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Sodium Pyruvate Alters the Immune Response to Influenza A Virus Infection in Macrophages

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**SODIUM PYRUVATE ALTERS THE IMMUNE RESPONSE TO INFLUENZA A
VIRUS INFECTION IN MACROPHAGES**

A Masters Thesis

Presented to

The Graduate College of
Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Hazzar Marwan Abysalamah

August 2018

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Biology

Missouri State University, August 2018

Master of Science

Hazzar Marwan Abysalamah

ABSTRACT

Pyruvate is the end product of glycolysis. It can either be transported into the mitochondria for use in the TCA cycle or be used to regenerate NAD^+ during fermentation or aerobic glycolysis (also called the Warburg Effect). I recently discovered that addition of sodium pyruvate to the culture medium during infection of macrophages with influenza A virus affects the production of cytokines involved in immune signaling. While infection of macrophages with influenza A virus resulted in high levels of cytokines (IL-6, IL-1 β , and TNF- α) in the absence of sodium pyruvate, the addition of sodium pyruvate significantly impaired cytokine production. I hypothesized that sodium pyruvate may directly inhibit virus entry or replication resulting in less immune stimulation. Alternatively, the addition of sodium pyruvate may alter metabolic pathways in the macrophages and affect the immune response to the infection. However, sodium pyruvate did not affect virus growth. Instead, the addition of pyruvate resulted in reduced reactive oxygen species production in the mitochondria resulting in diminished immune signaling. Overall, the effects of sodium pyruvate are on the immune response produced by the macrophages and not the growth of the virus.

KEYWORDS: immune response, macrophages, influenza a virus, sodium pyruvate, and metabolism

This abstract is approved as to form and content

Dr. Christopher Lupfer
Chairperson, Advisory Committee
Missouri State University

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Approved:

Christopher Lupfer, PhD

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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.

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INTRODUCTION

Increasing our understanding of the immune system, in general has the potential to provide vaccines, treatments and diagnostics that can improve the quality of life and reduce the number of deaths around the world because of infectious and immune diseases. Finding products that can support the immune response and fight infections is a major problem because every day many people around the world die from severe acute infections like Ebola or “Bird Flu”, chronic diseases like Hepatitis C virus, and other viral infections causing inflammation. Vaccines do not exist for many of these infections; therefore, understanding how the immune system is regulated in response to such infections is essential for developing treatments.

Recently, immunologists have come to appreciate that immune cells require specific metabolic pathways in order to function correctly. Specifically, glycolysis is required by T cells and macrophages in order to respond to a viral infection or to fight cancer (1). In addition, several reports demonstrate that inhibiting glycolysis blocks the ability of influenza A virus (IAV) to replicate in cells. During the course of infecting macrophages with IAV, we noted that using different brands of cell culture media with different nutrient compositions had an effect on the magnitude of the immune response. Thus, understanding what affects nutrients play on the immune response to IAV will help us better understand the immune response in general, but it will also help us determine if certain nutrients or nutritional supplements can improve or impair the immune response to IAV.

Influenza A Virus

Respiratory viruses in general, and IAV in particular, represent continuing global threats to human health (2). Seasonal and pandemic Influenza both play major roles in the health and economy of countries worldwide (3). Even though the IAV vaccine has existed for over 70 years, IAV continues causing seasonal epidemics and occasional pandemics around the world (4). IAV is easily transmitted in different ways. For instance, it can be transmitted by inhaling contaminated air if someone sneezes, by shaking hands with sick people and then touching your face, and by touching contaminated surfaces like desks or door knobs and then touching your face. Symptoms are various but include cough, sneeze, shaking chills, headache, stuffy and runny nose, sore throat, fatigue, and often a high fever. IAV can cause respiratory problems and death especially in those age 65 and older (5).

IAV is a member of the Orthomyxoviridae family. IAV can infect both humans and animals including horses, dogs, whales, seals, birds, ducks and more (6). It is a negative sense single-stranded virus (-) ssRNA, which has eight genome segments encoding 10-12 genes, depending on the strain. The two main surface glycoproteins are the hemagglutinin (HA) and neuraminidase (NA), and they are the main targets for antibodies made by the host during the immune response or during vaccination. In general, antibodies against HA are considered for both therapeutic and diagnostic potential (7). HA performs two main functions: cell attachment via binding sialic acids (SA) on host cell glycoproteins and fusion of cellular membranes and viral membranes following virion internalization into endosomes (8). Membrane fusion also requires a transmembrane protein in IAV called the M2 ion channel, which allows for the passage of H^+ ions in the acidifying endosome

to enter the virion resulting in conformational changes in the HA protein that induces membrane fusion and also results in unpackaging of the virus genome from the virus matrix. The NA protein is an enzyme that removes sialic acid from the surface of infected cells preventing the new virions from infecting the cell they just left and allowing the virus to infect other healthy cells in the host or spread to a new host (9, 10, 11). There are actually 18 major variants of HA and 11 variants of NA among IAV strains (12, 13). Virus strains are designated based on their HA and NA proteins (for example H3N2, H5N1 or H1N1).

