IL-10. Finally, it is possible that these lectins only affect certain stimuli, and future studies could investigate the effects of dietary lectins on different diseases, especially gastrointestinal diseases like celiac disease or irritable bowel disease.

References

1. Chen L, H Deng, H Cui, et al. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2017; 9: 7204–7218.

2. Gong T, X Wang, Y Yang, et al. Plant Lectins Activate the NLRP3 Inflammasome To Promote Inflammatory Disorders. J Immunol. 2017; 198: 2082–2092.

3. Ren K, R Torres. Role of interleukin-1beta during pain and inflammation. Brain Res Rev. 2009; 60: 57–64.

4. Gu Y, X Zuo, S Zhang, et al. The Mechanism behind Influenza Virus Cytokine Storm. Viruses. 2021; 13: 1362.

5. Naugler WE, M Karin. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. Trends Mol Med. 2008; 14: 109–119.

6. Dienz O, JG Rud, SM Eaton, et al. Essential role of IL-6 in protection against H1N1 influenza virus by promoting neutrophil survival in the lung. Muc Immunol. 2012; 5: 258–266.

7. de Punder K, L Pruimboom. The Dietary Intake of Wheat and other Cereal Grains and Their Role in Inflammation. Nutrients. 2013; 5: 771–787.

8. Prajitha N, PV Mohanan. Intracellular inflammatory signalling cascades in human monocytic cells on challenge with phytohemagglutinin and 2,4,6-trinitrophenol. Mol Cell Biochem. 2022; 477: 395–414.

9. Abusalamah H, JM Reel, CR Lupfer. Pyruvate affects inflammatory responses of macrophages during influenza A virus infection. Virus Res. 2020; 286: 198088.

10. Cordain L, L Toohey, MJ Smith, et al. Modulation of immune function by dietary lectins in rheumatoid arthritis. British J Nutri. 2000; 83: 207–217.

11. Sodhi A, V Kesherwani. Production of TNF-α, IL-1β, IL-12 and IFN-γ in murine peritoneal macrophages on treatment with wheat germ agglutinin in vitro: involvement of tyrosine kinase pathways. Glycoconj J. 2007; 24: 573–582.

INHALATION OF SODIUM PYRUVATE TO REDUCE THE SYMPTOMS AND SEVERITY OF RESPIRATORY DISEASES INCLUDING COVID-19, LONG COVID, AND PULMONARY FIBROSIS

Abstract

Background. To combat the continuing COVID-19 pandemic, and to treat the symptoms in Long COVID patients safe, effective, and inexpensive treatments are needed. Patients recovering from severe COVID-19 are at serious risk of developing pulmonary fibrosis. Conversely, patients with pulmonary fibrosis have an increased risk and susceptibility to COVID-19 infection, demonstrating the need to treat both.

Design. Three separate clinical trials were conducted 1) in COVID-19 infected patients, 2) in Long COVID patients, and 3) in patients with Pulmonary Fibrosis to determine the efficacy of N115, a sodium pyruvate based nasal spray. Patient symptoms, vital signs and respiratory function were evaluated compared to a placebo control or a no treatment baseline control.

Findings. During active COVID-19 infection, N115 decreased viral titers and produced a significant improvement over saline in coughing/sneezing and fatigue. In Long COVID patients, N115 significantly reduced headache, coughing/sneezing and increased SaO_2 levels (decreased hypoxemia) and improved breathing (dyspnea). In patients with Pulmonary Fibrosis, there was a significant improvement in all lung functions, compared to baseline, as determined by changes in SaO_2, FVC, FEV_1, PEF, and FEV_1/FVC ratio.

Conclusions. N115 is safe and effective at reducing symptoms of active COVID-19 infection and improves disease condition in Long COVID patients. Furthermore, N115 significantly improves lung function in Pulmonary Fibrosis patients. As COVID-19 and

Pulmonary Fibrosis are associated with each other, our clinical research demonstrates that N115 is a promising treatment for both and adds to the current 19 human clinical trials where N115 has shown efficacy in thousands of patients, regardless of the etiology of the lung disease (COPD, CF, allergic rhinitis, sinusitis, the flu, COVID-19 infected patients, Long COVID and patients with Pulmonary Fibrosis).

Introduction

COVID19 is a disease caused by the novel SARS-CoV-2 virus [1]. In the last 1.5 years since the spread of this virus began a world-wide pandemic, hundreds of millions of people have become infected and millions have died [2]. Although the advent of any novel pathogen is likely to result in widespread infection and mortality, SARS-CoV-2 induces a particularly severe and rapid form of pneumonia in some patients, concomitant with an overall cytokine storm but diminished interferon responses [3]. Although case severity varies by sex, age and comorbidities, some of the most severe comorbidities include high blood pressure, diabetes and interstitial lung disease [4].

Interstitial lung disease encompasses a large group of chronic lung disorders associated with excessive tissue remodeling, scarring, fibrosis, decreased FEV_1 values, decreased SaO_2 and decreased Nitric Oxide (NO) associated with nasal inflammation that causes congestion, coughing and sleep disorders [5, 6]. Researchers have demonstrated that pulmonary fibrosis increases risk and susceptibility to COVID-19 infection [7]. Acute exacerbations of idiopathic pulmonary fibrosis (IPF) are known as serious events, which can reach a mortality rate of 50% when viral infections play a role [8]. This isn't surprising considering pulmonary fibrosis and severe cases of COVID-19 share a few common risk factors, including: increasing age, male sex,

diabetes and hypertension [9, 10]. Given that pulmonary fibrosis (PF) debilitates lung function, it makes sense that PF would only increase the risk of having a severe case of COVID-19.

Understandably so, these overlapping risk factors are cause for concern when it comes to mitigating a double attack on the lungs, should a patient become exposed to COVID-19. Inversely, people recovering from severe COVID-19 are at serious risk of developing PF [9, 10] clearly demonstrating the two-way relationship between COVID-19 and PF, which calls for specific considerations in how they interact. There are millions of patients worldwide with Long COVID symptoms, (patients that had COVID with lingering symptoms), including coughing, fatigue, low SaO_2, and many with respiratory issues like PF and interstitial lung disease [8, 11]. In 2015, there were over thousands of complaints to the FDA stating that steroids, and all the available nasal spray products, failed to provide relief from nasal inflammation or treat the symptoms of IPF [12]. With many Long COVID patients developing PF and interstitial lung disease, new therapies are needed.

In the COVID-19 infection arm of this research study, we show that N115 is slightly better than saline at reducing viral loads, but N115 was clinically superior over saline in reducing some symptoms of COVID-19 infections, including coughing/sneezing and fatigue. In long COVID, N115 significantly reduced hypoxemia (low SaO_2), coughing/sneezing, trouble breathing, and headaches. In our current and ongoing clinical trials examining the effects of sodium pyruvate nasal spray (N115), we discovered that patients with PF with COPD and IPF without a COPD component experienced significant improvement, including less coughing, improved nasal irritation/erythema, increased average expelled-NO, higher SaO_2, and improved lung function (FVC, FEV_1, PEF, and FEV_1: FVC ratio).

Materials and Methods

COVID-19 Animal Research. Animal research was conducted at the Regional Biocontainment Laboratory at the University of Tennessee Health Sciences Center, Memphis, TN under Institutional Animal Care and Use Committee (IACUC) protocol 2021.013A according to IACUC guidelines, AVMA Guidelines on Euthanasia, NIH regulations (Guide for the Care and Use of Laboratory Animals), and the U.S. Animal Welfare Act of 1966. Two groups of ten 5 – 6 weeks old female K18-hACE2 transgenic mice were infected by intranasal installation of 800pfu of SARS CoV-2 P3 isolate USA-WA1/2020 in 50µl saline. Mice were then treated from day 0-9 with nebulized saline (control) or N115 3x daily for 30 minutes each treatment. Mice were weighed and monitored for survival daily for 14 days.

COVID-19 Infected Clinical Trial. Prior to conducting human clinical trials, IRB approval was obtained (CIRBI:Pro00049340, Appendix B) and the trials were registered on www.clinicaltrials.gov (NCT04824365, NCT04871815). The study protocol was prepared in accordance with the revised Helsinki Declaration for Biomedical Research Involving Human Subjects and Guidelines for Good Clinical Practice and patients included in this study were provided written informed consent. This was a two-phase study. In the first phase, thirty adults with confirmed active COVID-19 infections (by qRT-PCR) were randomly, and blindly, assigned to either a saline nasal spray or a saline + sodium pyruvate nasal spray (N115) treatment group. Patients were instructed to use their spray 3x daily for 14 days. Patient's vital signs (BP, SaO_2, HR, RR, Temp.) were monitored and nasal swabs tested for SARS-CoV-2 levels every 2 days for 14 days. Patients were asked to complete a Daily Symptoms Log every day for 14 days, scoring the symptoms on a Likert scale from 0-10 with 10 representing the most severe symptoms. Symptoms included fatigue, coughing/sneezing, sore throat, chills, congestion,

trouble breathing, headache and body ache. Patients also recorded their body temperature 2x daily for the 14 days.

Long COVID Clinical Trial. In the second phase of the study, 22 patients were enrolled and served as their own negative controls. Patient's vital signs (BP, SaO_2, HR, RR, Temp.) were recorded on the first day, and patients were asked to complete daily symptoms log every day for 7 days without the use of the study medication. Patient's symptoms (coughing/sneezing, chills, trouble breathing, body aches, headaches, fatigue, anxiety, loss of taste/smell and sore throat) were scored on the log using a Likert scale from 0-10 with 10 representing the most severe symptoms. On day 8, patient's vital signs were recorded again as a baseline and the patient administered the first dose of N115. Fifteen minutes later, the patient's vital signs were again tested, and the patients were asked to complete the same daily symptoms log every day for 7 more days while using N115 3x daily as a nasal spray. After the second week, the patients returned for a final collection of vital signs.

Pulmonary Fibrosis Clinical Trial. An initial twenty-one-day sub-chronic clinical trial was conducted that included fifteen patients with PF (9 with PF and COPD and 6 with IPF without COPD) that remained on their normal medications but were also administered the 20mM sodium pyruvate nasal spray (N115). If the patients were also on nasal sprays as part of their normal therapy, that nasal spray was eliminated. In all 15 patients, the test results were compared to their previous three-week screening and baseline data on their current therapies as the baseline control for each variable for all their lung functions (FEV_1, FVC, PEF, FEV_1/FVC ratios, SaO_2, Nitric oxide, coughing rates, and nasal inflammation). Following this, five new patients with PF and COPD had their medications removed and were administered N115 for three days in order to assess its effect without any other medication.

Results

COVID-19: Acute infections in animals. Previously, we demonstrated that treatment with sodium pyruvate can improve inflammation and decrease viral loads in mice during infection with influenza A virus and HSV1 [13, 14]. As pyruvate acts on the host immune response, through metabolic pathways and not directly on the virus [15], our data demonstrate that sodium pyruvate is a promising treatment option that is safe, effective, and unlikely to elicit antiviral resistance. We, therefore, examined the effects of N115 treatment in hACE2 transgenic mice infected with SARS-CoV-2 to determine safety and efficacy. Mice treated with nebulized N115 lost significantly less weight compared to mice treated with nebulized saline (Figure 1). Mortality was similar between groups, but the infectious dose was not expected to result in high mortality. From this preliminary animal study, and in conjunction with multitudes of previously reported safety data [13, 14], we proceeded with a clinical trial in humans with active infection of SARS-CoV-2.

Figure 1

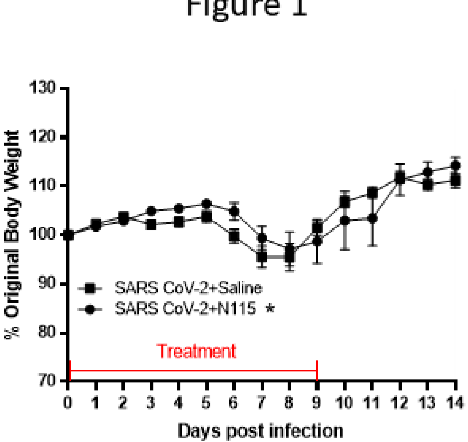


Figure 1: Effects of N115 treatment in mice infected with SARS CoV-2.
Two groups of ten mice each were infected with 800pfu of SARS CoV-2 P3 isolate USA-WA1/2020 in 50µl saline intranasally. One group of ten mice was then treated with PBS 3x daily and the other group of ten mice treated with N115 3x daily. Mice were weighed daily and monitored for malaise and mortality for 14 days. Statistical analysis was performed by two-way ANOVA (*$p<0.05$).

COVID-19 Infected Patients. This clinical trial was designed to determine the safety and efficacy of N115 against saline in COVID-19 infected patients. Thirty adults (Demographics presented in Table 1) with confirmed (positive RT-qPCR test) active COVID-19 infections were randomly, and double blindly, assigned to either a saline nasal spray or a saline + 20mM sodium pyruvate nasal spray (N115) treatment. Patients self-administered the sprays 3x daily for 14 days. Saline is acknowledged (Edenborough ELVIS project) to physically reduce nasal viral titers by 0.5 logs to 0.7 logs over untreated patients and reduces mucus and allergens which subsequently reduces congestion, trouble breathing, and sore throats [16]. Therefore, saline is not a true placebo for this study but a standard of care. Viral titers in N115 treated patients were lower compared to saline treated patients through day 8 as measured by RT-qPCR from nasal swabs ($p<0.0197$) (Figure 2A). N115 lowered viral titers below 10,000, the value that has been reported to significantly decrease transmission of the virus [17]. The mean day for patients to drop below 10,000 viral genome copies as measured by RT-qPCR from nasal swabs was day 6.4 for N115 vs. day 7.7 for saline.

Patient's vital signs (BP, SaO_2, HR, RR, Temp.) were monitored every 2 days for 14 days. Patients were asked to complete a Daily Symptoms Log every day for 14 days, scoring the symptoms on a Likert scale from 0-10 with 10 representing the most severe symptoms. Patients also recorded their body temperature 2x daily for the 14 days. Over the fourteen-day trial, there was no significant change in blood pressure (BP), heart rate (HR), or respiratory rate (RR). We observed similar improvements in patients treated 3x daily with either saline or N115 in SaO_2, trouble breathing, and sore throat (Figure 2B and Table 1). However, N115 performed significantly better with coughing/sneezing ($p<0.0435$) and fatigue ($p<0.0001$) symptoms over saline. (Figure 2C-D and Table 1). We observed significant improvements in patients treated 3x

daily with either saline or N115 over the 14 days, for fever, body aches, headaches and chills that resolved and returned to normal levels by day 14 as viral numbers decreased, but conversely, N115 treatment resulted in higher body temperature (Fever, $p<0.0030$) and higher scores for body aches ($p<0.0001$), headaches ($p<0.0001$), and chills ($p<0.0001$) over saline (Figure 2E-H and Table 1). No adverse events were reported from the use of either saline or N115 by patients or clinical staff.

Table 1. Patient Demographics and Symptom Data

	N115 Treated (15 patients)	Mean	Saline Treated (15 Patients)	Mean	SED	P value
Age (Stdev)	53.2 (±17.57)		54.73 (±19.51)			
Sex (Number)	Female (11) Male (4)		Female (12) Male (3)			
Ethnicity (Number)	Latino (15)		Latino (15)			
Symptoms (Number of patients exhibiting symptoms)	Fever (14)	99.65	Fever (14)	99.34	0.1049	0.0030
	Body Aches (10)	5.186	Body Aches (8)	3.482	0.3035	<0.0001
	Headaches (5)	3.571	Headaches (7)	1.939	0.3362	<0.0001
	Chills (8)	3.929	Chills (8)	1.964	0.2473	<0.0001
	Congestion (8)	3.241	Congestion (10)	3.107	0.3177	0.6738
	Coughing/Sneezi	2.543	Coughing/Sneezi	3.026	0.2382	0.0435
	ng (9)		ng (11)		0.2832	0.2753
	Trouble	3.310	Trouble	3.000	0.2636	<0.0001
	Breathing (12)		Breathing (11)		0.2711	0.8509
	Fatigue (7)	4.020	Fatigue (4)	5.446		
	Sore Throat (8)	3.184	Sore Throat (8)	3.235		

Mean body temperature and patient scores for each sign or symptom over the 14 days of the trial. (SED) standard error of differences, (Stdev) standard deviation. Statistical analysis was performed using two-way ANOVA. $p<0.05$ was considered statistically significant.