IAV has the ability to reassort and create a new strain if a host is infected by two different strains of IAV simultaneously. For instance, a new H1N1 strain was introduced into the human population in 2009 that came about when a pig was infected with different strains of IAV originating from a pig, a human and a duck (14). This new IAV strain was antigenically unique meaning that no one in the human population had immunity to it, and it was able to spread rapidly causing the first IAV pandemic of the 21st century (15). This process of reassortment is possible due to the fact that the genome of IAV is segmented. If two different viruses infect the same cell at the same time, the segments from one virus can be packaged randomly into new virions as they leave the cell. The ability of IAV to reassort and make antigenically distinct viruses leads to periodic changes in the viruses that circulate among humans and results in pandemics including 1918, 1957, 1968, 1977, and 2009 pandemics. However, not all IAVs have the same virulence. The 1918 “Spanish Flu” killed as many as 230 million people, but the 2009 “Swine Flu” only killed about 284,500-500,000 people (16, 17). There are clearly genetic differences between these viruses that account for some differences in virulence,

but the overall health of the human population has also been implicated in the severity of new pandemics (18, 19). Obviously, in 2009, the healthcare available was far superior to that in 1918. For instance, the IAV vaccine was also not available in 1918. The nutritional status of humans has also improved over the last 100 years and may play a role in susceptibility to severe IAV infection (20). Thus, we are interested in understanding how metabolism or altering metabolic pathways can affect the immune response and outcomes of IAV infection.

The Innate Immune System

Cells of the Innate Immune System. The immune system is a defense system to fight pathogens causing diseases in the body. The immune system has two parts: innate immunity and adaptive immunity. The innate immune system is the first line of defense and consists of a variety of cells that can detect many foreign particles. White blood cells (WBCs) or leukocytes, which are derived from hematopoietic stem cells from bone marrow, can differentiate into many types of cells. For instance, monocytes, neutrophils, basophils, eosinophils, NK cells and lymphocytes are all leukocytes. Macrophages can play a major role in the immune system to defend against different types of pathogens. They have the ability to phagocytose and digest pathogens or any foreign particles that enter the body (21). They also have the ability to activate the adaptive immune system by carrying digested pathogen material to lymph nodes and presenting antigens to the T lymphocytes through MHC class I and II proteins on the macrophage cell surface (22). Finally, macrophages are activated during infection and produce immune signaling proteins called cytokines that can travel throughout the body and induce the production of

more leukocytes from the bone marrow (23), cause fever (24), recruit more leukocytes to the sight of infection (25, 26), and cause blood vessels to become leaky resulting in inflammation (27). The types of cytokines produced by macrophages depend on several things. One factor affecting cytokine production is the pathogen causing the infection (virus, bacteria, or parasite). Another factor is the metabolic environment at the site of infection. There are a variety of conditions that can alter metabolism and alter innate inflammatory conditions. Examples include obesity, diabetes, and other metabolic syndromes. Based on the metabolic environment and the pathogen, macrophages are classified into two cell types: M1 or classically activated macrophages (CAM), and M2 or alternatively activated macrophages (AAM). M1 macrophages are pro-inflammatory and are prevalent during viral and bacterial infections where glycolysis can function. M2 macrophages are common during parasite infections or when oxidative phosphorylation is dominant (28, 29).

Pathogen Detection by the Innate Immune System. The innate immune system has germline-encoded pattern-recognition receptors (PRRs). These sensors are capable of recognizing microorganisms that invade the host (30). PRRs are receptor proteins found on the surface of immune cells, for example, dendritic cells, macrophages, and lymphocytes, as well as some epithelial cells. These proteins can bind to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide or peptidoglycan from bacteria and double-stranded RNA from virus genomes (31). When IAV infects cells, the virus genomic RNA is the major PAMP detected by PRRs. Toll-like receptors (TLRs) are a class of PRRs that are on the membranes of cells or in endosome membranes. TLRs consists of 13 family members, each has the ability to detect PAMPs that have been

derived from different pathogens such as bacteria, fungi, and virus (32). TLR3 can detect double-stranded RNA from IAV in the endosomes when the virus enters the cell by endocytosis. TLR7 is located in endosomes (33, 34, 35) and it can detect single-stranded RNA. TLR7 can play an important role in immunity against ssRNA viruses such as IAV and vesicular stomatitis virus (VSV). TLR7 is required to generate immunity against ssRNA viruses (36). Other sensors of viral replication are RIG-I-like receptors and are found in the cytoplasm of cells. RIG-I can detect the presence of viral RNA and 5'-triphosphorylated RNA species (37). In general, they can detect a variety of intracellular pathogens (38). Detection of PAMPs by PRRs can activate a variety of immune signaling pathways resulting in the production of cytokines or increased phagocytosis.

Transcription Factor Activation by PRRs. When RIG-I is activated, it moves to the mitochondria, where it interacts with Mitochondrial Antiviral Signaling protein (MAVS) (39, 40). MAVS then recruits different adaptors to gather at the mitochondria forming the MAVS signalosome, which includes TRAF family member proteins that associate with the transcription factor NF- κ B (41). NF- κ B is a transcription factor that controls transcription of genes for various cytokines including pro-interleukin-1 β (pro-IL-1 β), IL-6 and tumor necrosis factor- α (TNF- α) (42, 43). Similarly, TLR3 and TLR7 detect the genetic material dsRNA or ssRNA from intracellular pathogens like viruses, fungi, or bacteria (44). The activation of TLR3 or TLR7 will initiate a cascade of events leading to activation of the same transcription factor NF- κ B. Activation of NF- κ B requires activation of two kinases, I κ B kinase alpha and beta (IKK- α , - β), which phosphorylates the inhibitory κ B (I κ B) proteins leading to their degradation and releasing NF- κ B to enter the nucleus and activate transcription (45).