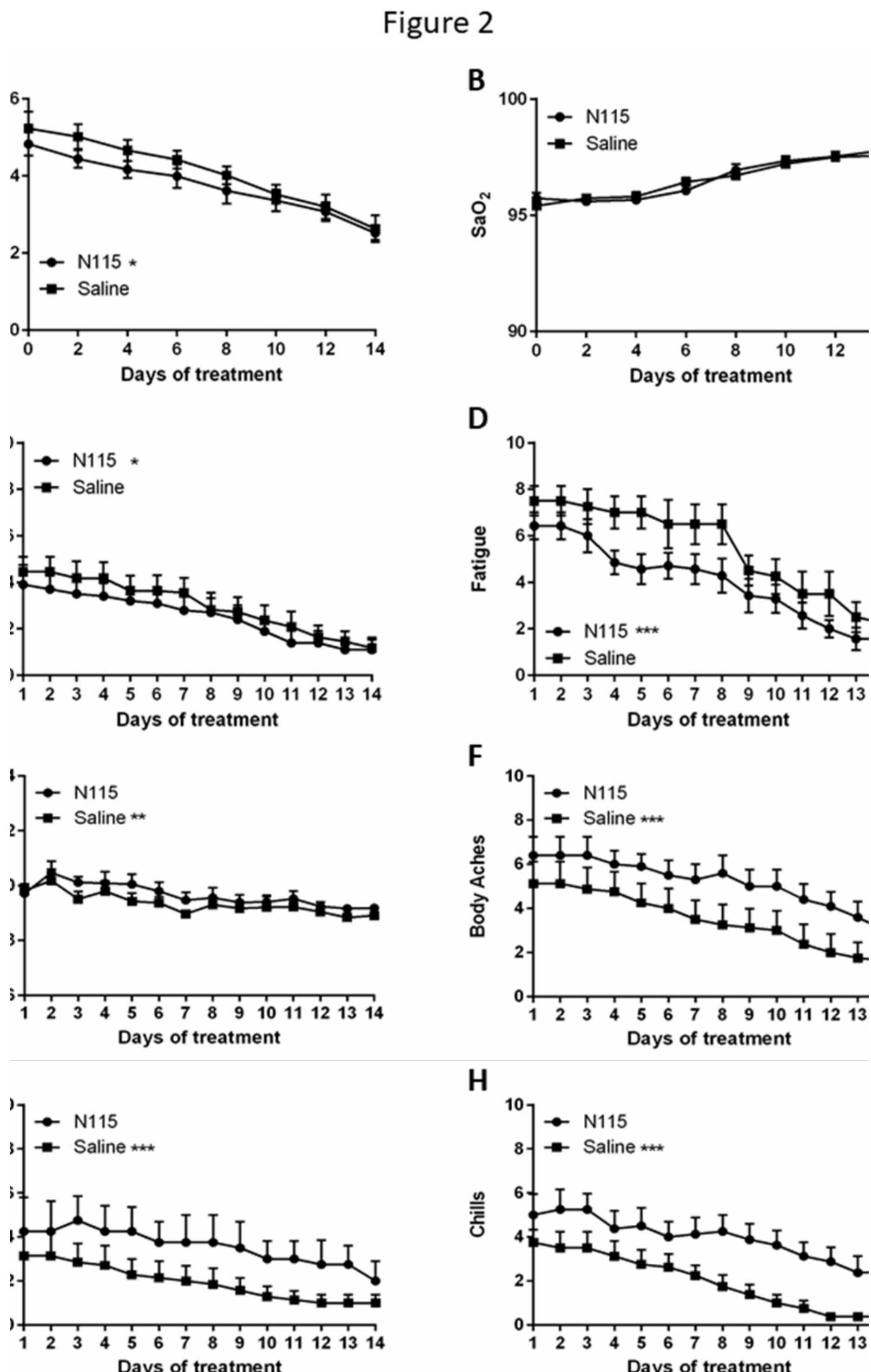


Figure 2: Effects of N115 treatment in Active COVID-19 Infection.
Thirty patients were randomly assigned to either the saline control or the N115 treatment group. Viral titers (A) and SaO_2 (B) were measured every 2 days. Coughing (C), Fatigue (D), Fever (E), Body Aches (F), Headaches (G), and Chills (H) were measured or scored daily on a Likert Scale (0-10, 10=most severe). Data were analyzed for statistical significance by two-way ANOVA. (*p<0.05, **p<0.01, ***p<0.0001)

Long COVID. We next examined the effects of N115 in patients that were experiencing long-term symptoms after recovering from active COVID-19. These patients, known as Long-COVID patients, were monitored for symptoms for one week with no treatment followed by one week of treatment with N115. Patients were not randomized but served as their own negative controls. During the initial 7 days when patients received no treatment, there was no significant change in SaO_2 or heart rate, but there was a slight improvement in BP 1.25mmHg ($p=0.015$) (Figure 3A-D). Heart rate remained stable throughout the trial (Figure 3B). There was additional improvement in BP after N115 treatment within 15 minutes after the first treatment (Day 8 post vs. Day 1, -2.25mmHg, $p<0.0001$) and BP remained lower on day 14 (Day 14 vs Day 1, -2.0mmHg, $p=0.0026$) (Figure 3C-D). Importantly, 15 minutes after the first dose of N115 was administered on day 8, SaO_2 improved by 0.5% from the pretreatment reading on the same day ($p=0.0114$). It continued to improve, and on day 14, SaO_2 levels improved by 1.63% over day 1 and 1.5% over day 8 pretreatment ($p<0.0001$ and $p<0.0001$) (Figure 3A).

During the first 7 days, when there was no treatment, patients reported little to no change in symptoms including body ache, headache, coughing/sneezing, and trouble breathing. However, after N115 treatment for 7 days, patients reported a significant 1.143-point improvement in headaches ($p=0.0373$), a 2.455-point improvement in coughing/sneezing ($p=0.0091$), and a 3.5-point improvement in trouble breathing ($p<0.0001$) (Figure 3E-H). Fatigue, anxiety, loss of taste/smell, congestion and body aches also showed some improvement, but the changes were not significant due to a lack of power from too few patients presenting with these symptoms enrolling in the study (Table 2). Overall, our results demonstrate that N115 significantly improves respiratory function in as little as 15 minutes with substantial improvement within 7 days compared to no treatment controls.

Pulmonary Fibrosis. Many long COVID patients develop PF [9, 10]. Therefore, we include here our data on N115 treatment of PF. Treatment of 15 patients with pulmonary fibrosis with N115, in addition to their standard medication, resulted in a significant (p=0.010) improvement in lung function (breathing) in all patients with IPF without COPD by day eight, and further increasing by day 22 compared to baseline (p=0.0005), as determined by changes in FVC, FEV_1, PEF, and FEV_1/FVC ratios (Figure 4A-C). The improved FEV_1/FVC ratio from 52% to 86% was clinically significant. N115 treatment also showed that coughing was significantly reduced in all patients (p=0.007) (Figure 4D), a significant improvement in nasal irritation/erythema with most patients being free of irritation by day 22 (p=0.0001) (Figure 4E), and a significant increase in the group average expelled NO by day 8 (p=0.010) (Figure 4F). These results indicated that current therapies in use are inadequate alone to treat patient with IPF.

Table 2: Patient Demographics and Symptom Prevalence

	(22 patients)	
Age	31.68 (±9.25)	
Sex	Female (11) Male (11)	
Symptoms (Number of patients exhibiting symptoms)	Fever (<99.5˚F)	(1)
	Body Aches	(3)
	Headaches	(7)
	Chills	(0)
	Congestion	(4)
	Coughing/Sneezing	(11)
	Trouble Breathing	(16)
	Fatigue	(3)
	Sore Throat	(1)
	Smell/ Taste	(4)
	Anxiety	(2)

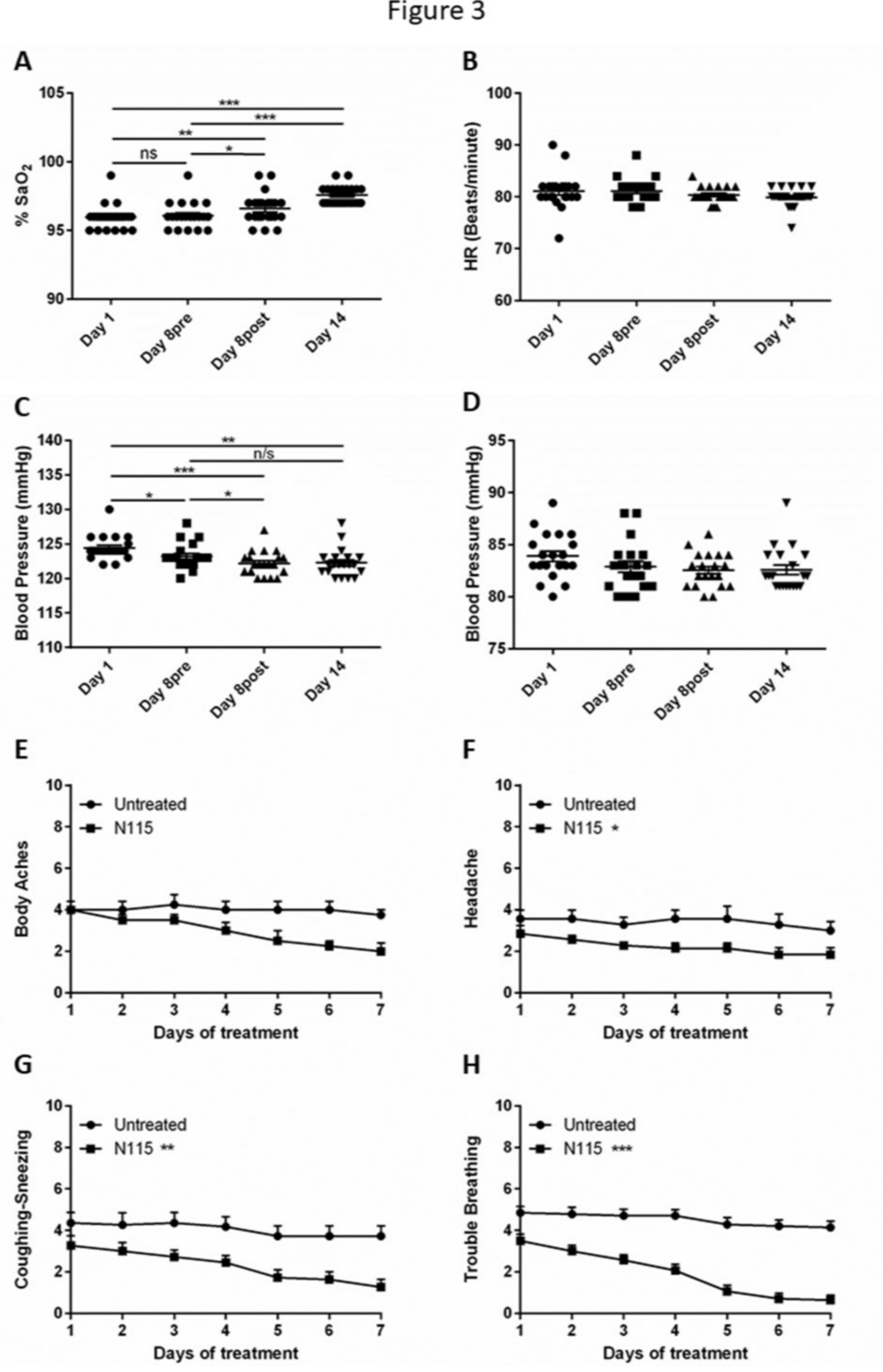


Figure 3: Results from N115 treatment of Long COVID Patients. Twenty-two patients were monitored for signs and symptoms for 7 days without treatment and then for an addition 7 days with N115 treatment. SaO_2 (A), Heart Rate (B), Systolic Blood Pressure (C) and Diastolic Blood Pressure (D) were measured on day 1, day 8 before treatment, day 8 after treatment (15 minutes after treatment), and day 14 (7 days of treatment). All symptoms including Body Aches (E), Headaches (F), Coughing/Sneezing (G) and Trouble Breathing (H) were measured or scored daily on a Likert Scale (0-10, 10=most severe) for 7 days prior to treatment and measured again from day 8-14 with N115 treatment. Data were analyzed for statistical significance by one-way ANOVA (A-D) or two-way ANOVA (E-H). (*$p<0.05$, **$p<0.01$, ***$p<0.0001$).

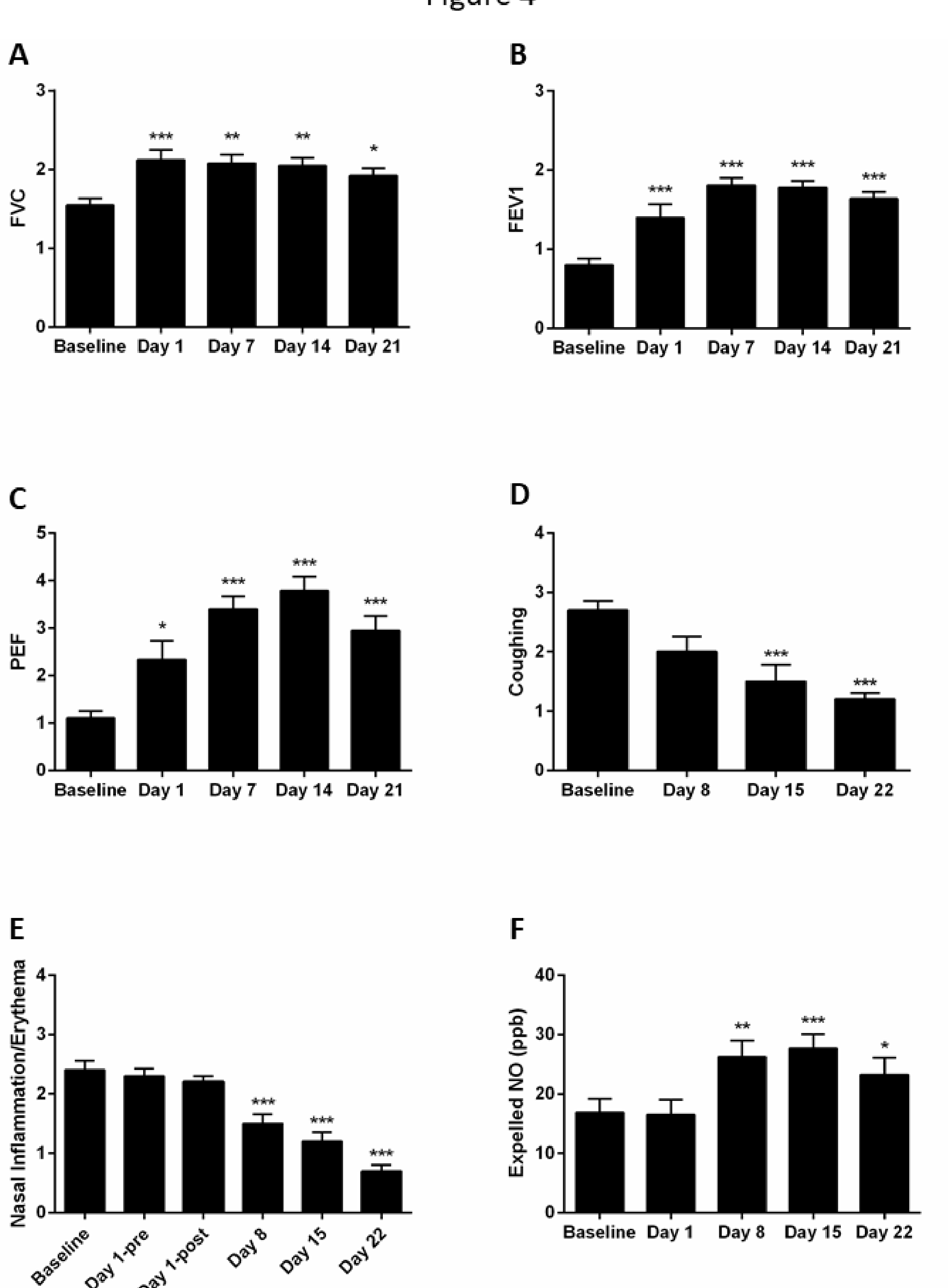


Figure 4: Sub-Chronic treatment of PF patients with N115. Fifteen patients (9 with PF and COPD and 6 with IPF without COPD) were monitored during a three-week screening to establish a baseline. Patients were then treated with N115 for 21 days and signs or symptoms collected on the indicated days. Data were analyzed for statistical significance by one-way ANOVA. (* $p<0.05$, ** $p<0.01$, *** $p<0.0001$).

In a second round, five patients with PF and COPD had their medications removed and were administered only N115 nasal spray solution for three days in order to assess its effects. The data from the three-day trial indicated a statistically and clinically significant improvement in lung function compared to baseline with increases that ranged from 12.0% to 43% in FVC, FEV_1, PEF, and FEV_1/FVC ratios (Figure 5A-C). A significant improvement was also seen in SaO_2 levels, compared to baseline, such that all subjects had SaO_2 levels of ≥97, which persisted throughout the trial (p-values <0.001 at all time points) (Figure 5D).

Figure 5

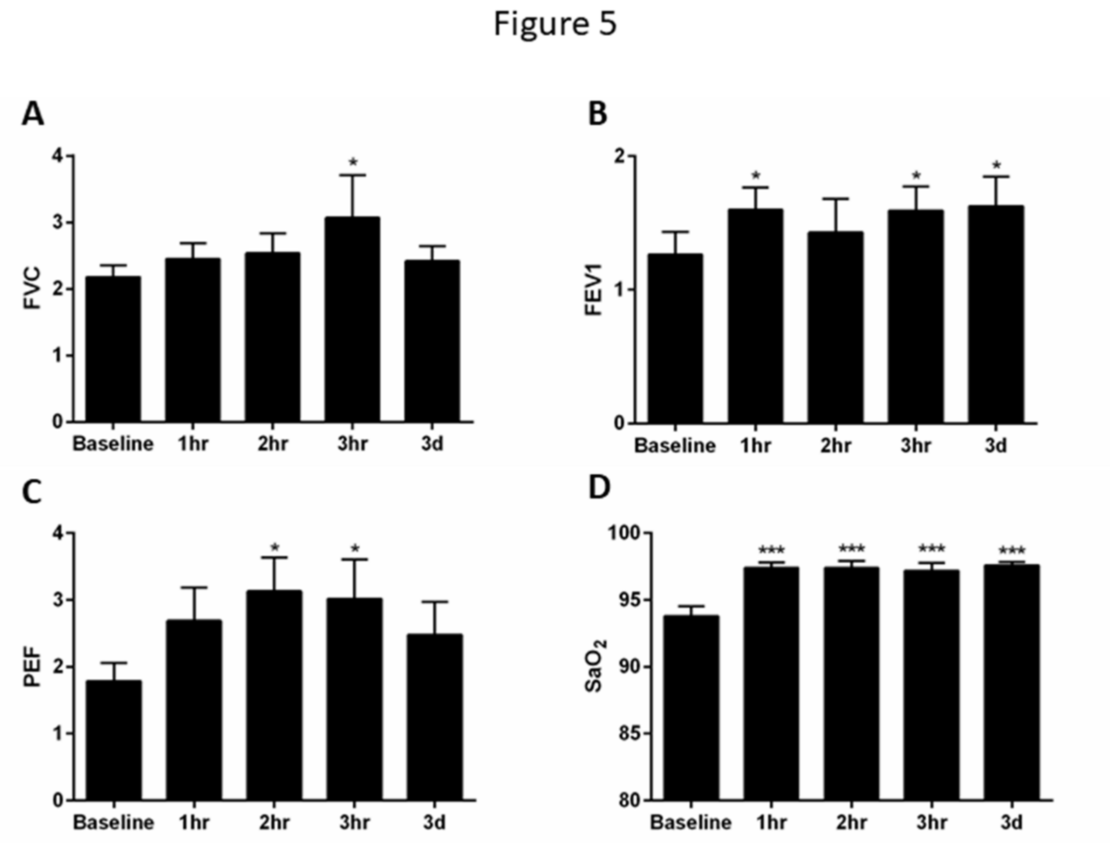


Figure 5: Acute treatment of PF with N115.
Following the 1-3-day pre-study period, eligible moderate PF patients returned to the clinic and were admitted to the Acute Phase three-day study. On day one of the study, patients were removed from their current therapies and patient data were recorded as the baseline prior to treatment. Then, patients were treated with N115 nasal spray and data recorded at the indicated times. Data were analyzed for statistical significance by one-way ANOVA. (*p<0.05, **p<0.01, ***p<0.0001)

Discussion and Conclusions

To combat the continuing COVID-19 pandemic, and to treat the symptoms in long COVID (hypoxemia (low SaO_2), fatigue, coughing/sneezing, trouble breathing, body aches, headaches and pulmonary fibrosis), N115 (sodium pyruvate) was chosen because of its safety and efficacy profile after treating 3.5 million patients in over 200 hospitals globally with no adverse events reported. In 19 Phase I, II, III FDA human clinical trials, against a saline placebo, only N115 reduced inflammation and oxygen radicals and inflammatory cytokines including IL-6, a cause of the cytokine storm in patients with an active COVID-19 infection [18]. In prior clinical trial, N115, not the saline placebo, reduced congestion and coughing while increasing lung functions, increasing the synthesis of NO, and increasing SaO_2 levels in thousands of patients including patients with varying lung diseases like COPD, pulmonary fibrosis, cystic fibrosis, allergic rhinitis, sinusitis and influenza infected patients [18]. Numerous studies have shown oxidative stress to be associated with pulmonary fibrosis, including Long COVID patients with PF, and that antioxidants are effective in attenuating fibroproliferative responses in the lungs of animals and humans [18-22]. Sodium Pyruvate is a natural antioxidant of the human body that inhibits fibrosis and received Orphan Drug Designations for the treatment of Cystic Fibrosis and Pulmonary Fibrosis [14, 18, 21]. The objective of the clinical trials reported here was to study the safety and efficacy of N115 and changes in lung function and COVID symptoms in acute virally infected COVID-19 patients, patients with chronic symptoms after COVID-19 (Long COVID), and patients with PF.