Inflammasome Activation by PRRs. The inflammasome is a multiprotein complex, and it has a biochemical function which that results in cleavage and activation of the cysteine protease caspase-1 (46). Inflammasomes are present in a variety of cells such as macrophages, dendritic cells, neutrophil, B cells, and T cells (47). Active caspase-1 leads to activation of the inflammatory cytokines IL-1 β and IL-18 (48, 49). Inflammasome activation is regulated by several PRRs that detect PAMPs such as DNA or flagellin in the cytoplasm (AIM2 and NLRC4 respectively). NOD-like receptor containing a pyrin 3 (NLRP3) can also cause inflammasome formation (50). However, NLRP3 is somewhat unique in how it senses pathogens. NLRP3 does not directly detect pathogen molecules like viral RNA. Instead, NLRP3 responds to defined microbial components gaining access to the cytosol (51) and requires microbial stimuli or cytokines expressed through the activation of NF- κ B. NLRP3 activation forms the inflammasome complex with the adaptor molecule ASC, which controls the activation of caspase-1 (52). The activation of caspase-1 cleaves pro-IL-1 β and pro-IL-18 into the biologically active, secreted forms of these cytokines (53).

Metabolism and the Immune System

Glycolysis is a central pathway in cellular glucose metabolism that produces energy and intermediate metabolites for use in other biosynthesis pathways (54, 55, 56). Intracellular glucose is phosphorylated into glucose-6-phosphate to enter glycolysis and the final products are ATP, NADH and pyruvate. Pyruvate then feeds into the tricarboxylic acid cycle (TCA) in the mitochondria, where additional ATP, NADH and FADH₂ are produced and CO₂ is a byproduct. The NADH and FADH₂ produced in

glycolysis and the TCA cycle donate electrons to the electron transport chain in the inner mitochondrial membrane where O_2 is the final electron acceptor. The energy from the electrons is then converted into a proton-motive force to drive ATP synthesis (57, 58).

The chemical formula for sodium pyruvate (NaPyr) is $NaC_3H_3O_3$. Pyr can play a role in energy metabolism as just discussed. In addition to the typical glycolysis-to-TCA pathway, Pyr can be derived from lactate taken up from outside the cells, or it can be made intracellularly from amino acids (59). Although Pyr is an important metabolite, it has additional characteristics that are sometimes overlooked. NaPyr is often added to cell culture media due to its pH buffering abilities, and it is a carbon source similar to glucose (60). NaPyr has been discovered to help in clinical applications due to its antioxidant properties in such diseases as cardiac failure, cardiopulmonary resuscitation, myocardial stunning, and cardiopulmonary bypass surgery (61). Instead of entering the TCA cycle, anaerobic glycolysis can also occur (fermentation) where pyruvate is reduced into lactate in order to regenerate NAD^+ . In cancer cells, this also occurs even when oxygen is present (aerobic glycolysis/Warburg effect). The Warburg effect occurs when cancer cells decide to rely on aerobic glycolysis, although it is an inefficient way to generate adenosine 5'-triphosphate (ATP), instead of relying on mitochondrial oxidative phosphorylation to generate the energy, because it allows for other metabolites necessary for rapid cell division to be made, such as amino acids (62).

The reason why these metabolic pathways are essential for energy production as well as intermediate metabolites for other biosynthetic pathways, thus it is not surprising that alterations in metabolism alter immune function. For instance, as mentioned above, altering metabolism can affect M1 or M2 macrophages. One mechanism is through

regulation of immune signaling pathways like inflammasome activation. Glycolysis in general is important for cellular activation and it is involved in the immune response (63). So, the interaction between immune signaling pathways and metabolic pathways leads to plethora of signaling pathways and cellular activation mechanisms (64). Since the immune system is composed of various cell types and subtypes that are essential to defending the host against foreign particles, these metabolic pathways play cell-specific roles. For instance: metabolic pathways regulate T cells and macrophages by glycolysis-mediated upregulation of surface markers for convenient effector response against pathogens. So, regulation of metabolism inside immune cells is required to activate immune cells necessary to protect the host and keep homeostasis. Dysfunction of immunological metabolic function and metabolic products such as reactive oxygen species can also lead to diseases. One of the hallmarks of cancer cells is the high level of glycolysis even in the presence of oxygen (65). Also, excessive glucose uptake can promote hyperactive immune responses and possible immune pathology (66). Glucose restriction can affect inflammasome activation and NF- κ B activation (67). In the absence of glucose, induced by the addition of inhibitors of ATPase, cells will apoptosis (68). Many cells of the immune system use aerobic glycolysis as a rapid energy source and as a way to regulate immune function. Glucose provides energy that lymphocytes need through glycolysis or by the pentose phosphate pathway to generate more NADPH (69). In addition, glucose can supply a carbon source for the synthesis of other macronutrients like nucleic acids and phospholipids. Another way is that glucose can be metabolized during aerobic glycolysis is not found exclusively in cancer cells, but it also can be found in rapidly dividing normal cells even under conditions of normoxia (70, 71). Not only

macrophages use glucose as an energy source, but T cells use glucose as a primary fuel source (72). Understanding this point was the first step in thinking about how sodium pyruvate could affect macrophages during IAV infection.

Problem Statement and Hypothesis

I discovered through preliminary experiments that the addition of sodium pyruvate to the culture medium during infection of macrophages with influenza A virus has an inhibitory effect. This leads to a decrease in the production of pro-inflammatory cytokines (IL-1 β , TNF α , IL-6) which are involved in immune signaling.

The object of my research is to determine what effect sodium pyruvate has on the immune response during influenza A virus infection, to make sure that sodium pyruvate can alter immune response, and finally, to determine if NaPyr can control the infection to protect the body from pathogens.

There are two possibilities that can explain how pyruvate functions: first is pyruvate will go to mitochondria and make more energy. The second possibility is adding more pyruvate will inhibit the glycolysis pathway due to an excess of the end product.

The question that we are trying to find a scientific answer to is what is the impact of using NaPyr on the immune response? I hypothesize that adding NaPyr to culture media during influenza A virus infection of macrophages will alter energy production in macrophages and affect the immune response. Alternatively, NaPyr may directly inhibit the influenza A virus infection and lessen the immune response.

MATERIALS AND METHODS

Overall Experimental Design

In the current study, my focus is on the role of NaPyr in altering the immune response in macrophages. There are several overall objectives: First is determining if NaPyr affects macrophages directly or if it affects virus replication and inhibits the immune response by inhibiting the virus. This will be done by checking virus titer and virus RNA levels in macrophages. Second is adding different inhibitors such as 2-deoxyglucose (2DG), heptelidic acid (HA), and NaPyr to see if inhibitors of glycolysis (2DG, HA) also affect the immune response of macrophages infected with IAV. For this type of experiment, I examined immune signaling by western blot, cytokine gene expression by qRT-PCR, cytokine production by ELISA, and cell death or mitochondrial damage by flow cytometry. All of these experiments helped me determine the mechanism that NaPyr uses to alter the immune response in macrophages.

Animal Welfare

All mice used in these experiments were C57BL/6J mice that were bred and raised in the Temple Hall Vivarium according to IACUC protocol 16.015. They were then transferred to IACUC protocol 16.009 prior to experimental use. All breeding and experiments were performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines under protocol (January 8, 2016; approval #16.009 and February 17, 2016; approval #16.015), the AVMA Guidelines on Euthanasia, NIH regulations (Guide for the Care and Use of Laboratory Animals), and the U.S. Animal

Welfare Act of 1966. All associated work with cell culture was completed inside a sterilized biosafety cabinet.

Making Bone Marrow Macrophages

In order to do these experiments, I needed to generate macrophages (white blood cells) from bone marrow stem cells and infect them with influenza A virus (IAV).

Macrophages are made by taking bone marrow from the femur and tibia of 7-14-week-old C57BL/6J mice. Bone marrow cells were then grown for 5 days in cell culture dishes (150 mm x 25mm) having bone marrow differentiation media (BMDM), and cells were fed with additional BMDM on day 3. To make 500ml of BMDM media, I use 300ml Dulbecco's Modified Eagle Medium (DMEM), 50ml heat inactivated Fetal Bovine Serum (FBS), 150 ml L929 cell conditioned media, 5 ml Non-essential amino acids (NEAA), and 5ml penicillin–streptomycin (Pen/Strep). L929 cell conditioned medium contains Macrophage colony-stimulating factor (M-CSF) and is made by growing L929 cells in DMEM+10% FBS+Pen/Strep.

On day 5 of BMDM growth, I prepared cells to plate them by sucking the media out of the dishes, then adding 10 ml of phosphate-buffer saline (PBS) and letting it incubate for three minutes. Macrophages are adherent cells and must be scraped off the cell culture dish using a sterile plastic cell scraper. Macrophages in the PBS were then transferred to a 50ml tube. 5 ml of extra PBS was added to the dishes to rinse them to make sure that all macrophages are removed and then transferred to the 50ml tube. 50ml tubes of macrophages were centrifuged at 400xg for 7 minutes. Supernatants were removed and replaced with 10 ml BMDM. Cells were counted using a hemocytometer

and plated in 12 well plates at 1×10^6 cells/well in 1ml BMDM media and incubated overnight to allow cells time to adhere to the new plates. Macrophages were then used the following day for infection experiments.

Production of Influenza A Virus in Chicken Eggs

The strain of IAV used in all experiments is the influenza A/PR/8/34 H1N1 strain. Prior approval for this project was obtained from the Institutional Biosafety Committee (IBC) on October 2nd, 2015. In order to generate virus, specific pathogen-free chicken eggs were ordered from Charles River. Eggs arrived on day 0. Eggs were incubated at 38 °C (99 °F) for 10 days. Location of the embryo was determined by shining a light on the eggs to make sure that the embryo is viable and mark its location (candling). On the opposite side of the egg from the embryo, a spot was marked 1/4 inch from the air sack membrane. Eggs were sterilized by spraying them with 70 % ethanol. A 20-gauge needle was used to make a hole in the shell at the mark. (It is important to not poke a hole through the embryo, only through the shell). The virus used to infect the eggs was diluted at 10^{-4} in PBS+ antibiotics pen/strep + gentamycin. Using virus too highly concentrated will kill the embryo and result in poor virus titer. A 1-inch 25gauge needle and syringe were used to inject 100ul of virus per egg by inserting the needle through the hole at about a 45-60° angle. After injection, the hole in the eggshells was covered with super glue. Eggs were then incubated at 38°C for 3 days. Eggs were checked daily for embryo viability by candling them. On day 3, eggs were placed in the refrigerator to chill at least 3h. This is done to stop the blood flow and reduce the amount of blood in allantoic fluid during the collection process, which can bind to IAV and reduce the virus titer. Eggs

were again disinfected with 70% ethanol and the shell cracked above the air sack with tweezers and opened over the air sack to access the allantoic fluid. Allantoic fluid was then removed via pipette and placed in 15ml centrifuge tubes on ice. After harvesting the eggs, the allantoic fluid was centrifuged at 3000xg for 10 minutes to remove any cells or debris. Clarified allantoic fluid of new virus stocks was aliquoted into 100 μ l tubes and virus stored at -80°C for future use and determination of stock titer by plaque assay.