In the COVID-19 infections study, saline nasal spray was used as a control. However, saline is acknowledged (Edenborough ELVIS project) to physically reduce other Coronavirus titers by 0.5 logs to 0.7 logs over untreated patients, and saline also reduces mucus and allergens

which subsequently reduce congestion, trouble breathing, and sore throats [16]. Therefore, saline is not a true placebo. Still, N115 lowered viral titers to below 10,000 by day 6.4 versus day 7.7 for saline. As titers below 10,000 reduce the transmission of COVID-19, this may help decrease virus spread in N115 treated patients [17]. N115 was also significantly better at reducing some of the symptoms of COVID-19 infections including coughing/sneezing and fatigue. Unfortunately, other drugs tested for COVID-19 treatment delivered in saline have reported increased coughing, sore throat, irritation, and other negative symptoms. As reported by the WHO, steroids increased SARS CoV-2 titers over untreated patients, potentially exacerbating infection. Unlike steroids that down-regulate nasal nitric oxide synthesis, sodium pyruvate reduces nasal inflammation while increasing the synthesis of nitric oxide in the nasal passages that is released into the lungs. Nitric oxide is then available to the lungs to fight infections, maintain bronchodilation, increase lung functions and decrease lung fibrosis [23-29].

Over the fourteen-day trial, patients treated with either saline or N115 showed improvement in headaches, trouble breathing, body aches, chills, sore throats, coughing/sneezing and fatigue. N115 treated patients did have slightly higher fever and took longer for chills, body aches and headaches to return to normal. As N115 works by modulating the immune response, including increasing NO etc., and not by direct antiviral activity, some increase in immune responses is anticipated while others are anticipated to decrease [13-15]. However, saline, does not affect inflammation or inflammatory cytokines, does not decrease oxygen radicals, or decrease coughing or increase lung functions, which are all desirable for treatment of COVID-19 and Long COVID or patients with PF and are documented with N115. Furthermore, N115 is not likely to elicit antiviral resistance as it targets the host response and not the virus directly. As SARS CoV-2 variants continue to immerge to the vaccine and are likely to immerge to antiviral

drugs, the development of immune modulators like N115 that can treat COVID-19 patients is essential.

Long COVID patients were monitored for symptoms for one week with no treatment followed by one week with treatment with N115. Patients were not randomized but served as their own negative controls. During the first 7 days, when there was no treatment, patients reported little to no change in symptoms. However, after N115 treatment for 7 days, patients reported a significant improvement in headache, coughing/sneezing, and trouble breathing. Most importantly, N115 treatment improved SaO_2 from the pretreatment reading on the same day and continued to improve blood oxygenation through day 14 as well as lowering blood pressure. Overall, N115 significantly improves respiratory function, which was supported by the patient's scores on trouble breathing and SaO_2.

Numerous reports indicate that long COVID patients develop pulmonary fibrosis, associated with excessive tissue remodeling, scarring, fibrosis, decreased FEV_1 values, decreased SaO_2 and decreased Nitric Oxide (NO) associated with nasal inflammation that causes congestion, coughing, trouble breathing, and sleep disorders [10, 11, 30-33]. Also, patients with pulmonary fibrosis have an increased risk and susceptibility to COVID-19 infection, which can reach a mortality rate of 50% [7, 34]. Thus, N115 was used to determine its efficacy and safety in these patients too. During the acute treatment of patients with PF with a COPD component, their regular therapy was removed, and they were treated for three days with only N115 nasal spray, which demonstrated a statistically and clinically significant improvement in all lung functions compared to baseline as determined by changes in FVC, FEV_1, PEF, and FEV_1/FVC ratios, which persisted throughout the three-day trial. A significant immediate average improvement was also seen in SaO_2 levels.

The results in the sub-chronic 21-day clinical test of patients with PF with a COPD component and patients with IPF without a COPD component, showed that inhalation of N115 for 21 days provides a significant reduction in coughing by day eight of the treatment, and continued to decrease over the course of the 21-day treatment. There was a significant improvement in nasal irritation/erythema with most patients being free of irritation by day 22. The group average expelled NO was higher, with 14 of 15 patients showing an increase during the study. Patients with PF with a COPD component remained on their regular therapy. Thus, no improvement in some lung functions was anticipated. However, there was a significant ($p=0.010$) improvement in lung function observed in all patients with IPF without COPD, while on their current medications as determined by changes in FVC, FEV_1, PEF, and FEV_1/FVC ratios. The improved FEV_1/FVC ratios from 52% to 86% was clinically significant and indicated that current therapies in use are inadequate to treat patient with IPF. This study confirmed the thousands of complaints to the FDA stating that steroids, and all the available nasal spray products, are inadequate to provide relief from nasal inflammation or treat the symptoms of IPF [12]. Importantly, very high patient acceptance of the N115 drug was reported (8 to 10 out of 10: "very good" or "excellent").

Although the number of patients enrolled in these trials was small, thus decreasing the power of the statistical analysis, the results all indicate that N115 is able to improve lung function, especially in patients suffering from chronic conditions such as PF and Long COVID. Seventeen other clinical trials with data submitted to the U.S. FDA also demonstrate similar findings in patients treated with N115 including COPD, cystic fibrosis, allergic rhinitis, sinusitis and influenza infected patients where lung function, NO and SaO_2 improved with N115 treatment. In these studies, as in the previous 17 studies, N115 must be inhaled, and our previous

work shows that the route of administration is important for the function of sodium pyruvate [13, 14]. Overall, our results demonstrate that N115 is a promising treatment that warrants further investigation.

Acknowledgements

We thank the staff at Family First Medical Research Clinic, Virginia Gardens, FL for patient recruitment and collection of patient data. We also thank Trinity Healthcare and Dynamic DNA Labs, Springfield, MO for collection of patient data.

References

1. Chen N, M Zhou, X Dong, et al. 2020. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020; 395: 507-513.

2. Organization, WH. 2021. Coronavirus disease (COVID-19) Weekly Epidemiological Update and Weekly Operational Update. 2021.

3. Karki R, BR Sharma, S Tuladhar, et al. Synergism of TNF-alpha and IFN-gamma Triggers Inflammatory Cell Death, Tissue Damage, and Mortality in SARS-CoV-2 Infection and Cytokine Shock Syndromes. Cell. 2021; 184: 149-168 e117.

4. Williamson EJ, AJ Walker, K Bhaskaran, et al. Factors associated with COVID-19-related death using OpenSAFELY. Nature. 2020; 584: 430-436.

5. Kalchiem-Dekel O, JR Galvin, AP Burke, et al. Interstitial Lung Disease and Pulmonary Fibrosis: A Practical Approach for General Medicine Physicians with Focus on the Medical History. J Clin Med 2018; 7.

6. Todd NW, IG Luzina, SP Atamas. Molecular and cellular mechanisms of pulmonary fibrosis. Fibrogenesis Tissue Repair. 2012; 5: 11.

7. Naqvi SF, DA Lakhani, AH Sohail, et al. Patients with idiopathic pulmonary fibrosis have poor clinical outcomes with COVID-19 disease: a propensity matched multicentre research network analysis. BMJ Open Respir Res. 2021; 8.

8. Uzel FI, S Iliaz, F Karatas, et al. COVID-19 Pneumonia and Idiopathic Pulmonary

Fibrosis: A Novel Combination. Turk Thorac J. 2020; 21: 451-453.

9. Naik PK, BB Moore.Viral infection and aging as cofactors for the development of pulmonary fibrosis. Expert Rev Respir Med. 2010; 4: 759-771.

10. Ojo AS, SA Balogun, OT Williams, et al. Pulmonary Fibrosis in COVID-19 Survivors: Predictive Factors and Risk Reduction Strategies. Pulm Med. 2020: 6175964.

11. Groff D, A Sun, AE Ssentongo, et al. Short-term and Long-term Rates of Postacute Sequelae of SARS-CoV-2 Infection: A Systematic Review. JAMA Netw Open. 2021; 4: e2128568.

12. Administration, U. S. F. a. D. 2015. The Voice of the Patient: Idiopathic Pulmonary Fibrosis. 2015.

13. Reel J, C Lupfer. Sodium Pyruvate Ameliorates Influenza A Virus Infection In Vivo. Microbiol Res. 2021; 12: 258-267.

14. Sheridan J, E Kern, A Martin, et al. Evaluation of antioxidant healing formulations in topical therapy of experimental cutaneous and genital herpes simplex virus infections. Antiviral Res. 1997; 36: 157-166.

15. Abusalamah H, JM Reel, CR Lupfer. Pyruvate affects inflammatory responses of macrophages during influenza A virus infection. Virus Res. 2020; 286: 198088.

16. Ramalingam S, C Graham, J Dove, et al. Hypertonic saline nasal irrigation and gargling should be considered as a treatment option for COVID-19. J Glob Health. 2020; 10: 010332.

17. Wolfel R, VM Corman, W Guggemos, et al. Virological assessment of hospitalized patients with COVID-2019. Nature. 2020; 581: 465-469.

18. Inc., E. 2021. N115 FDA Submissions.

19. Kelly FJ, A Blomberg, A Frew, et al. Antioxidant kinetics in lung lavage fluid following exposure of humans to nitrogen dioxide. Am J Respir Crit Care Med. 1996; 154: 1700-1705.

20. Mogensen TH, J Melchjorsen, P Hollsberg, et al. Activation of NF-kappa B in virus-infected macrophages is dependent on mitochondrial oxidative stress and intracellular calcium: downstream involvement of the kinases TGF-beta-activated kinase 1, mitogen-activated kinase/extracellular signal-regulated kinase kinase 1, and I kappa B kinase. J Immunol. 2003; 170: 6224-6233.

21. Stanko RT, L O'Hare. The power of pyruvate: the natural way to better health and well

being. Keats Pub., Los Angeles. 1999.

22. Suhail S, J Zajac, C Fossum, et al. Role of Oxidative Stress on SARS-CoV (SARS) and SARS-CoV-2 (COVID-19) Infection: A Review. Protein J. 2020; 39: 644-656.

23. Artlich A, T Busch, K Lewandowski, et al. Childhood asthma: exhaled nitric oxide in relation to clinical symptoms. Eur Respir J. 1999; 13: 1396-1401.

24. Dhar A, JM Brindley, C Stark, et al. Nitric oxide does not mediate but inhibits transformation and tumor phenotype. Mol Cancer Ther. 2003; 2: 1285-1293.

25. Djupesland PG, JM Chatkin, W Qian, et al. Nitric oxide in the nasal airway: a new dimension in otorhinolaryngology. Am J Otolaryngol. 2001; 22: 19-32.

26. Gallai V, P Sarchielli. Nitric oxide in primary headaches. J Headache Pain. 2000; 1: 145-154.

27. Gerstberger R. Nitric Oxide and Body Temperature Control. News Physiol Sci. 1999; 14: 30-36.

28. Gouw, PE. Stimuli affecting exhaled nitric oxide in asthma. European Respiratory Review. 1999; 9: 219-222.

29. Jobsis Q, HC Raatgeep, SL Schellekens, et al. Hydrogen peroxide and nitric oxide in exhaled air of children with cystic fibrosis during antibiotic treatment. Eur Respir J. 2000; 16: 95-100.

30. Bui LT, NI Winters, MI Chung, et al. Chronic lung diseases are associated with gene expression programs favoring SARS-CoV-2 entry and severity. Nat Commun. 2021; 12: 4314.

31. Deng L, A Khan, W Zhou, et al. Follow-up study of clinical and chest CT scans in confirmed COVID-19 patients. Radiol Infect Dis. 2020; 7: 106-113.

32. McGroder CF, D Zhang, MA Choudhury, et al. Pulmonary fibrosis 4 months after COVID-19 is associated with severity of illness and blood leucocyte telomere length. Thorax. 2021.

33. Wang Y, C Dong, Y Hu, et al. Temporal Changes of CT Findings in 90 Patients with COVID-19 Pneumonia: A Longitudinal Study. Radiology. 2020; 296: E55-E64.

34. Mahmud SMH, M Al-Mustanjid, F Akter, et al. Bioinformatics and system biology approach to identify the influences of SARS-CoV-2 infections to idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease patients. Brief Bioinform. 2021; 22.

OVERALL CONCLUSIONS

Diet and metabolism significantly affect the immune system and should be studied extensively as every nutrient that is consumed affects the body in some way. In this study, I found that in the absence of infection, treatment with bean lectins resulted in an increase in IL-6 production by macrophages. This is consistent with previous studies that have shown that PHA is inflammatory, activates T cells, and induces proliferation and differentiation of lymphocytes. It was also reported in a previous study that PHA treatment increased the expression of inflammatory cytokines like IL-6 and TNF-α in human PBMCs. [36]. While I did not see a statically significant increase in IL-1β or IL-6 when cells were treated with wheat lectin, it has been reported in previous studies that WGA induces the release of pro-inflammatory cytokines and can stimulate monocytes and macrophages. Importantly, WGA has been shown to increase permeability of cells in the gastrointestinal tract which could lead to inflammation in the gut and could contribute to diseases like irritable bowel syndrome [37]. While this study examined the effects of red kidney bean lectin and wheat lectin on the levels of IL-1β and IL-6 cytokines produced by mouse macrophages, there are many other dietary lectins that could be studied as well.

Endogenous metabolic products also play a critical role in the regulation and modulation of the immune response. Pyruvate is a metabolite that plays a critical role in energy production. It is the end product of glycolysis and the starting material for the tricarboxylic acid (TCA) cycle [38]. Sodium pyruvate is an endogenous antioxidant that is secreted by cells and can react with oxygen radicals to detoxify them and prevent them from damaging organs [39]. Sodium pyruvate has been shown to decrease inflammasome activation during influenza A infection [34]. In a

previous study, it was found that inhalation of sodium pyruvate nasal spray can decrease nasal inflammation and congestion in patients with allergic rhinitis [39]. Nasal inflammation was a symptom studied briefly in the pulmonary fibrosis leg of the clinical trial discussed earlier herein. 40 to 80% of COPD patients also have nasal symptoms like allergic rhinitis, and some of the clinical trial patients had both pulmonary fibrosis and COPD [39]. Therefore, sodium pyruvate could possibly provide these patients with much needed relief, without the risk of adverse effects. In addition to these findings, hypertonic sodium pyruvate was found to be more effective in protection against inflammation and stress injury events than Ringer's ethyl pyruvate [40]. Sodium pyruvate is also useful in the storage of organs for transplant surgeries because it decreases the amount of cell death and increases graft metabolism [41]. While there has already been a lot of research on the therapeutic potential of sodium pyruvate, more research needs to be done to explore the entirety of its uses.

Lectins and pyruvate are just 2 of thousands of dietary and metabolic products that impact the immune system. In general, diet and metabolism play a significant role in the regulation and modulation of the immune response. There are many different "fad diets" that on the surface seem like their goal is to make the body healthier. However, these diets may provide inadequate nutrition for a properly functioning immune system and could exacerbate prior chronic diseases [42]. Preventing and treating obesity is important, as being obese or overweight increases the risk of cardiovascular disease, high blood pressure, diabetes, and many other diseases and disorders [43]. However, it is also important to provide the body with enough nutrients so that it can function optimally. This is especially important for the immune system since it requires more nutrients due to increased metabolic rate during an infection. In addition, diet plays a critical role in the maintenance and health of the gut microbiome. Recent studies

have suggested that the composition of the gut microbiome is pivotal in the regulation of chronic diseases like obesity, type 2 diabetes, cardiovascular disease, inflammatory bowel disease, and inflammatory skin conditions [44]. Since diet is easily changeable and can rapidly alter the gut microbiome, it is important to understand how specific foods affect the bacterial species present and what the implications of those alterations are. In general, it is vital that we continue to research and investigate the essential roles that diet and metabolism play in a healthy immune system.

References

1. Childs CE, PC Calder, EA Miles. Diet and Immune Function. Nutrients. 2019; 11: 1933.

2. Camell C, E Goldberg, VD Dixit. Regulation of Nlrp3 inflammasome by dietary metabolites. Seminars in Immunol. 2015; 27: 334–342.