Preparation of Virus Stocks from MDCK Cells by Ultracentrifugation

An alternative method for virus preparation was also used. Madin-Darby Canine Kidney cells were grown in T175 flasks until confluent (about 3 days). Cells were then washed 2x with PBS and infected with 2.5×10^6 PFU of influenza A/PR/8/34 H1N1 diluted in 5 ml of 1x plaque assay media per flask. Flasks were incubated for one hour at 37°C and 5% CO₂ with shaking every 10 minutes. After an hour, the media was removed and an additional 20 ml of 1x plaque media was added to each flask. Then 20 μ l TPCK trypsin was added to each flask to help the virus mature. Flasks were incubated at 37°C and 5% CO₂ for three days and checked under the microscope to make sure that at least 85% of the cells are dead.

Media was then transferred from flasks to 50 ml centrifuge tubes and vortexed for 1 min, then centrifuged at 2000 x G for 10 minutes to remove MDCK cells. Ultra-centrifuge tubes were preloaded with 3 ml of 5% sucrose in MHN buffer (Table1). This was overlaid with the virus infected media once the MDCK cells had been removed. Each tube was weighed and balanced with PBS if needed and put in the ultra-centrifuge rotor (JS-24). The centrifuge was set to a temperature of 4°C and speed of 23,000 RPM

for 1 hour. When it was done, the centrifuge tubes were placed on ice in the biosafety cabinet before opening. The virus was pelleted at this point, so all but 3ml of the media was sucked out. The remaining 3ml of media from each tube was then combined into 1 tube and weighed again, and a balance tube was made. The pooled virus was then centrifuged again with the same conditions. The tubes were again returned to the ice in the biosafety cabinet and all but 3ml of media removed. The virus pellet and the remaining 3ml media were then vortexed in a 15ml centrifuge tube for 10 minutes to resuspend the virus pellet. The virus stock was then aliquoted at 60 μ l for each 1.5 ml tube and stored at -80°C.

Flu Plaque Assay

Plaque assay is a process to determine the number of infectious virions in a sample based on the ability of the virus to kill infected cells and create a hole in the cell culture monolayer (73, 74). For the IAV plaque assay, two days before starting the plaque assay, MDCK cells were plated at 3×10^5 cells/well in 1ml in 12-well plates with DMEM medium (1x Plaque Assay Medium) (Table 2) that has 5% FBS, pen/strep, and L-glutamine. Dilutions of the virus grown in eggs or concentrated by ultracentrifugation were prepared in 1x plaque assay medium by making 10-fold dilutions. MDCK cells were then washed with PBS twice by adding 1 ml/well. PBS was removed and 100 μ l/well of the virus dilutions were added to duplicate wells in the 12-well plates. Plates were incubated at 37 °C and 5% CO₂ for one hour, plates were shaken every 10 min to keep cells from drying out. After 40 minutes of incubation, the overlay was prepared. 2% SeaPlaque low melting point agarose (Bio Whittaker, Cat. No.50100) in diH₂O was

microwaved to melt the agarose. This was allowed to cool to 37-42 °C. Then 2X plaque assay medium (Table 3) was warmed to 37-42 °C and the 2% agarose was mixed together at a 1:1 ratio. TPCK-trypsin was added to a final concentration of 1.0 µg/ml. After the full hour of incubation, the 12-well plates were removed from the incubator and the infection medium was removed by aspiration. 2ml of the warm overlay was added to each well and allowed to harden in the biosafety cabinet with lids ajar on the plates to prevent condensation. Once the agar was hard, the plates were turned upside down and incubated for 3 days. After 3 days, the overlaid agar plugs were removed. Then, the wells were stained with 1% crystal violet in methanol. The stain was removed and wells rinsed with water. After drying, the plaques were read by counting the white spots.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA uses antibodies specific to a protein of interest to determine the concentration of that protein in a sample. It is often used to examine cytokines (IL-1 β , TNF- α , IL-18, and IL-6). ELISA kits were purchased from thermos fisher scientific (IL-1 β , TNF- α , and IL-6, catalog #s 88-7013-88, 88-7324-88, 88-7064-88). To perform the ELISA, Corning Costar 9018 ELISA plates were coated with 100µl/well of capture antibody diluted 1:500 in 1x coating buffer provided by the manufacturer. Plates were then sealed and incubated overnight at 4 °C. The next day, the coating buffer was dumped and plates washed with 200µl wash buffer (Table 4, 5) three times. Wells in the plate were then blocked to prevent unwanted protein binding to the plate by adding 150µl/well of 1x ELISA assay diluent (also provided in the kit). Plates were then incubated at room temperature for 1 hour. During this time, 1x ELISA assay diluent was used to dilute the

control stock by 2-fold dilutions to generate a standard curve from 1000pg/ml to 31.25pg/ml (Figure 1). After 1-hour, the 1x ELISA assay diluent used to block the plates was removed by dumping and replaced with 100µl of each sample, the 6 standards for the standard curve, and 1 blank well (1x ELISA assay diluent). Plates were incubated for 2 hours at room temperature then washed with 200µl wash buffer three times. Biotin-conjugated-detection antibody stock was diluted 1:500 in 1x ELISA assay diluent and 100µl added to each well and plates incubated for another 45 minutes. Wells were then washed 3 times with 200µl wash buffer and streptavidin-HRP was diluted 1:500 in 1x ELISA assay diluent. Then, 100 µl was added to each well and plates incubated for another 45 minutes. After this incubation, plates were washed four times with 200µl wash buffer. 100µl of TMB substrate was added to each well in the plates and plates incubated until the 1000pg/ml standard turned dark blue. The reaction was then stopped by adding 100 µl of 1M H₂SO₄ (wells then turned yellow). Plates were read at 450nm on a microplate reader (BioTek ELx808).

Western Blotting

Western blotting was used in this project to measure the activation of caspase-1 (caspase-1 p20) and NF-κB (phosphorylated IκBα) as well as total pro-IL1β and total IκBα in both infected and uninfected samples with and without treatment. Samples were collected by adding RIPA buffer to lyse cells. 4x protein loading dye was then added to samples and they were boiled for 20 minutes. Samples were loaded on 12% polyacrylamide gels and gels were run at 100V for 2 hours in 1xTris/Glycine/SDS buffer (Running Buffer) (Table 6). Gels were transferred to Polyvinylidene difluoride (PVDF)

membranes already soaked in methanol for a few seconds and incubated for 5 minutes in 1xTris/Glycine buffer (Transfer Buffer) (Table 7). The transfer system was set up for 45 minutes at 40 Volts. When the transfer was completed, the membranes were blocked in blocking buffer (5% milk in wash buffer) on a shaker for 1 hour. The blocking buffer was removed and replaced with primary antibody diluted in 5% milk in wash buffer overnight at 4 °C. The next day, the membranes were washed 3x in wash buffer and incubated 45 minutes in secondary antibody diluted in 5% milk in wash buffer. Then, the membranes were washed 4x in wash buffer. 1 ml of Substrate (Super Signal West Femto; ThermoFisher, A53225) was added to each membrane for 1 minute and then the membrane was imaged on an Azure C300 digital imaging system. (See table 8 for a list of antibodies).

Cell Death and Mitochondrial Damage

To determine if NaPyr, 2DG or HA altered the immune response by affecting cell death or mitochondrial function, macrophages were stained with fluorescent dyes designed to detect cell death by holes in the cell membrane (SYTOX red; ThermoFisher, S34859) or mitochondrial damage through mitochondrial reactive oxygen species production (MITOSOX; ThermoFisher, M36008). Macrophages were plated in 12-well plates and infected and/or treated as described for the different inhibitor experiments. After 24 hours, and 30 minutes before collecting samples, 25 µl of each dye diluted in RPMI was added to each well (2.5nM Mitosox, 5 mM SYTOX). One control well was also unstained as a control. One control well each was stained with only one of the two dyes as a control. After 30 minutes, the media was removed and saved for ELISA and

1ml of PBS was added to each well and the macrophages were scraped off the wells with a 1ml pipet tip. Cells and PBS were transferred to a 1.5ml tube and analyzed on an ACURI C6 flow cytometer using channel FL3 for MITOSOX and channel FL4 for SYTOX red. 10,000 cells per sample were analyzed for fluorescence intensity and positivity of each dye.

RNA Isolation

In order to test the gene expression of immune system genes, β -Actin was used as a control and IL-1 β , TNF- α , and IL-6 gene expression was examined. Viral gene expression was also examined with β -Actin as the control and influenza A virus M1 and NP genes were tested. BMDM were infected and treated as before, and samples were collected at 6, 12, and 24 hours after infection. Media was removed and 500 μ l of Trizol was added to samples and incubated for 5 minutes at the room temperature. Samples were transferred to 1.5ml centrifuge tubes and 100 μ l of chloroform was added to each sample. Then, tubes were shaken for 15 seconds by hands and incubated at room temperature for 3 minutes. Tubes were centrifuged at 12,000xg for 15 minutes at 4⁰C. The clear upper aqueous layer was transferred to new tubes. 250 μ l of 2-propanol was added to each sample. After shaking the tubes, they were incubated at room temperature for 10 minutes. Samples were centrifuged at 12,000xg for 10 minutes at 4⁰C. Supernatants were removed, but about 5 μ l of it was left in each tube to avoid accidentally sucking up the RNA pellet. To wash the pellet, 500 μ l of 75% ethanol in molecular biology grade water was added to each tube. Tubes were vortexed briefly for 5 seconds. They were centrifuged at 7500xg for 5 minutes at 4⁰C. Supernatants were

removed and the tubes were dried in the vacuum centrifuge with the cap open. After drying the liquid, 20µl of the nuclease free water was added to reconstitute the RNA. Concentrations were checked by using the Implen Nanophotometer. Samples were then normalized to 200ng/µl by adding additional nuclease-free water to each sample.