3. Christ A, M Lauterbach, E Latz. Western Diet and the Immune System: An Inflammatory Connection. Immunity. 2019; 51: 794–811.

4. Kiran S, V Kumar, EA Murphy, et al. High Fat Diet-Induced CD8+ T Cells in Adipose Tissue Mediate Macrophages to Sustain Low-Grade Chronic Inflammation. Front in Immunol. 2021; 12.

5. Pahwa R, A Goyal, I Jialal. Chronic Inflammation. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. 2022.

6. WHO Coronavirus (COVID-19) Dashboard. n.d. With Vaccination Data. https://covid19.who.int

7. de Frel DL, DE Atsma, H Pijl, et al. The Impact of Obesity and Lifestyle on the Immune System and Susceptibility to Infections Such as COVID-19. Front in Nutri. 2020; 7.

8. Chen L, H Deng, H Cui, et al. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2017; 9: 7204–7218.

9. Gu Y, X Zuo, S Zhang, et al. The Mechanism behind Influenza Virus Cytokine Storm. Viruses. 2021; 13: 1362.

10. Hughes CE, R Nibbs. A guide to chemokines and their receptors. FEBS J. 2018; 285: 2944–2971.

11. Wu Xianli, A Schauss. Mitigation of Inflammation with Foods. J Agri Food Chem. 2012; 60.

12. Lester SN, K Li. Toll-like receptors in antiviral innate immunity. J Mol Biol. 2014; 426: 1246–1264.

13. El-Zayat SR, H Sibaii, FA Mannaa. Micronutrients and many important factors that affect the physiological functions of toll-like receptors. Bull Natl Res Cent. 2019; 43: 123.

14. Wan Z, C Durrer, D Mah, et al. One-week high-fat diet leads to reduced toll-like receptor 2 expression and function in young healthy men. Nutri Res. 2014; 34: 1045–1051.

15. Lee JY, KH Sohn, SH Rhee, et al. Saturated Fatty Acids, but Not Unsaturated Fatty Acids, Induce the Expression of Cyclooxygenase-2 Mediated through Toll-like Receptor 4. J Biol Chem. 2001; 276: 16683–16689.

16. Rehwinkel J, MU Gack. RIG-I-like receptors: their regulation and roles in RNA sensing. Nat Rev Immunol. 2020; 20: 537–551.

17. Yang G, HE Lee, JK Seok, et al. RIG-I Deficiency Promotes Obesity-Induced Insulin Resistance. Pharmaceuticals (Basel, Switzerland). 2021; 14: 1178.

18. Liu T, L Zhang, D Joo, et al. NF-κB signaling in inflammation. Signal Transduction and Targeted Therapy. 2017; 2: 17023–.

19. Carlsen H, F Haugen, S Zadelaar, et al. Diet-induced obesity increases NF-kappaB signaling in reporter mice. Genes and Nutrition. 2009; 4: 215–222.

20. Capece D, D Verzella, I Flati, et al. NF-κB: blending metabolism, immunity, and inflammation. Trends in Immunol. 2022; 43: 757–775.

21. Franchi L, N Warner, K Viani, et al. Function of Nod-like receptors in microbial recognition and host defense. Immunol Rev. 2009; 227: 106–128.

22. Caruso R, N Warner, N Inohara, et al. NOD1 and NOD2: Signaling, host defense, and inflammatory disease. Immunity. 2013; 41: 898–908.

23. Zangara MT, I Johnston, EE Johnson, et al. Mediators of Metabolism: An Unconventional Role for NOD1 and NOD2. Int J Mol Sci. 2021; 22: 1156.

24. Zhong Y, A Kinio, M Saleh. Functions of NOD-Like Receptors in Human Diseases. Frontiers in Immunol. 2013; 4.

25. Ren K, R Torres. Role of interleukin-1beta during pain and inflammation. Brain Res Revs. 2009; 60: 57–64.

26. Watanabe S, M Alexander, AV Misharin, et al. The role of macrophages in the resolution of inflammation. J Clin Invest. 2019; 129: 2619–2628.

27. Tay MZ, CM Poh, L Rénia, et al. The trinity of COVID-19: immunity, inflammation and intervention. Nature Rev Immunol. 2020; 20: 363–374.

28. Olwal CO, NN Nganyewo, K Tapela, et al. Parallels in Sepsis and COVID-19 Conditions: Implications for Managing Severe COVID-19. Frontiers in Immunol. 2021; 12.

29. Eisinger GJ, W Osman, ER Prather, et al. Inflammasome Activation in an In Vitro Sepsis Model Recapitulates Increased Monocyte Distribution Width Seen in Patients With Sepsis. Critical Care Explorations. 2022; 4: e0631.

30. Nedeva C, J Menassa, H Puthalakath. Sepsis: Inflammation Is a Necessary Evil. Front Cell Dev Biol. 2019; 7.

31. Allergy Facts. Asthma and Allergy Foundation of America. https://aafa.org/allergies/allergy-facts/

32. Gong T, X Wang, Y Yang, et al. Plant Lectins Activate the NLRP3 Inflammasome To Promote Inflammatory Disorders. J Immunol. 2017; 198: 2082–2092.

33. Lectins. 2022. The Nutrition Source. https://www.hsph.harvard.edu/nutritionsource/anti-nutrients/lectins/

34. Reel JM, CR Lupfer. Sodium Pyruvate Ameliorates Influenza A Virus Infection In Vivo. Microbiology Research. 2021; 12: 258–267.

35. Lupfer CR, R Nadler, R Amen, et al. Inhalation of Sodium Pyruvate to Reduce the Symptoms and Severity of Respiratory Diseases Including COVID-19, Long COVID, and Pulmonary Fibrosis. Eur J Res Med. 2021; 3: 3.

36. Prajitha N, PV Mohanan. Intracellular inflammatory signaling cascades in human monocytic cells on challenge with phytohemagglutinin and 2,4,6-trinitrophenol. Mol Cell Biochem. 2022; 477: 395–414.

37. de Punder K, L Pruimboom. The Dietary Intake of Wheat and other Cereal Grains and Their Role in Inflammation. Nutrients. 2013; 5: 771–787.

38. Fink MP. The Therapeutic Potential of Pyruvate. J Surg Res. 2010; 164: 218–220.

39. Martin A, C Lupfer, R Amen. Sodium Pyruvate Nasal Spray Reduces the Severity of Nasal Inflammation and Congestion in Patients with Allergic Rhinitis. J Aerosol Medicine and Pulmonary Drug Delivery. 2022.

40. Sharma P, PG Mongan. Hypertonic sodium pyruvate solution is more effective than Ringer's ethyl pyruvate in the treatment of hemorrhagic shock. Shock. 2010; 33: 532-40.

41. Cobert ML, M Peltz, LM West, et al. Glucose is an ineffective substrate for preservation of machine perfused donor hearts. J Surg Res. 2012; 173: 198-205.

42. Tahreem A, A Rakha, R Rabail, et al. Diets: Facts and Fiction. Front Nutri. 2022; 9: 960922.

43. Fruh SM. Obesity: Risk factors, complications, and strategies for sustainable long-term weight management. J Am Assoc Nurse Pract. 2017; 29: S3–S14.

44. Singh RK, HW Chang, D Yan, et al. Influence of diet on the gut microbiome and implications for human health. J Translat Med. 2017; 15: 73.

APPENDICES

Appendix A

PI: Christopher L Lupfer Page: 1 of 15

Application to Use Live Vertebrate Animals Dept: Biology

IACUC ID: 19-019.0 Web ID: 500

Title: Anti-inflammatory function of sodium pyruvate during influenza A virus infection
Species: Mouse (Other)
Application Type: New Application
Multiple Species: No
Total Animal Number: 264 (Non-ORC - Bred)

Office Use Only

IACUC ID: 19-019.0-A

Renewal Date:

Yes 4.1 REQUIRED - Check this box in order to access Section 4.1, Alternatives to Proposed Procedures. Failure to check this box may result in protocol review delays.

Submission History for New Application:

06/13/2019 - Submitted
06/26/2019 - Under Review
07/03/2019 - Approved
07/03/2019 - Complete

Approval Date: 6/28/2019

PI: Christopher L Lupfer Page: 2 of 15

Application to Use Live Vertebrate Animals Dept: Biology

IACUC ID: 19-019.0 Web ID: 500

1. Personnel Information

Personnel		Roles	Techniques
Name:	**Christopher L Lupfer**	Email Contact Laboratory Coordinator Official Contact Principal Investigator	Anesthesia - Administering Anesthesia - Monitoring CO2 with Physical Euthanasia Handling and Restraint Injectable Anesthesia Intraperitoneal Injection Weighing and Measuring Intranasal infection with influenza A virus. administration of treatment in drinking water and food.
Dept:	152024 - Biology		
Campus Box:	901 S National Ave Temple 254 Springfield MO 65897-0027		
Phone:	417-836-6887		
Email:	christopherlupfer@missouristate.edu		
Name:	**Jessica Reel**	Student Investigator	Anesthesia - Administering Anesthesia - Monitoring CO2 with Physical Euthanasia Handling and Restraint Injectable Anesthesia Intraperitoneal Injection Weighing and Measuring Intranasal infection with influenza A virus. administration of treatment in drinking water and food.
Dept:	152024 - Biology		
Campus Box:	901 S National Avenue Springfield MO 65897-0027		
Phone:			
Email:	jessica19@live.missouristate.edu		
Name:	**Jordan Fleetwood**	Student Investigator	Anesthesia - Administering Anesthesia - Monitoring CO2 with Physical Euthanasia Handling and Restraint Injectable Anesthesia Intraperitoneal Injection Weighing and Measuring Intranasal infection with influenza A virus. administration of treatment in drinking water and food.
Dept:	152024 - Biology		
Campus Box:	901 S National Ave Springfield MO 65897-0027		
Phone:			
Email:	jordan987@live.missouristate.edu		

2. Funding

Funding Source	Agency Deadline	Funding Period	Grant Number
National Institutes of Health	10/25/2019	3 years	

3. Scientific Justification for Animal Species

1. **Justify the species to be used by indicating:**

   This is a new model. (Veterinarians available for consultation on new model development.) **No**

   The results will be directly applicable to the health, care or welfare of this species. **No**

   Other Justification? **Yes**

Approval Date: 6/28/2019

If Yes, Explain:

The purpose of this study is to examine the effects of the natural metobolite sodium pyruvate on the immune response to influenza A virus infection. In immunology, the mouse is the prefered species as there is a wealth of knowledge regarding the mouse immune system and there are established molecular and diagnostic tools, such as antibodies, for detecting mouse cytokines.

2. Features of the species (e.g. anatomic, physiologic, genetic, etc.) that make it desirable for this model.

The mouse is prefered for the study of infectious disease and immunology for several reasons. First, mice are easy to handle, house and physicaly manipulate. Second, mice are the prefered species as there is a wealth ofknowledge regarding the mouse immune system and there are established molecular and diagnostic tools such as antibodies for detecting mouse cytokines. Finally, the mouse has already been established as a model for the study of multiple infectious diseases of human importance, including influenza A virus.

3. Will the PI conduct the same experiment in multiple species? No
If Yes, Explain:

4. Reduction, Refinement, Replacement, and Animal Numbers

1. Reduction, Refinement, and Replacement

a. Replacing vertebrate animals

No Are there computer simulation, non-living, or in vitro alternatives to the proposed use of animals described in your application?

If Yes, Explain:

Although we have performed initial examination of the immune signaling pathways in vitro in cell lines, cells in isolation cannot recapitulate the complexity of the immune system of an entire living organism. Therefore, the conclusions of any in vitro immunology experiment must be validated in vivo. The determination of therapeutic efficacy requires the examination of these drugs in an animal model.

b. Refining experimental procedures to minimize pain or distress

Yesendpoints in the design of the experiment? Did you consider the use of pain-relieving drugs, or procedures that avoid or minimize discomfort, distress and pain, and humane

If No, Explain:

The proposed research will examine the anti-inflammatory effects of sodium pyruvate on the immune response to influenza A virus and determine if there is any therapeutic benifit. Due to the nature of the experiments, the use of drugs to minimize distress or pain would also affect inflammation and the immune response or add a confounding variable to the examination of therapeutic efficacy. Therefore, no such drugs can be employed in this protocol while still acheiving the research objectives. However, we have incorporated the following humane end points. Any animal that loses greater that 30% starting body weight or is moribund will be immediately euthanized. In addition, we have conducted preliminary experiments in cell culture in vitro to determine the potential signaling pathways involved and will only perform in vivo experiments to examine those

pathways. Finally, drug doses will be based upon previously published research to avoid toxicity.

c. Reduction in the number of animals Specify the methods used for reducing the number of animals that were considered in the design of the proposed experiments.

Yes Rational selection of group size (e.g., pilot studies to estimate variability, power analysis) **Yes**

Careful experimental design (e.g., appropriate choice of control groups)

Yessharing tissues with other investigators) Maximize use of animals (e.g., selecting the minimal number of animals per group required for statistical verification,

Yes Minimize the loss of animals (e.g., good post-operative care, avoidance of unintended breeding)

For any of the above items not checked, please provide a brief comment about why the option is not appropriate.

2. Using the specifics of your experimental plan, justify the number of animals requested for each pain category (B, C, D, E).

All animal models using intranasal infection such as influenza A virus or S. pneumoniae are inherently variable with standard deviations consistently around 30% of the mean. To arrive at the numbers of mice proposed here, we have taken into account past experience with intranasal infection models over the last 10 years as well as the number of animals typically used in the published literature. The effect size for the type of research designs employed in this protocol averages a Cohen's f = .40; therefore, using G*Power for the various designs proposed, with a Power = .80; Alpha = .05, a minimum of 6 animals is required per experiment. In the case of survival challenge experiments or experiments with multiple groups, more animals may be required for confidence in the interpretation of the results (e.g. n=10 per group for survival experiments). The justification for mice is as follows. Category D:

All mice in category D will be used to investigate the in vivo efficacy of treating mice with sodium pyruvate as an anti-inflammatory drug. Two groups will be examined during infection with influenza A virus: (1): a placebo group (daily subQ saline injection), (2): sodium pyruvate treatment group (daily subQ injection of 2mg/kg). This dose of sodium pyruvate has already been shown to be safe in mice in other disease models. We will need 66 WT mice per treatment or control group. However, the placebo group (66 mice) will be included in category E, the remaining
66 mice are included here in category D. The 66 mice in each treatment group will be used for examination of survival (3 independent replicates x 10
mice per replicate = 30 mice) and examination of immune responses on day 3 and day 7 after
infection (3 independent replicates x 6 mice per time point x 2 non-repeat measure time points = 36). It is possible that the subQ route of sodium pyruvate administration is not ideal for influenza A virus infection. Thus, we request an additional 66 mice to examine the treatment of mice with nebulized sodium pyruvate. Therefore, a total of 132 mice are classified as category D for the examination of the anti-inflammatory effects of sodium pyruvate. WT mice used in this portion of the proposed research will be bred in house.

Category E:

All mice in category E will be used to investigate the in vivo efficacy of treating mice with sodium pyruvate as an anti-inflammatory drug. As mentioned for category D, Two groups of mice will be examined during infection with influenza A virus: (1): a placebo group (daily subQ saline injection), (2): sodium pyruvate treatment group (daily subQ injection of 2mg/kg). The placebo control mice are included in category E, as they will recieve no interventional treatment. We will need 66 WT mice for the control group. The 66 mice in will be used for examination of survival (3 independent replicates x 10 mice per replicate = 30 mice) and examination of immune responses on day 3 and day 7 after infection (3 independent
replicates x 6 mice per time point x 2 non-repeat measure time points = 36). It is possible that the subQ route of sodium pyruvate administration is not ideal for influenza A virus infection. Thus, we request an additional 66 mice to examine the treatment of mice with nebulized sodium pyruvate. Therefore, a total of 132 mice are classified as category E for the examination of the anti-inflammatory effects of sodium pyruvate. WT mice used in this portion of the proposed research will be bred in house.

3. Estimate the following animal number totals required for this study during the three-year approval period.

Pain Category	Animals
B	0
C	0
D	132
E	132
	Total = 264

Justification for Category E:

The proposed research will examine the effects of sodium pyruvate on the immune response to influenza A virus infections. Due to the nature of the experiments, the use of drugs to minimize distress or pain would also affect inflammation and the immune response or add a confounding variable to the examination of therapeutic efficacy. Therefore, no such drugs can be employed in this protocol. We have incorporated the following humane end points. Any animal that loses greater that 30% starting body weight or is moribund will be immediately euthanized. In addition, we have conducted preliminary experiments in cell culture in vitro to determine the potential signaling pathways involved and will only perform in vivo experiments relative to those pathways. Finally, drug doses will be based upon previously published research to avoid toxicity.

4. **Transfer of Existing Animals:** Yes If Yes, Indicate the IACUC ID: 19.005

4.1 Alternatives to Proposed Procedures

1. Details about the search for alternatives

a. Names of searched databases and the date when the search was conducted:

No Agricola:
No AWIC:
No Biosis:
No Cab Abstracts:
No Crisis:
No Embase:
Yes Pub Med: 06/11/19 No Medline: No
NTIS:
No Psychlit:
No Sciscarch:
No Toxline:
No Pascal: No
Other:

If Yes, Explain:

b. Keywords used in the database searches:

Influenza and pyruvate: This search returned 28 results, and none have examined the affect of treating influenza A virus infection with pyruvate. one article (Respir Investig. 2016 Sep;54(5):312-8.) discusses that treatment of patients with drugs that enhance pyruvate metabolism (inhibitors of pyruvate dehydrogenase kinase 4) may have therapeutic benifit, but no one has examined the dirrect treatment with sodium pyruvate. c. Years Searched: 1900-2019

d. Resources used in addition to the computer database search:

Information Services and other Literature Sources:

No Animal Welfare Information Center
No Lab Animal Welfare Bibliography (NLM)
No Laboratory Animal Science Journal
No Alternatives to Laboratory Animals Journal (FRAME, U.K.)
No Quick Bibliography Series (AGRICOLA)
No Peer Review
If Yes, Explain:
No Other
If Yes, Explain:

Other Methods or Sources Used:
No Direct contact with investigators in field
If Yes, Explain:
No Consultation with expert in the area of investigation
If Yes, Explain:
No Other methods or sources
If Yes, Explain:

5. Details of Animal Use:

1. Goals and objectives of your research

Influenza A virus infection results in hundreds of thousands of deaths world wide every year. Although a vaccine exists, it is often poorly effective, and is too expensive for use in the developing world. We accidentally discovered that the addition of sodium pyruvate to the cell culture medium of cells infected with influenza A virus results in supression of the immune response, with lower levels of immune signaling molecules called cytokines. Subsequent research is needed to determine if injection or inhalation of sodium pyruvate may help to diminish the inflammation and symptoms associated with influenza A virus infection. We propose to examine the effects of sodium pyruvate treatment during influenza A virus infection in mice. If sodium pyruvate proves benificial, then it is a natural and inexpensive treatment for a disease that is a global problem.

2. If this application is a continuation of an ongoing project, state concisely how these goals differ from those in the original application and what was accomplished during the prior approval period. If this is a new project, please indicate so.

This is a new project.

3. Provide a concise overview of the experimental manipulations and treatments conducted on animals. This description should allow the IACUC reviewer to understand exactly what will be done to all animals from entry into the experiment to the endpoint of the study.

For the infection of mice with influenza A virus, 8-12 week old mice will be anesthetized by intraperitoneal injection of 80 mg/kg ketamine and 8 mg/kg xylazine. Following injection of mice with ketamine/xylazine, mice are allowed to become completely sedated for 5 minutes and sedation verified by pinching the paws. If no response is elicited, then mice are

inoculated intranasally with influenza A virus in 30µl phosphate buffered saline. Mice will be monitored daily. In allexperiments mice will recieve daily subQ injections of saline or 2mg/kg sodium pyruvate in saline. For survival experiments,

mice will be monitored for 14 days. Mice that lose >30% of their starting body weight or that are moribund will be euthanized immediately. Additional, mice will also be euthanized by CO2 asphyxiation on day 3 or day 7 after infection and blood collected by cardiac puncture. Lung, spleen and lymph nodes will also be collected for examination of pathology, cellular infiltration and immune cytokine responses and viral titers.

6. Animal Care

1. Animal ID Method:

No Ear Tag
No Ear Punch
No Microchip

No Not Applicable

NoNo TattooToe Clip

Yes Other
If Yes, Explain:

Marking the tail with a sharpie.

2. How will animals be monitored and maintained?

All animals will be housed in the Missouri State Iniversity Managed Vivarium and maintained under the standard operating proceedures establishedd for that facility and species. Facility conditions and monitoring typically includes:

Temperature ~72-75F

12h/12h light/dark cycle

Humidity between 30-70%
Air changes from 10-15 per hour

Food and water provided ad libitum

Cage changes once per week

Animals that are infected with influenza A virus will be monitored daily by the PI or student on the IACUC protocol. The PI or emergency contact will perform after hours, weekend and holiday monitoring as needed during infection studies.

If special monitoring has been arranged with DLAM facility supervisor, provide DLAM contact name:

3. Should ORC contact the PI or the emergency contact if animals are found dead? **Yes**

4. Indicate requests for special handling of sick and dead animals. **As mice used in these experiment will be infected with influenza A virus, they are expected**

to become sick and will be monitored at least daily for. No additional contact is necessary for infected sick animals. However, if infected animals are moribund, please contact me immediately and such animals will be euthanized.

5. Special Housing

Will any special housing or care be necessary? **Yes**
If Yes, describe and list any deviations from standard ORC husbandry procedures, Guide recommendations or special animal care needs.

All animals need to be housed in microisolator cages and all infected animals need to be housed under ABSL2 conditions.

6. Special Diets

Are special diets, additives to food and/or water, or antibiotics needed? **No**
If Yes, Describe and List Agents:

7. Describe endpoints (time points, tumor sizes etc.) and/or the maximum time length of study.

Mice used for infections will be infected at 8-12 weeks of age and the maximum duration of infection will be 14 days.
Some mice will be euthanized on day 3 or day 7 after infection with Influenza A virus to collect blood,

lung and lymphoid organs for examination of the immune system response and pathology. Mice to be used for survival experiments will be kept for a maximum of 14 days. All surviving mice will then be euthanized. During infections, mice that are moribund or that lose >30% of their starting body weight will be euthanized immediately.

8. Describe the criteria used to determine when an animal should be removed from the study prior its endpoint. During infections, I have occasionally (1/100 infected mice) observed ataxia, presumably due to encephalitis. Such mice will be immediately euthanized. Mice with severe infection will also present with hypothermia just prior to death. These mice will also be euthanized immediately.

9. Will animals be euthanized as part of the study? Yes If No, Describe the final disposition:

If Yes, Answer all of the following questions:

Euthanasia Methods

Yes CO_2-compressed carbon dioxide gas in cylinders and a physical method

No Barbiturate overdose

If Yes, Specify Dosage and Route:

No **Overdose of Gas Anesthetic**

If Yes, Specify Agent:

No **Anesthesia - followed by physical euthanasia**

If Yes, Specify Agent, Dosage, and Route:

If any of the above have been checked, indicate the physical methods that are used to ensure animals are dead:
Cervical Dislocation,Thoracotomy (Open the chest cavity using sharp scissors or scalpel),Collection of vital organs performed if tissues are needed for experimental purposes

No **Cervical Dislocation performed with no anesthesia**

If Yes, Justify:

No **Decapitation performed with no anesthesia**

If Yes, Justify:

No **Other Methods**

If Yes, Describe:

10. Would the PI be willing to make available extra animal tissues or organs to other PI's?
Yes

7. Anticipated Animal Pain & Distress

1. Are there any clinical, behavioral, or physiological manifestations expected to result from experimental manipulation?
Yes

If Yes, Answer all questions in this section.

a. Expected clinical and/or behavioral signs of pain and distress in animals:

Yes **Decreased weight**
Yes **Changes in food/water consumption**
Yes **Decreased ambulation**
Yes **Ruffled fur**
No **Skin abnormality**
No **Urinary problems**
Yes **Hunched posture**
No **Paw guarding**
No **Porphyrin Staining**
Yes **Lethargy**
Yes **Diarrhea**
No **Other**

If Yes, Explain:

b. Methods of dealing with the above complications:

No **Analgesics**
No **Anesthetics**

No Sedation or tranquilization
Yes No Increased beddingOther

If Yes, Explain:
Agents used in dealing with complications:

Animals experiencing unrelieved pain or distress prior to the endpoint, as defined by institutional policy, must be humanely
2. euthanized, unless an exception to policy is requested and approved. Is exception required?
Yes

If Yes, Answer all questions in this section.

a. Criteria for euthanasia that will be used in this exception:

Mice will be euthanized if they are moribund or lose >30% of their starting body weight. Additionally, just prior to mortality, mice become hypothermic. Any mice that do not meet the qualifications of being moribund or losing 30% of their weight will be euthanized if they are hypothermic. Hypothermic mice will be examined by handling (they feel cold). Finally, some mice (about 1/100) will develop ataxia as a result of the infection and ensuing encephalitis. These mice will also be euthanized immediately.

b. Scientific justification for not using an earlier endpoint:In our studies, we are examining the anti-inflammatory affects of sodium pyruvate. The use of
pain relieving or anti-inflammatory drugs, in addition to sodium pyruvate, would confound the interpretation of our results in these studies.

A weight loss of 30% or moribund behavior are commonly accepted endpoints in the field of influenza Avirus

infection, as mice that lose less than 30% of their starting weight routinely recover and

the assessment of mortality cannot be correlated. Although death as an endpoint is not used here, we do need a reliable correlate, for which the 30% weight loss cutoff has been shown to correlate well. In addition, mice may become moribund prior to death but before the 30% weight loss cutoff and will thus be euthanized.

8. Request for Exception to Regulation or Policy

8.1 Exception Information

1. Description of exception:
Due to the examination of the immune response and the testing of novel therapeutics during influenza A virus infection, it is not possible to treat animals with any additional anti-inflammatory or analgesic drugs other

thansodium pyruvate, to alleviate the pain or discomfort of the infections.

2. Rationale (provide scientific justification and/or justification based on animal welfare):
In the human population, influenza A virus infection results in increased morbidity and mortality
[1]. Although vaccines and antiviral drugs exist to treat or prevent influenza A virus
infections, these are still of limited efficacy due to the fact that the virus constantly mutates, and there is a disregulated immune response leading to immune cell and edema infiltration into the lung exacerbates pneumonia [2]. Multiple lines of evidence point to exacerbated inflammation as a key factor. Additionally, influenza A virus infection results in altered metabolism [3]. This requires more energy/ATP production. Based on its known functions, and our preliminary experiments in cell culture in vitro, sodium pyruvate may be able to increase ATP production from cells and decrease inflammatory cytokine production.

Because of the nature of these experiments, the study of the immune response and examination of therapeutic benefit of sodium pyruvate, we cannot administer any treatment of drugs that would alter or inhibit the immune response or
inflammation. All analgesic and anti-inflammatory drugs affect the immune response and cannot be used. As mortality is a potential outcome from influenza A virus infection in the human clinical setting, it is necessary to determine if the immune signaling pathways or treatments proposed in this study affect mortality and morbidity. We will use 30% weight loss or moribund responses as surrogates of mortality. Again, the inclusion of drugs to alleviate pain or discomfort would impair the interpretation of the proposed experiments.

References

1. **Matias, G., R. Taylor, F. Haguinet, C. Schuck-Paim, R. Lustig, and V. Shinde. 2014. Estimates of mortality attributable to influenza and RSV in the United States during 1997-2009 by influenza type or subtype, age, cause of death, and risk status.** *Influenza and Other Respiratory Viruses* **8: 507–515**
2. **Zheng, J., and S. Perlman. 2018. Immune responses in influenza A virusand human coronavirus infections: an ongoing battle between the virus and host.** *Current Opinion in Virology* **28: 43–52.**
3. Kido H, Indalao IL, Kim H, Kimoto T, Sakai S, Takahashi E. (2016) Energy metabolic disorder is a major risk factor in severe influenza virus infection: Proposals for new therapeutic options based on animal model experiments. Respir Investig. 2016 Sep;54(5):312-9.

3. Potential adverse effects/clinical signs resulting from exception:

Animals that are infected with influenza A virus will experience flu-like-symptoms, including impaired breathing, weight loss, ruffled fur, decreased movement, malaise,hunched posture and in some instances diarrhea and ataxia.

4. Specify which animals in the approved protocol will be affected:

All animals infected with influenza A virus will be included in this exception.

Exception Approval Status: Approved 6/29/2019

12. Items not covered in other parts of the application

none

Application Certification

I agree to the following statements. Signify your agreement by signing at the bottom.

- I certify that I am familiar with and assure compliance in this Project with the legal standards of animal care and use established under the Federal and State laws and the policies on animal welfare of the National Institutes of Health and the University of Cayuse.
- I assume responsibility for ensuring that all persons working with animals on this project are familiar with and are trained in relevant animal procedures and that they will comply with established laws and policies regarding animal care and use. Applications will not be approved for investigators that have taken the IACUC orientation but have not completed required Laboratory Animal Coordinator certification. Contact the IACUC office to arrange training.
- I will appoint a Laboratory Coordinator to manage all animal use in the lab. I will ensure that the Coordinator receives required training and certification. I will ensure that after being certified, the Coordinator or IACUC representative will train and certify all individuals working with animals in the lab.
- I certify that all individuals working with animals on this project will register with the University Employee Occupational Health Clinic (UEOHC) by completing and submitting the "Research Animal Handlers & Animal Caretakers" medical history questionnaire (each individual who works with animals must complete the questionnaire during online orientation - UEOHC will assess the PI a processing fee).
- I certify the following: the research proposed herein is not unnecessarily duplicative of previously reported research; appropriate non-animal alternatives for this research do not exist; no alternatives to the potentially painful and/or distressful procedures conducted in this project exist. I have indicated methods used to make these determinations in the appropriate section of this animal use
- application. I will secure IACUC approval before changing procedures or personnel associated with this study (including adding
- personnel) I assure that I and personnel under by direct supervision will use the animals acquired for the activity described herein solely for said purpose. I also certify that if live animals are shared with other PIs or are used in any procedure other than those described in this application, I will provide the details in the form of a written amendment to the original application prior to their use.
- I acknowledge that veterinary care will be administered to moribund animals or animals experiencing more than momentary or slight pain or distress. Division of Laboratory Animal Medicine (DLAM) veterinary staff will attempt to contact me regarding the care of treatment of a moribund animal, but will institute treatment or euthanasia, as needed, if PI cannot be reached.
- I assure the IACUC and the Missouri State University that the general procedures involving animals described in my grant application have been described in the animal use application and submitted to the IACUC for review.
- I assure that I have read "Notes on Euthanasia" of animals used in research and understand how it applies to animals in this animal use application.

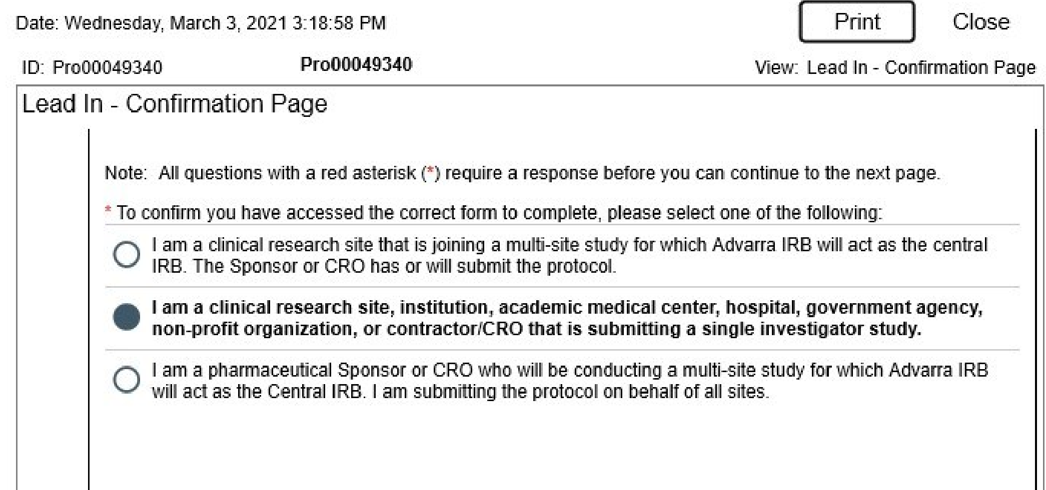
NOTE: Consultation of a DLAM veterinarian regarding space allocation is recommended prior to submission of application. IACUC approval of application does not assure DLAM space availability. Please contact DLAM for pre-study strategy meeting prior to ordering animals to disuss availability of housing.

NOTE: Cell lines that have been passaged in animals or maintained using animal serum may contain murine viruses that can alter the outcome of the study and may cause an outbreak of disease among other mice. ATTCC does not screen cell lines for murine pathogens. Cell lines that have been passaged in animals or grown in media containing rodent serum should be tested for murine pathogens prior to use in animals. Please contact DLAM for more information on testing of your cell lines.

PI Signature	Date
Co-PI Signature	Date

Approval Date: 6/28/2019

Appendix B


Date: Wednesday, March 3, 2021 3:18:58 PM

Print Close

ID: Pro00049340 **Pro00049340** View: Lead In - Confirmation Page

Lead In - Confirmation Page

Note: All questions with a red asterisk (*) require a response before you can continue to the next page.

* To confirm you have accessed the correct form to complete, please select one of the following:

- ○ I am a clinical research site that is joining a multi-site study for which Advarra IRB will act as the central IRB. The Sponsor or CRO has or will submit the protocol.
- ● **I am a clinical research site, institution, academic medical center, hospital, government agency, non-profit organization, or contractor/CRO that is submitting a single investigator study.**
- ○ I am a pharmaceutical Sponsor or CRO who will be conducting a multi-site study for which Advarra IRB will act as the Central IRB. I am submitting the protocol on behalf of all sites.

Protocol Information

1

* Full Protocol Title:

Two Week Sub-Chronic Double-Blinded, Placebo Controlled Trial Designed to Determine if Sodium Pyruvate Nasal Spray Will Reduce the Symptoms, Duration and Replication of COVID-19 and Influenza Infections.

2

Protocol Number: Pro00049340

* Do you have your own internal tracking number **(different than the protocol number above)** that you want to provide? ○ Yes ● **No**

3

* Enter the Sponsor of the study: _Other Organization

If 'Other Organization' - please enter the Sponsor's name: Cellular Sciences/Emphycorp

4

* Funding Source - select the appropriate funding source: Industry

If 'Other, please enter funding source:

Date Submitted:1/28/2021

Investigator and Administrative Personnel

1

* Please click **'Select'** to choose your Investigator:Christopher Lupfer

*Note: If you **do not** see the Investigator listed, then you will need to create an account/register the person. To create an account/register the Investigator, you will need to exit out of the application, logoff, and go to the CIRBI home page and click on the Sign Up link*

2

* Do you want to submit sub-investigator/co-investigator information for IRB review (note: this is **not** an IRB requirement) — ● **Yes** ○ No

* Please note that changes to sub-investigator information after IRB approval will result in additional fees upon submission. Do you still want to proceed with submission of this information? — ● **Yes** ○ No

* Please provide your sub-investigator/co-investigator information here:

	Sub-Investigator	CV of Sub-Investigator
View	John Abraham	CV 2020.doc(0.01)

3

To give staff members **access to this submission**, please click the Add button and complete the information in the pop-up form presented.

Name	Email	Role	Has Editing Privileges	PRO Notifications
Alain martin	dr.martin@erols.com	Study Coordinator	yes	PRO,MOD,PRE,CR

4

* Who is the primary point of contact (POC) for this research study? Christopher Lupfer

5

Provide the contact information of the Accounting/Accounts Payable Department/Project Coordinator who should receive invoices (***Please note:*** The invoice contact listed is the party responsible for issuing payment for IRB Services):

* Title: Dr.
* First Name: Alain
* Last Name: Martin

* Company Name: Emphycorp/Cellular Sciences, inc.
* Address 1: 84 Park Avenue
Address 2: Atrium, Suite E-102
* City: Flemington
* State: NJ
* Zip Code: 08822
* Country: United States

* Phone Number: (908) 237-1561
* Email Address: dr.martin@erols.com

If there is a **different** person to contact regarding invoice follow-up questions, please provide the person's

contact information below:

First Name:
Last Name:
Phone Number:
Email Address:

* Does the party responsible for paying invoices require a Purchase Order to be in place? Yes **No**

* Does the Purchase Order need to be in place **before** proceeding with IRB review? Yes **No**

Please provide the Purchase Order #:

Date Submitted:1/28/2021

IRB Review and Study Type

1

* Are you requesting a review to determine if the research is **exempt** from IRB oversight? ○ Yes ● **No**

* Are you requesting a review to determine if the research is **not** human subjects research (NHSR)? ○ Yes ● **No**

Note: these are **not common requests for drug, biologic, or device research*

2

* What type of research study are you submitting?