CDNA synthesis and Real-Time PCR

High capacity cDNA reverse transcriptase kit (Thermo Fisher Scientific 436881) was used. 10 µl of the master mix were mixed with 10 µl of the isolated RNA. PCR tubes were centrifuged for 5 seconds and run on the thermocycler according to the manufacturer's instruction. Once the cycle was complete, the cDNA was diluted 1:5 by adding 80µl of molecular biology grade water. RT-PCR was performed by making a standard curve was by 2-fold dilution of the 12-hour infection sample without any inhibitors or treatments. 5µl of the standards were mixed with 15 µl of 2x DyNAmo HS SYBR Green qPCR master mix (Thermo Scientific 00596849) and added to control wells in the PCR plate. 5µl of the samples were mixed with 15 µl of master mix and were added to sample wells in the plate. Primers for β -actin, IL-1 β , TNF- α , IL-6, M1, and NP, were used to test gene expression (A15612T). MX program in STRATAGENE-Mx3005P PCR machine was used for data acquisition. (See table 9 for all Primer Sequences).

Effects of Different Inhibitors on the Immune Response

Glucose is the starting substrate for glycolysis and NaPyr is the product. 2DG and HA are glycolysis inhibitors (75). I wanted to determine if glucose or inhibitors of

glycolysis also affected the immune response to IAV. 1×10^6 macrophages were plated in 12-well plates overnight. The next day, cells were washed 2x with ml of PBS. Then 200 μ l of RPMI 1640 with 1x L-glutamine media was added to macrophages. 10 μ l of IAV (2.5×10^7 PFU, 25MOI) was added to wells and they were treated with 2 μ l of NaPyr or glycolysis inhibitors or mock treated. Final concentrations were 1mM NaPyr, 10 μ M H.A, and 250 μ M 2DG. RPMI with inhibitors and no virus was also a control. Plates were incubated at 37 $^{\circ}$ C and 5% CO₂ for two hours with shaking. Then, 200 μ l RPMI +20% FBS was added to each well, and 2 μ l additional NaPyr or glycolysis inhibitors added to maintain the same concentration. Samples were collected at 6, 12, 24, and 29 hours.

Comparing the Effects of Inhibitors on Different Pathogens or Treatments

To determine if the effects of NaPyr, 2DG or HA were specific to IAV or general effects on the immune response of macrophages, I plated 1×10^6 macrophages per well in 12-well plates. The next day, macrophages were infected by adding 200 μ l of RPMI 1640, with 1x L-glutamine media. Then 10 μ l of IAV (2.5×10^7 PFU, 25MOI) was added to some wells, 10 μ l of *E. coli* (1MOI) or 1 μ l of *Aspergillus fumigatus* (0.35MOI) were also added to other wells. A final set of wells were treated with 1 μ g/ml LPS and 5mM ATP, but this was done the following day, as treatment with LPS only lasts for 4 hours and ATP only 30 minutes. Some wells were also treated with 2 μ l of NaPyr or inhibitors (1mM NaPyr, 10 μ M H.A, and 250 μ M 2DG). RPMI alone or with inhibitors and no pathogen was the control. Plates were incubated at 37 $^{\circ}$ C and 5% CO₂ for two hours with shaking. Then, 200 μ l RPMI +20% FBS was added to each well. An additional 2 μ l of

NaPyr or inhibitors was then added to maintain the same concentration. Samples were collected at 24 hours for IAV, *E. coli* and *A. fumigatus*, and 4 hours for LPS+ATP.

Statistical Analysis

Statistical analysis was performed using GraphPad PRISM6. Comparison of 2 conditions was performed using the 2-sided student's t-test. Comparison of multiple conditions was performed using the One-Way ANOVA with Tukey's posthoc test. A p-value <0.05 was considered statistically significant.

RESULTS

NaPyr Affects the Immune Response, Not virus Replication

I hypothesized that the addition of NaPyr to cell culture medium would inhibit the immune response to IAV infection. To examine the immune response, I looked at cytokines (IL-1 β , TNF α , and IL-6) produced by macrophages infected with IAV. Infected macrophages were treated with NaPyr at the same time as infection with IAV and samples collected 24 hours later. Cell culture supernatants were examined by ELISA, and the results indicate that NaPyr significantly inhibits the production of IL-1 β , TNF α , and IL-6 (Figure 2A-C). I decided there were two possible explanations for the lower amounts of cytokines. Either NaPyr was inhibiting IAV from infecting or replicating in the macrophages, or NaPyr was altering the ability of the macrophages to make IL-1 β , TNF α , and IL-6. I tested virus replication by collecting cell culture media from infected cells and performing viral plaque assays to determine the number of infectious particles produced after the 24-hour infection. I found that NaPyr did not affect the number of infectious particles (Figure 3A). To further confirm that NaPyr did not affect virus growth or its ability to infect macrophages, I infected macrophages with IAV and collected the macrophages to purify RNA and performed qRT-PCR for two different virus genes (M1 and NP). NaPyr also did not inhibit the production of virus RNA demonstrating that NaPyr does not affect the immune response by inhibiting IAV replication (Figure 3B-C).

Examination of Possible Metabolic Effects of NaPyr on Macrophage Immune Responses

To understand how NaPyr affects the immune response, I compared the immune response of macrophages infected with IAV and treated with either NaPyr or glycolysis inhibitors 2-deoxy-D-glucose (2DG) or heptelidic acid (HA) compared to control samples with RPMI+ Flu with no additive treatments. I discovered that treatment with 2DG and HA could also decrease IL-1 β cytokine production during IAV infection (Figure 4 A-C). With IL-6, 2DG and NaPyr decreased cytokine production (Figure 4D, E). However, HA treatment (Figure 4F) showed increased IL-6 production compared to RPMI+Flu. TNF- α cytokine production was lower with NaPyr and both glycolysis inhibitors compared to control samples too (Figure 4G-I). To understand how NaPyr, 2DG, and HA affected cytokine production in macrophages, we examined cytokine gene expression by RT-PCR at different time points 6, 12, 24, and 29 hours after IAV infection (Figure 5). I saw that glycolysis inhibitors (especially HA) could, at some time points, inhibit gene expression, but NaPyr did not have a dramatic effect on gene expression. These data indicate that inhibiting glycolysis and altering cellular metabolism can affect cytokine production during IAV infection, but this did not mimic NaPyr treatment.