- [x] **Drug**
- [ ] Biologic
- [ ] Device
- [ ] Social Science/Behavioral
- [ ] Natural Health Product (NHP)/Dietary Supplement
- [ ] Planned Emergency Research
- [ ] Other

If Other, enter research study type:

3

* Has this research study been disapproved by or withdrawn from another IRB? ○ Yes ● **No**

4

* Are you requesting transfer of IRB oversight? ○ Yes ● **No**

5

* What is the approximate **end** date of the study? 4/30/2022

Date Submitted:1/28/2021

Drug/Biologic/Natural Health Product Research Studies

1

* What is the research study phase?Phase 2

2

* Will a placebo be used in this research study? ● **Yes** ○ No

If placebo controlled, please provide justification and any provisions to reduce risks to subjects who receive placebo:
Only young (<40 years old) healthy (no pre-existing health conditions that exacerbate COVID19 or influenza) patients with mild symptoms will be included in the study.

3

* Does this study involve the use of an In-Vitro Diagnostic Device (e.g., biomarker assay, chemistry tests, spirometry tests, etc.)? ○ Yes ● **No**

* Does the study involve a use of a mobile application/software/wearable device? ○ Yes ● **No**

4

* Are approved drugs/products being used in this study and being compared to the study drug/product under investigation (e.g. an Active Comparator)? ○ Yes ● **No**

5

* Does the investigational product contain genetically modified material (including genetically modified cells and gene therapy studies)? ○ Yes ● **No**

6

* Drug/Biologic/Natural Health Product Profile(s):

Drug Name
Sodium Pyruvate

Date Submitted:1/28/2021

Data Monitoring Plan

The research plan should make adequate provision for monitoring the data collected to ensure the safety of subjects.

1

* Does the protocol outline the plan for monitoring data to ensure subject safety (e.g. serious adverse event and unanticipated problem reporting, a description of how risks are minimized, plan to monitor progress and safety)? ● **Yes** ○ No

If no, provide a summary of the data monitoring plan:

2

* Is there a formal Data Monitoring Committee (DMC) for this research? ○ Yes ● **No**

If **no**, and this study involves intervention that places subjects at serious risk, compares blinded treatments over a long time period, or which may call for 'stopping rules' at certain endpoints, provide the rationale for not including a DMC:

Data will be monitored by the PI and the clinical coordinator, but there is not a formal committee. As only young otherwise healthy patients will be enrolled in the study, there is minimal risk. This is also a small trial with limited enrolment.

Date Submitted:1/28/2021

Informed Consent and Authorization

1

* Indicate the types of consent and/or authorization that will be used in this research study:

- [ ] No consent document required (i.e., requesting a waiver of consent)
- [x] **Written/signed consent by subject**
- [ ] Written/signed consent by a legally authorized representative (for an adult)
- [ ] Written permission for a minor by a parent or legal guardian
- [ ] Written/signed assent by minor
- [ ] Written/signed authorization agreement (HIPAA)
- [ ] Online/Website, Verbal consent or written information sheet (i.e., requesting a waiver of documentation of signed consent)
- [ ] Electronic Consent (eConsent) - Electronic systems and processes employing multiple electronic media to obtain informed consent)
- [ ] Requesting a Full or Partial HIPAA Waiver
- [ ] Exception from informed consent for planned emergency research

2

* Please select the following registry(s) that will list this protocol?

- [x] **clinicaltrials.gov**
- [ ] EU Clinical Trials Registry
- [ ] WHO or WHO Registry Network
- [ ] Not Applicable
- [ ] Other

If 'Other', please list registry here:

3

* Please select one of the following regarding HIPAA Authorization:

- (●) **Insert the Advarra IRB's HIPAA Authorization into the ICF**
- ( ) HIPAA Authorization has previously been negotiated with the IRB

- ◯ Site(s) will manage HIPAA Authorization through a separate document
- ◯ HIPAA Authorization is already in the Sponsor's ICF
- ◯ Not Applicable (e.g., not a covered entity, study is being done in Canada)

Note: If the ICF contains HIPAA Language, then the IRB will review that HIPAA Language to ensure all required elements are included.

4

* Do you have or have you applied for an NIH Certificate of Confidentiality (CoC) for this study? ◯ Yes ● **No**

5

* Will individuals (subjects) signing the Informed Consent Form document(s) have limited or no reading skills? ◯ Yes ● **No**

6

* Will participants have to pay to participate in the research, excluding co-pays relating to insurance coverage? ◯ Yes ● **No**

7

* Will the Sponsor/CRO authorize translations of the ICF & AA for this research study? ◯ Yes ● **No**

If yes, once your protocol is approved, submit a modification to request the translation of the ICF document(s).

8

* Are there any additional arrangements by the Sponsor or the Institution (if you are a site), besides what is in the Informed Consent Form, to provide medical care, respective payment for medical care, or to provide any compensation beyond the costs of medical care to any subject who has had a research related injury? ◯ Yes ● **No**

If 'yes', please explain:

Date Submitted:1/28/2021

Protocol Procedures

1 * Are there any protocol procedures in this research study which require storage of samples over an **extended** period of time? (e.g. for future research) ◯ Yes ● **No**

2 * In this research study, will there be any sub-studies? ◯ Yes ● **No**

Date Submitted:1/28/2021

Investigational/Research Location(s) and Subject Recruitment

1

* How many subjects will be enrolled at your site(s)? 60

2

* Select the investigational/research location(s) this study will use, or click 'Add' to enter data if it is not shown below. If you need to make an Update to a location, select the location first and you will then be able to do an Update:

Add

		Company Name	Address
☑	Update	Dynamic DNA Labs	2144 E Republic Rd B204, Springfield, MO, 65804, USA
☑	Update	Trinity Healthcare	2740 N Mayfair Ave, Springfield, MO, 65803, USA

3

* Which of the following subject populations may be enrolled in this study? (check all that apply):

- ☐ Hospitalized
- ☐ HIV Positive
- ☐ Decisionally Impaired
- ☐ Educationally Disadvantaged
- ☐ Pregnant Women, Human Fetuses, or Neonates
- ☑ **Military Personnel**
- ☑ **Economically Disadvantaged**
- ☐ Prisoners
- ☐ Terminally Ill
- ☑ **Adults**
- ☐ Minors (subjects under the age of majority)
- ☐ Individuals with Limited or No Reading Skills
- ☐ Non-English Speakers
- ☐ Blind or Visually Impaired

- [ ] Healthy Subjects
- [ ] Institutional/Nursing Home
- [ ] Other
- [ ] Males Only (No Females)
- [ ] Females Only (No Males)
- [ ] Students of Researcher
- [ ] Employees/Colleagues

If 'Other', please explain:

4

* This research study, by design, **excludes** the following ethnic groups (check all that apply)

- [x] **None**
- [ ] American Indian or Alaskan Native
- [ ] Asian
- [ ] Black, not of Hispanic Origin
- [ ] Hispanic or Latino
- [ ] White, not of Hispanic Origin
- [ ] Other

If 'Other' please explain:

5

Provide justification for any ethnic, language, age, or gender-based exclusion criteria (as applicable)
To limit potential risk, children (<18 years old) and adults over the age of 40 will be excluded due to either increased risk of severe COVID19 or influenza.

6

* Are you submitting this protocol from an institution (i.e., university hospital or medical school?) ● **Yes** ○ No

Date Submitted:1/28/2021

Regulatory Inspection Information

1

We have the following ***regulatory*** inspections on file for the Investigator and/or your investigational/research location(s):

Type	Date	Audit Finding	Address

We do not have any Audit information on file for either the listed PI or for any of the Research Locations indicated for this submission

2

Please enter any ***regulatory*** inspections not listed above that have occurred in the last 5 years by clicking '**Add**':

Type	Date	Audit Finding	Address

There are no items to display

Date Submitted:1/28/2021

Multiple Investigational/Research Locations Questions

Because you have indicated that subjects may be seen at more than one location, respond to the following questions:

1

* How often will the PI communicate with the research staff at each location?
Daily

If Other, specify:

2

* Choose all the methods that the PI and the research staff will use to communicate:

E-mail

Telephone

Regularly scheduled meetings

Other

If Other, specify:

Zoom

3

* Are any of your locations a nursing home/care facility, school, or facility where the subject may be a student or resident? ● **Yes** ○ No

If yes, has the facility documented in writing that they will allow this research study to be conducted there? ● **Yes** ○ No

Date Submitted:1/28/2021

Individual Conflict of Interest

The following questions apply to any investigator, including PI, sub-I, research staff, and any other person who is responsible for the design, conduct, or reporting of the research.

The questions also apply to the immediate families of investigators (meaning their spouses and any dependent children)

"Relevant company" refers to an entity that sponsors provide support for, or owns or produces the technology being investigated.

1

* Have any of the above individuals received compensation from a relevant company (e.g., in exchange for consulting, speaking, or serving on an advisory board) that when aggregated for the immediate family for the prior 12 months is $5,000 or greater? *(Please note that salary paid to an investigator or research staff is NOT considered a reportable payment, UNLESS that salary is contingent upon the result of this study.)* ○ Yes ● **No**

2

* Do any of the above individuals have an ownership interest (e.g., stock) in a **publicly-held** relevant company that when aggregated for the immediate family for the prior 12 months is $5,000 or greater? ○ Yes ● **No**

3

* Do any of the above individuals have any ownership interest (e.g., stock, stock options) in a relevant company that is **privately-held**? ○ Yes ● **No**

* Do any of the above individuals have a proprietary interest being investigated in the research study (e.g., patent or licensing agreement) ○ Yes ● **No**

* Do any of the above individuals have a financial agreement with any company in which they receive, or will receive, compensation that is linked to the outcome of the research study? ○ Yes ● **No**

* Do any of the above individuals serve as in an executive position or on the board of directors for a relevant company? ○ Yes ● **No**

* Do any of the above individuals have any other financial or non-financial interests not listed above that could appear to potentially influence the conduct or outcome of this research study at the investigational/research location(s) or interfere with the ability to adequately protect research subjects? ○ Yes ● **No**

4

* Has an in-house Institutional Conflict of Interest Committee made any determinations and/or required any specific management plans related to this research for any of the above individuals? ○ Yes ● **No**

Institutional Conflict of Interest

5

* Are you reporting an Institutional Conflict of Interest (where the financial interest of an institution and an external entity may affect or appear to affect research conducted at the institution)? ◯ Yes ◯ No

Date Submitted:1/28/2021

Informed Consent Document

The IRB will provide an Informed Consent Form (ICF) document(s) formatted with your information. Indicate below the information that you want included:

1

* Place a checkmark next to each address you want listed on the ICF document(s):

	Address
☑	**2144 E Republic Rd B204, Springfield, MO, 65804, USA**
☑	**2740 N Mayfair Ave, Springfield, MO, 65803, USA**

2

* Primary phone number to be listed on the ICF document(s): (417) 521-3925

* 24-Hour phone number to be listed on the ICF document(s): (908) 399- 3426

3

* The following are questions related to monetary and non-monetary compensation, to include payment for participation and re-imbursement for expenses (travel, parking, etc.). Upon receipt of your application, **the IRB assumes that the amounts have been finalized and will proceed with review** unless further notification is received.

Provide the breakdown of compensation **or** reimbursement to subjects, including any gift cards, toys, or movie tickets. If you are **not** compensating and/or reimbursing subjects, then you can just indicate N/A:
Visit 1 (Study enrollment visit) $50
Final Visit (Day 14 visit or final swab visit after testing negative by PCR) $50
The following are questions related to monetary and non-monetary compensation, to include payment for participation and re-imbursement for expenses (travel, parking, etc.). Upon receipt of your application, **the IRB assumes that the amounts have been finalized and will proceed with review** unless further notification is received.

Provide the breakdown of compensation **or** reimbursement to subjects, including any gift cards, toys, or movie tickets. If you are **not** compensating and/or reimbursing subjects, then you can just indicate N/A:" _ald_errorLayout="EntityView:/ViewPages/MissingAttributeError" _ald_bindOverrideHook="" _ald_layoutOrder="3">

4

Timing of Compensation **and/or** Reimbursements to Subjects:

- ○ Subjects will be paid following each completed visit
- ○ Subjects will be paid monthly
- ○ Subjects will be paid quarterly
- ● **Subjects will be paid at the end of their participation in the research study**
- ○ Subjects will be paid following each completed visit or at the end of their participation in the research study, whichever they prefer
- ○ There will be no payment/reimbursement to subjects
- ○ Other

If 'Other', then please provide an explanation of the timing below:

5

List any visits for which subjects will **not** be paid:
After the initial visit to enroll patients, they will not be compensated until the final visit when the study finishes (day 14 or the patient tests negative for the virus).

6

Will you need the Informed Consent form translated into another language? ◯ Yes ● **No**

If yes, what language(s)?

Please note: The sponsor will need to approve the translation request before being released to your site.

Date Submitted:1/28/2021

ID: Pro00049340 **Pro00049340** View: Message to End User

Message to User

Changes Incorporated as of October 30, 2015:

To facilitate your application process, the next pages already display the current information we have on file for the investigator if you have submitted since October 30, 2015.

Update or edit as necessary. Any changes you make will be saved and available for future submissions.

Changes Incorporated as of December 9, 2016:

**New questions were added to question #3 on the next page on December 9, 2016. These will have to be completed
and saved on file even if you have submitted this since October 30, 2015.**

Investigator Experience and Qualifications

1. * How many years has the investigator been involved in the conduct of research? None (New to research)

2. What is the investigator's National Provider Identifier (NPI) Number (if applicable):

3. * What additional training, certifications, and/or degrees in the field of human research protections have been completed by the Investigator

- [ ] OHRP Human Subject Assurance Training
- [ ] NIH Online Course: Human Participant Protections Education for Research Teams (training must have occurred prior to September 27, 2018)
- [ ] Investigator Meeting(s)
- [x] **Collaborative Institutional Training Initiative (CITI) Program**
- [ ] APPI [Certified Physician Investigator (CPI™)]
- [ ] ACRP [CTI, CCRC, CCRA]
- [ ] SOCRA [CCRP]
- [ ] Graduate/Undergraduate researcher studies/degree(s)
- [ ] DIA [CCI]
- [ ] Tri Council Policy Statement Course on Research Ethics (CORE)
- [ ] Clinical Research Association of Canada (CRAC)
- [ ] Academy of Physicians in Clinical Research (APCR)
- [ ] Other

4.

* What is the current number of research studies supervised by the Investigator?	0
* What is the approximate number of active research subjects currently supervised by the Investigator?	0
* How many Sub-Investigators with clinical trials experience are assisting the Investigator?	1
* How many research staff members with clinical trials experience are assisting the Investigator?	0

If there are any other resources available at your site to support the administration of any active clinical trials, please provide them here:

Questions 4-9 ask about the investigator's specialties and research experience. The IRB may share this information with Sponsors or organizations acting on their behalf to identify investigator candidates for future research studies. You may opt out of those disclosures by checking the box here. ☐

5

* Specialty of the investigator (if applicable):None

6

* Sub-specialty(s) - if any

Sub-Specialty

None

7

* What phases of research has the investigator conducted (if applicable)?

- ☐ Phase 0
- ☐ Phase 1
- ☐ Phase 2
- ☐ Phase 3
- ☐ Phase 4
- ☑ **N/A**

8

* In which therapeutic areas does the investigator have experience?

Therapeutic Area

Immunology

Infectious Disease

9

* In which following disease/general areas does the investigator have research experience

Diseases/General Areas

- ☐ _None
- ☐ Blood, Blood-forming Organs Diseases
- ☐ Circulatory System Diseases
- ☐ Dental and Oral Health
- ☐ Digestive System Diseases
- ☐ Ear/Mastoid Process Diseases

Diseases/General Areas

- [ ] Endocrine Diseases
- [ ] Endocrine, Nutritional, and Metabolic Diseases
- [ ] Eye/Ocular Adnexa Diseases
- [ ] Genitourinary System Diseases
- [x] **Infectious and Parasitic Diseases**
- [ ] Mental/Behavioral Disorders
- [ ] Metabolic Diseases
- [ ] Musculoskeletal/Connective Tissue Diseases
- [ ] Neoplasms
- [ ] Nervous System Diseases
- [ ] Nutritional Diseases
- [ ] Pain Management
- [ ] Pelvis, Genital, and Breast Diseases
- [ ] Perinatal Diseases/Conditions
- [ ] Pregnancy-Related Diseases
- [x] **Respiratory System Diseases**
- [ ] Skin/Subcutaneous Tissue Diseases
- [ ] Social and Behavioral Research
- [ ] Urinary System Diseases

10

* What age groups does the investigator have research experience (if applicable)

Name

- [ ] Adolescents
- [ ] Adults
- [ ] Adults-Older

	Name
☐	Children
☐	Infants
☐	Neonates
☑	**None**

Date Submitted:1/28/2021

Site and Local Context Information

1

* Indicate any state or local laws having an impact on research at your investigational/research location(s) by checking all that apply:

- [x] **None**
- [ ] Mandatory IRB Site Visits
- [ ] Age of Majority is 19 years (US states of AL, NE & Canadian provinces of BC, NB, NL, NS) or 21 years for Puerto Rico
- [ ] California Experimental Subject's Bill of Rights
- [ ] State Privacy laws related to the use of Protected Health Information (PHI)
- [ ] Other

If 'Other', please explain:

2

* Which, if any, of the following pending or on-going actions or restrictions related to the practice of medicine or research apply at your location(s) [including the PI and the research staff]

- ○ Legal
- ○ Regulatory
- ○ Professional
- ○ Other
- ● **None of the above**

If any, please explain:

3

* What recruitment methods will be used in this research study?

- [x] **In conversation during routine office visits**
- [ ] Rollover or extension or participation from another research study
- [x] **Mass distributed print publication (ex: newspaper, magazine, newsletter)**
- [x] **Flyer, poster or bulletin board**
- [ ] Radio
- [ ] Television

- [ ] Direct Mailing
- [ ] Internet
- [ ] Database/Chart Review
- [ ] Telephone Screening Script
- [ ] Other

If 'Other', please explain

4

* Will you be paying any professionals for their assistance in the recruitment of potential subjects (for example: finder's fees, referral fees, etc.) ○ Yes ● **No**

If 'yes', please explain

5

* Do any of your investigational/research locations have a local IRB? ● **Yes** ○ No

* You indicated that there is a local IRB. Please select one of the following:
An oversight waiver will be attached on the Document Upload Page at the end of this submission form

6

* Does your site have an FWA? ● **Yes** ○ No

If you have an FWA, then please provide the FWA- 00004733

7

* How would you describe the attitudes about research held by potential research subjects in your community?

○ Positive

● **Neutral**

○ Negative

If 'Negative', please explain

8

* Has there been any recent media focus on research in your community? ● **Yes** ○ No

If 'Yes', give a brief explanation:
News station coverage of COVID19 vaccine trials.

Date Submitted:1/28/2021

Informed Consent Process, Data Privacy and Confidentiality

1

* The informed consent process is an ongoing, continuous process. It is the IRB's expectation that ongoing consent of the subject is ensured by the Investigator during the course of the research study.

To comply with the conditions of IRB Approval, the following procedures must be followed during the informed consent process at your location(s):

a. The Investigator will not involve any individual in the research study unless the Investigator has obtained the legally effective informed consent of the potential research subject (or legally authorized representative [LAR]).

b. The potential research subject (or LAR) is provided sufficient opportunity to consider whether to participate in the research study.

c. The consent process minimizes the possibility of coercion or undue influence.

d. The consent discussion is in a language understandable to the potential research subject (or LAR).

e. The consent discussion is free from the use of any exculpatory language.

f. Procedures required only for the research study will not be performed prior to obtaining consent

g. The most recent IRB Approved version of the ICF is used for enrollment.

h. The potential research subject (or LAR) is given adequate time and a place to read and review the ICF.

i. The potential research subject (or LAR) is given the opportunity to take the ICF home for review prior to signing the document, as appropriate.

j. The consent discussion provides ample opportunity for the Investigator (or sub-investigator with equivalent qualifications to serve as Investigator) to be available to answer questions the potential research subject (or LAR) may have.

k. Each person on the IRB Approved ICF signs and dates the form on the same visit, as appropriate. The potential research subject (or LAR) receives a signed and dated copy of the ICF

l. The consent discussion includes an assessment of the subject's understanding of the study following the consent process and before being enrolled in the study

The informed consent process is an ongoing, continuous process. It is the IRB's expectation that ongoing consent of the subject is ensured by the Investigator during the course of the research study.

To comply with the conditions of IRB Approval, the following procedures must be followed during the informed consent process at your location(s):

a. The Investigator will not involve any individual in the research study unless the Investigator has obtained the legally effective informed consent of the potential research subject (or legally authorized representative [LAR]).

b. The potential research subject (or LAR) is provided sufficient opportunity to consider whether to participate in the research study.

c. The consent process minimizes the possibility of coercion or undue influence.

d. The consent discussion is in a language understandable to the potential research subject (or LAR).

e. The consent discussion is free from the use of any exculpatory language.

f. Procedures required only for the research study will not be performed prior to obtaining consent

g. The most recent IRB Approved version of the ICF is used for enrollment.

h. The potential research subject (or LAR) is given adequate time and a place to read and review the ICF.

i. The potential research subject (or LAR) is given the opportunity to take the ICF home for review prior to signing the document, as appropriate.

j. The consent discussion provides ample opportunity for the Investigator (or sub-investigator with equivalent qualifications to serve as Investigator) to be available to answer questions the potential research subject (or LAR) may have.

k. Each person on the IRB Approved ICF signs and dates the form on the same visit, as appropriate. The potential research subject (or LAR) receives a signed and dated copy of the ICF

l. The consent discussion includes an assessment of the subject's understanding of the study following the consent process and before being enrolled in the study

- (●) **I agree with the process**
- ( ) I disagree.**

**If you do not agree, provide an explanation:

2

* Do you conduct competing research studies? *(This does not include research with healthy subjects)* ( ) Yes (●) **No**

3

* Please specify the location at your site where the informed consent process will be conducted with a potential subject (or their LAR) [check all that apply]:

- [x] **In a private room/area**
- [ ] In a group setting
- [ ] Other

If 'Other', please explain

4

* Please specify the steps taken by the Investigator and authorized research staff to minimize the possibility of coercion or undue influence during the informed consent process (check all that apply):

- [x] **The informed consent discussion is presented to the subject (or their LAR) by someone who is sufficiently knowledgeable about the research to properly interpret and correctly answer questions.**
- [x] **The subject (or their LAR) is not pressured to participate in the research and is not penalized or excessively questioned for deciding not to participate in the research.**
- [x] **The consent presentation is discussed in non-technical language understandable to the subject (or their LAR) and the subject's (or LAR's) understanding is confirmed through an unrushed two-way conversation.**
- [ ] Other

If 'Other', please explain

5

* Please specify the steps taken by the Investigator and authorized research staff to ensure that the subject (or their LAR) is provided sufficient opportunity to consider participation in the research (check all the apply):

- [x] **The subject (or their LAR) is given adequate time and place to read and review the Informed Consent Form and ask questions.**
- [x] **The subject (or their LAR) is given the opportunity to take the Informed Consent Form home for review prior to signing the document.**
- [x] **The subject (or their LAR) is provided a sufficient waiting period between being informed of the research and signing the consent form.**
- [ ] Other

If 'Other', please explain

6

* How will the subject's data identifiers be recorded?

- (●) **Identifiers will be anonymized, coded, or de-identified as outlined in the protocol or our standard operating procedures/policies**
- ( ) Other

If 'Other', please explain

7

* Choose all the mechanisms in place to ensure that the research records/data will be kept to protect the privacy and confidentiality of subject information

- [x] **Paper-based records will be kept in a secure location only accessible to authorized staff**
- [x] **Computer-based files will be available only to authorized staff using access privileges and passwords**
- [ ] Other

If 'Other', please explain

Date Submitted:1/28/2021

Document Upload Page

Please attach all documentation necessary for IRB review in the **correct areas as outlined below**:

1 Protocol Document:

Name	Created Date
COVID-19 Protocol_1-27-2021 CL.rtf(0.01)	1/28/2021 12:47 PM

2 Recruitment Materials and Subject Facing Materials:

	Type of Material	Name	Document Type	Submission Type	Document
View	Recruitment Material	Flyer for clinical2	Flyer, poster, or bulletin board	New Material	Flyer for clinical2.docx(0.01)
View	Subject Facing Materials	Patient log	Diary	New Material	Patient log.docx(0.01)

3 Informed Consent Form(s)

Name	Created Date
N115_consentform 1-28-2021.doc(0.01)	1/28/2021 12:47 PM

4 Translated Material(s):
There are no items to display

5 Drug/Biologic Profile(s):

Drug Name
Sodium Pyruvate

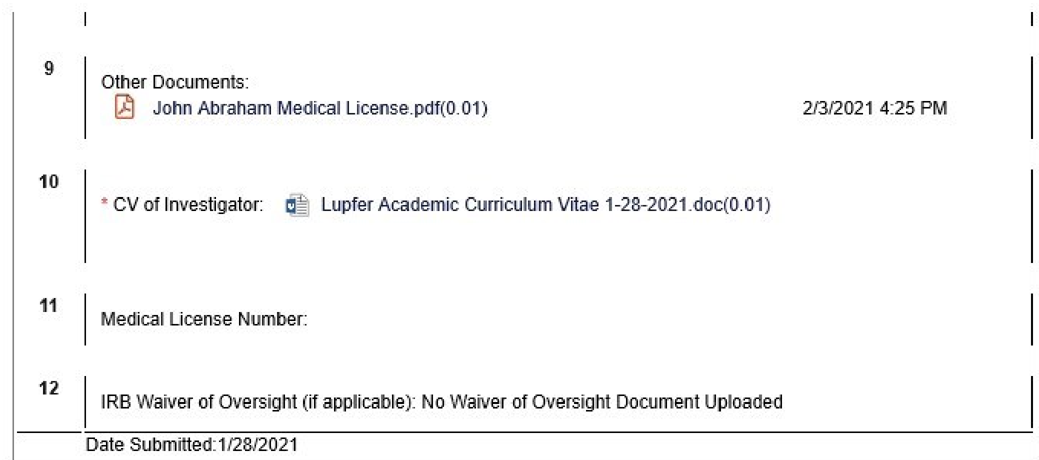
6 Device Profile(s):

Device Name	Manufacturer
No Device Profiles Selected	

7 In-Vitro Diagnostic Device Profile(s):
There are no items to display

8 Federal Grant Document(s):

Name	Created Date
No Federal Funding Grant Document Loaded	



9	Other Documents: John Abraham Medical License.pdf(0.01) 2/3/2021 4:25 PM
10	* CV of Investigator: Lupfer Academic Curriculum Vitae 1-28-2021.doc(0.01)
11	Medical License Number:
12	IRB Waiver of Oversight (if applicable): No Waiver of Oversight Document Uploaded
	Date Submitted:1/28/2021

End of Application

* Please select one of the options below and click **'Continue'.** If you select **'Submit Application',** the IRB review process will begin.

- (selected) **Submit Application**
- ( ) Save Application, but DO NOT submit

****Note if you select "Submit Application", then you are attesting to the following:**

For a Single-Site Protocol or Investigator Application, the Principal Investigator (PI) is responsible for and attests to the following:
a) Not starting the research study prior to receiving IRB Approval
b) Personally conducting or supervising the described investigation(s)
c) Ensuring that all associates, colleagues, and employees assisting in the conduct of the research study are informed about their obligations in the conduct of the research
d) Utilizing only the IRB Approved Informed Consent Form/eConsent to enroll subjects
e) Obtaining appropriate informed consent, as required by the IRB, from potential research subjects prior to performing any research procedures ("If changes are made due to immediate danger to a subject, immediately report these change to the IRB")
f) Making no changes in the research without IRB approval, except where necessary to eliminate apparent immediate hazards to human subjects
g) Complying with all federal, state, provincial, and local regulations regarding the conduct of research
h) Ensuring your investigative/research location(s) are conducting this research in compliance with the policies and procedures outlined in the IRB Handbook located in the Reference Materials Section of CIRBI.
i) Including in the contract (or other agreement) with the Sponsor that results/new findings from a research study directly affecting subject safety or their medical care will be communicated to subjects and how that communication will occur.
j) Including in the contract (or other agreement) with the Sponsor that any findings from a research study discovered by the Sponsor that could affect the safety of participants, affect the willingness of participants to continue participation, influence the conduct of the study, or alter the IRB's approval to continue the study will be communicated and subsequently reported to the IRB by the Investigator.
k) Including in the contract (or other agreement) with the Sponsor that routine and urgent data and safety monitoring reports will be communicated and subsequently reported to the IRB by the Investigator.

For Multi-Site studies, the Sponsor is responsible for and attests to the following:
1) Reporting to the IRB, during the study and/or after the study is complete, any findings that could:
  a) Affect the safety of participants.
  b) Affect the willingness of subjects to continue participation.
  c) Influence the conduct of the study.
  d) Alter the IRB's approval to continue the study.
2) The sponsor provides assurances that the manufacture and formulation of investigational or unlicensed health products (drugs, biologics, medical devices or natural health products) conform to federal regulations.

Date Submitted:1/28/2021

ID: Pro00049340 **Pro00049340** View: Create View for Protocol Submission

Sub-Investigator/Co-Investigator Information

1

* Type in the person you are adding as a sub-investigator/co-investigator for this study?John Abraham

2

Please select what training(s) this person has had:

- [ ] Reviewed FDA Information Sheets, TCPS Tutorial (CAN), GCP Guidelines and the Belmont Report
- [ ] Attended educational seminar(s) related to human subject protection provided by the sponsor/CRO/research site or other entity
- [x] **Completed formal education/training in human subject protection via web-based or published modules (e.g. NIH, ORHP video training series, or CITI)**
- [ ] Human subject protection training has not yet been completed, but is scheduled to be completed prior to study initiation at the site
- [ ] Other

3

* Please upload a copy of the Sub-Investigator's/Co-Investigator's CV here: CV 2020.doc(0.01)

Appendix C

ANIMAL CARE & USE APPLICATION

INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

v. July 2019

All Animal Care & Use Applications should be submitted electronically to IACUC@missouristate.edu.

A. Investigator Information		
Principal Investigator: Christopher Lupfer	Department: Biology	Office Address: Temple Hall 254
Office Phone: 6-6887	Emergency Phone: 901461-9215	Email: christopherlupfer@missouristate.edu

B. Project Information		
Project Title: Lupfer Breeding Colony		
Protocol Action: ☐ New Proposal ☐ Pilot Study ☒ Renewal (due to protocol expiration) ☐ Review for Exemption	**Protocol Type:** ☒ Research ☐ Teaching	**Protocol Class:** ☐ Agricultural ☐ Behavioral ☒ Biomedical ☐ Wildlife/Conservation
Is this project externally funded and/or do you anticipate future funding? ☒ **Yes** ☐ **No** **If Yes, what is the name of the Funding Agency and grant number/title? Emphycorp, NIH**		

C. Previous Approved Protocol	
For work that is similar to a previously approved protocol, provide the original protocol number and approval date. On the remainder of the forms, indicate changes to the originally approved protocol in bold font.	
Original Protocol Number: 19.005	Approval Date: 2/26/2019

D. Investigator Assurances

1. The information provided herein is accurate and, to the best of my knowledge, conforms to all applicable University, PHS, and USDA policies on the use of animals in research and teaching.
2. I affirm that all procedures involving vertebrate animals will be performed only by personnel trained in the humane care, handling, and use of animals and that all personnel will abide by the recommendations of the University's Occupational Health and Safety Program.
3. I agree not to proceed with any portion of this project until I receive written approval from the Missouri State University Institutional Animal Care and Use Committee.
4. I agree any changes in the procedures contained in this protocol will be promptly forwarded to the IACUC for review and approval prior to performing any revised procedures.
5. I agree to provide proper, current documentation (e.g., licenses, permits and additional approval forms), when applicable, to the Office of Research Compliance throughout the course of this project.
6. I agree to allow inspection of my research facilities by members of the IACUC, including the veterinarian, and to comply promptly if informed of any violations of the Missouri State University's Animal Care and Use Policy.
7. I have taken into consideration the three "Rs" (replacement, reduction, and refinement) for my study and provided adequate justification for the animal model chosen, animal numbers requested, and procedures to reduce pain and distress.
8. I have conducted a literature search to ensure that I am not unnecessarily duplicating previous experiments.

Christopher Lupfer	3-10-22
Signature of Principal Investigator	Date

ANIMAL CARE & USE PROTOCOL

Read all sections for instructions. Answer all questions or answer N/A if the question does not apply. Complete electronically, handwritten versions will not be accepted. Submit electronically to **IACUC@missouristate.edu** .

Section 1. Personnel Information

List all individuals, including the PI, performing manipulations or working with animals. Indicate each individual's role (PI, graduate assistant, undergraduate student, etc.) in the position column. Training should indicate both online training modules and lab-specific procedures. Experience should indicate length of involvement (months, years, etc.) in the relevant area of research.

Name	Title/Position	Degrees	*Training/Experience
Christopher Lupfer	PIr	Ph.D.	17 years experience in biomedical animal research including mice, rabbits and chickens. CITI training
Nayeon Son	GA	BS	CITI, 1.5 years in intranasal infections
Devyn Worthley	GA	BS	CITI, 1.5 years in intranasal infections

* All personnel must take the Online Animal Care & Use Training as well as enroll in the Occupation Health and Safety Program prior to animal related activities.

Section 2. Project Description

2.1 Nontechnical Summary

Provide in terms comprehensible to a nonscientist (abstracts or methods section of grant proposals are not acceptable):

A. The project's goals & objectives:

The primary goal is to produce mice to support other IACUC approved research protocols. Therefore, the mice on this protocol will be used strictly for breeding and no direct experimentation is planned. Mice to be used for experiments will be transferred accordingly.

For breeding, homozygous male mice will be mated with homozygous female mice for a particular genetic background. Mice will be bred starting at 8 weeks of age. Females will be rebred 2-6 weeks after weaning of the previous litter. To provide sufficient mice for our studies, we will need up to 12 females and 6 males for WT (C57BL/6J) mice. 2 females will be caged together in a harem and one male will be used to impregnate a harem of females. The males and harem females will need to be replaced every 6-9 months during the 3 years and this will be done by using non-sibling weanlings from previous litters. Mice will be used for breeding and pups will be used to support other projects. For weaning, pups will be weaned at 21 days of age. At weaning, pups will be sexed and will be transferred to other protocols. If mice will not be transferred to other protocols or used as future breeders, then they will be euthanized prior to 21 days of age. In some instances, mice will be transferred to other PI's approved IACUC protocols.

B. The project's benefit to society, education, or animals:

Mice on this protocol will not be used for research, only breading.

C. A summary of the experimental design/teaching plan:

Mice on this protocol will not be used for research, only breading.

D. What is the project duration dates (start and end) and the disposition of the animals at the end of the project?

3-11-22 to 3-10-25

2.2 Justification

This section should indicate consideration of the "three Rs."

- Replacement - replacing the use of animals with non-animal techniques (i.e. computer models , in vitro assays, or cell culture)
- Reduction - reducing the number of animals used (i.e. limiting group sizes, sharing tissues, or performing experiments simultaneously)
- Refinement - changing experiments or procedures to reduce pain and distress in animals (new anesthetics/analgesics or surgical procedures)

Briefly describe the following.

A. Why each species was chosen.

Most mice that will be bred on this protocol will be used on other IACUC approved protocols to examine the immune response to infectious pathogens. In immunology, the mouse is the preferred species as there is a wealth of knowledge regarding the mouse immune system, there are established molecular and
diagnostic tools such as antibodies for detecting mouse cytokines, and genetic manipulation of mice for the generation of knockout mice is more established than in any other species.

B. Why the number of animals requested is warranted. (Why the proposed number of animals is sufficient, but not excessive for achieving valid results)

In the past 6 years, 2 previous breeding protocols, 500 mice per year was sufficient to provide the mice necessary for transfer to research protocols. We currently have one active research protocol and one ready to submit. Therefore, we anticipate a continued need of about 500 mice per year ore 1500 mice over the 3 years of the breeding protocol.

2.3 Literature Search

Searches should provide justification of the three "Rs" and be conducted within 2 months of protocol submission.

Date of Search	Keywords	Resources Used	Years Covered
3-1-22	Influenza, Coinfection, Animals, inflammasome	Pubmed	all
3-1-22	Influenza, coinfection, animals, IL-1	Pubmed	all
3-1-22	Influenza, coinfection, animals	Pubmed	all
3-1-22	Influenza, aspergillus, animals	Pubmed	all

Results: Summarize how the searches indicate the necessity of an animal model, lack of duplication, the need to repeat previous studies.

Keywords:
Influenza, Coinfection, Animals, inflammasome (Pubmed) 1 publication found
Influenza, Coinfection, Animals, IL-1 (Pubmed) 3 publications found
Influenza, Coinfection, Animals (Pubmed) 216 publications found
Influenza, Aspergillus, Animals (Pubmed) 12 publications found

Summary of literature searched:
The animals in this protocol are for breeding of more animals to be used in support of my research on viral and fungal coinfections, pyruvate treatment or HOCL treatment. The breeding techniques are up-to-date. Although no experiments will be performed on the breeders, the following information supports the use of live animals and thus the need for breeding animals for my research.
Many deaths attributed to influenza A virus (IAV) infection are the result of secondary bacterial or fungal infections termed coinfections. Although some research has been performed with viral-bacterial infections, there is currently only 1 publication on viral-fungal coinfection [1] and no publications specifically looking at influenza and Aspergillus fumigatus coinfection.
Thus, the mice that will be breed on this protocol will support research that has no overlap with previous studies and the findings have direct clinical application as well as adding to our fundamental understanding of the pathogenesis of coinfections.
In examining alternatives to animals, my database search provided 1 example of a lung tissue explant model for the study of coinfections. However, this model was limited to examination of the physiology of the coinfection, namely tissue damage, and pathogen replication. It was not able to recapitulate the immune response to coinfection as a live animal model would. In our preliminary research, we have also generated a novel cell culture model to study the immune signaling pathways that are involved during coinfection. However, our in vitro model uses a single immune cell (Macrophage) in isolation and once again, this does not recapitulate the complexity of the entire immune system or the physiology of pneumonia. Based on our database search, we conclude that there are no alternatives to the use of animals for studying the immune response to coinfection. Furthermore, we have chosen the mouse as a model because of the availability of reagents for studying the immune response in mice and the availability of genetic knockout mice. Mice are also the most established animal model used for IAV coinfection studies with 166 of the 274 publications using mice.

References

1. Oliveira LVN, et al. (2017) Influenza A Virus as a Predisposing Factor for Cryptococcosis. Front Cell Infect Microbiol. 2017 Sep 26;7:419. doi: 10.3389/fcimb.2017.00419. eCollection 2017.
2. Seldeslachts L, Vanderbeke L, Fremau A, Reséndiz-Sharpe A, Jacobs C, Laeveren B, Ostyn T, Naesens L, Brock M, Van De Veerdonk FL, Humblet-Baron S, Verbeken E, Lagrou K, Wauters J, Vande Velde G. Early oseltamivir reduces risk for influenza-associated aspergillosis in a double-hit murine model. Virulence. 2021 Dec;12(1):2493-2508. doi: 10.1080/21505594.2021.1974327. PMID: 34546839.

3.Tobin JM, Nickolich KL, Ramanan K, Pilewski MJ, Lamens KD, Alcorn JF, Robinson KM. Influenza Suppresses Neutrophil Recruitment to the Lung and Exacerbates Secondary Invasive Pulmonary Aspergillosis. J Immunol. 2020 Jul 15;205(2):480-488. doi: 10.4049/jimmunol.2000067. Epub 2020 Jun 10. PMID: 32522833; PMCID: PMC7416629.

Section 3. Animal Use

3.1 Animal Sources

Provide number and source for each species used.

Species	Common Name	Approximate Number	Source
Mus musculus (C57BL/6J)	mouse	1500	Temple vivarium

3.2 Animal Facilities

Identify buildings and room numbers where species will be housed and procedures performed.

Species	Housing	Procedure Area
Mus musculus (C57BL/6J)	Temple vivarium	Temple vivarium

3.3 Animal Husbandry

Describe how animals will be maintained including feeding, cage or housing conditions, and laboratory environment.

All animals will be housed in the Missouri State University managed Vivarium and maintained under the standard operating procedures established for that facility and species. Facility conditions and monitoring typically includes:
Temperature ~72-75F
12h/12h light/dark cycle
Humidity between 30-70%
Air changes from 10-15 per hour
Food and water provided ad libitum, breeding diet for breeders, standard chow for weaned pups
Cage changes at least once per week
Daily monitoring by Vivarium staff and weekly by the Attending Veterinarian

3.4 Animal Procedures

Check all that apply. Please fill out the appropriate addendum if one is indicated.

☐ Behavioral Studies	☒ Marking, Microchip, Tattoo
☐ Blood Sampling/Tissue Collection	☐ Non-Standard Housing
☐ Capture of Wild Animals (Addendum 5)	☐ Non-Standard Husbandry
☐ Death as an Endpoint	☐ Physiological Studies
☐ Field Observation Only	☐ Sleep Deprivation
☐ Food Restriction/Special Diet	☐ Student Project Involving Animals (Addendum 4)
☐ Injections	☐ Surgery* (Addendum 3)
☐ Long Term Restraint	☐ Use of Hazardous Material(s) (**Fill out 3.4 A**)

A. List hazardous materials to be used on this project. Materials can be harmful to humans, animals, or both. It is the PI's responsibility to have, up-to-date Material Safety Data Sheets (MSDS) for materials included on this form. Some materials (e.g., radioactive materials, rDNA and biohazardous materials) require additional institutional approval; contact the Office of Research Compliance for more information.

Material(s):

*Disruption of any integumentary surface is considered surgery except when: hypodermic needle, biopsy needle, ear punch, or a tail snip is performed. Addendum 4 details major, minor, multiple, survival and non-survival surgeries.

3.5 Pain or Distress

Please complete the following table indicating the number of animals of each species used for each pain category. Pain categories are based on USDA criteria. USDA Categories:

- Category B Animals are those that are being bred, conditioned, or held for use in teaching, testing, experiments, research, or surgery but have not yet been used for such purposes, however minor.
- Category C Animals are those that are subjected to procedures that involve no pain or distress, or procedures that would not require the use of pain-relieving drugs. (i.e. animal behavior or routine injections and blood samples)
- Category D Animals are those subjected to potentially painful procedures for which anesthetics, analgesics, or tranquilizers will be used. (i.e. surgery with appropriate anesthesia and postoperative analgesia)
- **Category E*** Animals are those subjected to painful or stressful procedures without the use of anesthetics, analgesics, or tranquilizers. (i.e. Lethal dose studies or pain studies that do not allow pain-relieving agents)

Species	Category B	Category C	Category D	**Category E***
Mus musculus (C57BL/6J)	1350			
Mus musculus (C57BL/6J)		150		

*** Provide scientific justification for this pain category. (include criteria for moribundity and euthanasia)**

Mice in category C are those breeders that will be tattooed or ear punched to distinguish them for breeding.

3.6 Pain Alleviation

Complete the table below.

Species	Agent	Dose (mg/kg body weight)	Route	Frequency & Duration	Purpose
N/A					

Appendix D

IBC approved 11/4/2020 protocol # IBC 2020-10

MEMORANDUM OF UNDERSTANDING & AGREEMENT (MUA) FOR BIOHAZARDS OTHER THAN RECOMBINANT DNA EXPERIMENTS

All MUA'S can be submitted electronically to researchadministration@missouristate.edu or submitted as a hard copy to the ORA in Carrington 405. A signed copy must be provided. *Biosafety in Microbiological & Biomedical Laboratories (BMBL)* should be used as a reference when completing this MUA (see http://www.cdc.gov/biosafety/publications/bmbl5/).

A. General Information

Date: 10-14-2020

Researcher Name: Christopher Lupfer and Patrick Brooks

Researcher Title: Assistant Professor

Phone: 6-6887, 6-5279

Department: Biology and BMS

Office Bldg & Room #: Temple Hall 254, Professional Building 342

Laboratory Bldg & Room #: Temple Hall 232, Temple Hall vivarium

Granting Agency: Pre and Clean

Grant Number (if applicable):

Title of Grant or Project: Assessment of the antimicrobial effectiveness of hypochlorous acid in vivo and in vitro

B. Project Information

1. Describe the experiments involving biohazard(s). Your description is to be sufficiently complete so as to provide committee members an understanding of what you intend to do and how you will do it.

This research consists of three parts. Part one will test the in vivo effectiveness of hypochlorous acid to treat or prevent infection. Part two will test the ability of hypochlorous acid to disinfect or sterilize respiratory masks, such as N95 masks. Part 3 will test the ability of hypochlorous acid to prevent sepsis following surgical procedures.

1. Mice will be infected with a variety of pathogens including influenza A virus, *Streptococcus pneumoniae,* or *Aspergillus fumigatus*. Mice will then be treated with nebulized hypochlorous acid. Mice will be infected intranasally with the pathogens, treated, monitored for weight loss and food intake measured. Infection experiments in animals will take place in the high containment room in the Temple Hall Vivarium. Some mice will be euthanized and lungs or other organs collected to examine pathogen numbers, pathology and immune responses. Samples collected from animals will be processed in our BSL2 lab in Temple 232
2. N95 masks will be inoculated with either influenza A virus or *Escherichia coli* using a sterile cotton swab dipped in a solution containing the virus or bacteria. The N95 mask will then be swabbed with the virus or bacteria, allowed to dry, and then sprayed with hypochlorous acid. Microbes in the pieces of the mask will then be extracted with a mortar and pestle or a tissue homogenizer and the amount of virus or bacteria determined by plaque assay or ATP assay, respectively.
3. Rats will be treated with hypochlorous acid following surgery to perforate the cecum. This is a common procedure to simulate sepsis and test the ability of antimicrobials to prevent sepsis. After the surgery, fluid from the peritoneal cavity will be collected and the number of bacteria in the peritoneal fluid determined by colony forming unit assays on BHI agar petri dishes.

2. Provide an assessment of the physical containment required for the experiments.

Influenza A/PR/8/34 H1N1 virus and *Streptococcus pneumoniae* are respiratory pathogens that can cause pneumonia, especially in elderly individuals and are BSL 2 pathogens. *Escherichia coli and Aspergillus fumigatus*, although handled as BSL 2 organisms, are only pathogenic in immunocompromised individuals. Fecal microbes collected from peritoneal washes from rats will also be cultured. These are not likely to be pathogenic, but the overgrowth of even benign microbes could cause problems for immunocompromised individuals. Overall, the likely hood of transmission to laboratory personnel is low. In keeping with BSL2 guidelines, all experimental procedures will be conducted in a Class II biosafety cabinet when using or growing these pathogens or infecting animals. The cabinet will be decontaminated for 5 minutes with 10% bleach or 70% ethanol before and after procedures. All personnel working with these pathogens must be wearing appropriate personal protective equipment (Please see section 3 and 4). Proper handling of infectious cultures or samples must be observed. Proper decontamination of research equipment, and personal protective equipment must be followed. Also, proper personal hygiene in the laboratory environment must be maintained to prevent accidental contamination or infection. In the event of accidental contamination, an eye wash station is located in Temple 228 or the high barrier room in the Temple Hall vivarium. Contamination of skin will be treated with 70% ethanol for 5 minutes and then washed with soap and warm water for 1 minute. Accidental infection will be referred to the Mager's health clinic for immediate antibiotic or antiviral treatment. The influenza A/PR/8/34 H1N1 virus strain is a mouse adapted strain that has never been documented to cause infection in laboratory workers and was selected for its safety. However, students working with Influenza and *Streptococcus pneumoniae* will be required to receive their annual flu vaccine and the PnuemoVax23 vaccine for *Streptococcus pneumoniae.*

3. Describe the facilities and specific procedures which will be used to provide the required levels of containment.

Temple 232 contains the space and designated equipment necessary for working with influenza A virus and *Streptococcus pneumoniae, E. coli* bacteria and *Aspergillus fumigatus* fungus, as well as peritoneal washes. This includes a tissue incubator with sealed air-tight door, designated centrifuge, and a class II biosafety cabinet (BSC). In addition, for work involving animals, the vivarium in Temple Hall has a High Barrier room and procedure room both with a BSC and are available for work with BSL2 level pathogens.

All procedures will be performed in the BSC present in the lab in Temple 232 or in the procedure room/High Barrier room of the vivarium. All samples collected will only be opened and handled in a BSC. The BSC will be decontaminated prior to and following all procedures using either 70% ethanol or 10% bleach, which must remain on the surface for 5 minutes. All contaminated materials (pipet tips, gloves, tissue culture plates, old samples or cultures, etc.) will be disposed of in biohazard bags and autoclaved prior to being discarded. Any liquid cultures will be collected in sealed containers containing bleach at a final concentration of at least 10% to inactivate pathogens. Samples collected during experiments may be handled outside of the safety cabinet if contaminating pathogens have been killed by either incubation for a minimum of 5 minutes with formaldehyde or other fixative at a concentration > 1%, incubation in solutions containing at least 10% bleach >5 minutes can also be used to decontaminate samples. Finally, heating at >95 degrees Celsius for at least 15 minutes (for example, boiling samples in Laemmli buffer prior to SDS-PAGE analysis) may also be used to ensure pathogens are destroyed. In addition to regular decontamination after every procedure, weekly decontamination of workbenches and other surfaces in the lab will also be performed using a 10% bleach solution.

All personnel working with pathogens will wear disposable latex or nitrile gloves, a laboratory coat and eye protection. Lab coats will be sterilized in an autoclave and then washed as needed. These must be worn at all times while working with infectious agents or potentially contaminated samples or cultures. Long pants and close toed shoes are also required. Prior to exiting the BSC, gloves must be removed and placed in the biohazard bag or decontaminated with 70% ethanol or 10% bleach. Samples to be removed from the BSC for transport or storage must likewise be decontaminated. No eating (including chewing gum), drinking, applying cosmetics or contact lenses is allowed in the laboratory even when work with infectious cultures is not taking place. After experiments are complete or before leaving the lab, all PPE will be removed and hands washed for 30 seconds with soap and warm water.

In addition to these procedures and precautions, it is required that individuals working directly with these pathogens be current on all recommendations for vaccinations with seasonal influenza and pneumococcal.

4. Describe the procedures and precautions to be followed if biohazardous organisms or agents are to be transported between laboratories.

When samples need to be transported, they should first be inactivated by chemical (RIPA buffer) or heat inactivation as described above. A secondary container should also be used if the sample contains any liquid (small paint can filled with paper towels etc.). If live cultures or samples containing potentially live organisms must be transported, then samples must be sealed in a shatter resistant container (such as a threaded-cap polypropylene plastic test tube) and the outside of the container decontaminated with 70% ethanol or 10% bleach. PPE including lab coat, eye protection and nitrile gloves will be worn when transporting live cultures. After experiments are complete or before leaving the lab, PPE will be removed and hands washed for 30 seconds with soap and warm water.

5. Describe the waste disposal procedures expected to be used during this experiment.

For BSL2 conditions, the BSC will be decontaminated prior to and following all procedures using either 70% ethanol or 10% bleach, which must remain on the surface for 5 minutes. All contaminated materials (pipet tips, gloves, vials, old samples, etc.) will be disposed of in biohazard bags in the biosafety cabinet, sealed and autoclaved prior to being discarded. Any liquid cultures, stocks or samples will be collected in sealed containers containing bleach at a final concentration of at least 10% to inactivate pathogens and then autoclaved. In addition to regular decontamination after every procedure, weekly decontamination of workbenches and other surfaces in the lab will also be performed using a 10% bleach solution. PPE including lab coat, eye protection and nitrile gloves will be worn during any procedure using these pathogens or when decontaminating any surface or handling any waste. After experiments or decontamination procedures are complete or before leaving the lab, PPE will be removed, disposed of in the biohazard waste can and hands washed for 30 seconds with soap and warm water.

6. Is this a select agent? If yes, contact the Office of Research Administration (ORA).

No

7. Please list all students, staff and faculty involved with this project. CITI Biosafety training is mandatory for all personnel working with biohazards prior to final IBC approval.

Christopher Lupfer, PhD, Assistant Professor
Riley Nadler, BS, Master's Student
Jessica Reel, BS, Master's Student
Riley Marcinczyk, Undergraduate Student
Patrick Brooks, MD, Assistant Professor

8. The undersigned agree to certify the following conditions of the proposed research:

a. The information above is accurate and complete. We agree to accept responsibility for training of all laboratory workers involved in the project. We agree to comply with the CDC requirements pertaining to shipment and of hazardous biological materials. We are familiar with and agree to abide the provisions of the Missouri State University policies and procedures applicable to experiments involving biohazards.
b. We understand that only the organisms specified are covered by this MUA, and work with other organisms or types of biohazards may require other MUAs.

Christopher Lupfer	10/14/20	Via email 10/30/2020 JNP	
Principal Investigator	Date	Department Head	Date

9. The Institutional Biosafety Committee has determined, based on information provided the principal investigator, that:

a. No special medical surveillance (other than usual University health programs) is required for the project described in this MUA

b. The following specific medical surveillance procedures must be carried out, for individuals listed by name, before commencing the project described in this MUA:

10. We certify that the Missouri State University Institutional Biosafety Committee has reviewed the proposed project and has found in to be in compliance with Missouri State University's policies and procedures applicable to experiments involving biohazards.

[signature]	November 4, 2020
MSU IBC Chair or Representative	Date