As seen in Figure 5, there were no major differences in the gene expression of any of the cytokines with NaPyr treatment. I performed western blotting (Figure 6 A-B) on cell lysates from BMDM infected with IAV and treated with NaPyr or glycolysis inhibitors. I observed that the production of pro-IL-1 β was unaffected by NaPyr treatment, but was less in cells treated with 2DG or HA (Figure 6A-B). Furthermore, the activation of NF- κ B (Phospho-I κ B) was less in cells treated with HA, but not NaPyr or

2DG (Figure 6 D-F). In some western blots, it appears that HA treatment inhibited pro-IL-1 β and NF- κ B. However, there was also less Actin loading control in these samples indicating that HA was causing cell death. These data provide evidence that NaPyr is not inhibiting NF- κ B transcription factor and the transcription of cytokine genes.

Mechanism for NaPyr Inhibition of Cytokines

As NaPyr does not appear to inhibit NF- κ B signaling and gene expression like the glycolysis inhibitors do, and is not causing cell death, we examined other possible mechanisms for how NaPyr could inhibit cytokine production. One possibility is that NaPyr alters cell death or cell damage. I again infected macrophages with IAV and treated with NaPyr, 2DG, or HA. After 24 hours, then I stained cells with MitoSOX, which is incorporated into the mitochondria and fluoresces when it interacts with reactive oxygen species (ROS) like superoxide and hydrogen peroxide in damaged mitochondria (76). The flowcytometry analysis clearly showed that the median fluorescence intensity (MFI) was less in cells treated with NaPyr than RPMI media controls or with HA (Figure 7A). Less fluorescence indicates less mitochondrial damage in cells treated with NaPyr and 2DG. On the other hand, HA caused more mitochondrial damage to IAV infected macrophages (Figure 7A). I also stained macrophages with SYTOX red dye, which only stains cells with holes in the membrane (dead cells). I found that NaPyr does not cause cell death, but HA causes dramatic cell death (Figure 7B). This demonstrates that NaPyr does not work the same way to inhibit the immune response as glycolysis inhibitors and we had to reject our initial hypothesis. Instead, NaPyr is an antioxidant. In fact, some papers have shown this before (77).

NLRP3 activation has been shown in many instances to be dependent on ROS and mitochondrial damage (78, 79). If NaPyr does inhibit ROS and prevent mitochondrial damage, then this should also inhibit activation of the NLRP3 inflammasome and there should be less activation of the protease caspase-1. I examined caspase-1 activation by western blot and found that caspase-1 activation in cells infected with IAV and treated with NaPyr was less (Figure 8A-B). It was also less in cells treated with 2DG, but this could be due to the fact that NF- κ B activation is also needed to express NLRP3 and “prime” the inflammasome for activation or due to less mitochondrial damage as seen by MitoSOX staining (Figure 7A).

Effects of NaPyr on Other Infections

In order to see if the effect of NaPyr is only on IAV, we infected macrophages with *Escherichia coli*, or treated cells with lipopolysaccharide (LPS) and adenosine triphosphate (ATP), which is a potent activator of the NLRP3 inflammasome (80). Cells were infected and treated at the same time with the addition of NaPyr or the glycolysis inhibitors 2DG and HA. ELISA data for IL-1 β showed that samples treated with NaPyr and 2DG had nearly the same amount of cytokine as control samples (Figure 9-10). While HA treatment showed decreases in IL-1 β production (Figure 9A, 10A). For IL-6 production, only HA affected this cytokine (Figure 9B, 10B), probably due to cell death. TNF- α also decreased with 2DG and HA treatments (Figure 9C, 10C). Finally, flowcytometry data confirmed that NaPyr and both of the glycolysis inhibitors had no effect on mitochondrial damage (Figure 11) and caspase-1 activation was also not inhibited (Figure 12).

DISCUSSION

The ability of metabolites to affect the immune response to pathogens is an important area of research with implications for preventing and treating disease. Recent research shows that changes in metabolism in cells of the immune system can affect diseases such as influenza, cancer, diabetes and more (81,82,83). The goal of this project was to understand how NaPyr can affect the immune response. My data clearly indicate that treatment of IAV infected macrophages with NaPyr can reduce cytokines (IL-1 β , TNF- α , and IL-6). However, NaPyr does not affect virus growth or replication by either plaque assay or qRT-PCR for IAV matrix (M1) or nucleoprotein (NP). This indicates that NaPyr is altering the immune function of the macrophages.

As NaPyr is the end product of glycolysis, I thought that adding NaPyr to macrophages might alter the metabolic pathways like glycolysis or the TCA cycle. Other researchers showed that inhibition of glycolysis with 2DG suppresses lipopolysaccharide-induced IL-1 β (84), which is the reason why I tried to use different glucose inhibitors. However, in previous studies, inhibitors of glycolysis inhibit IAV replication by inhibiting virus entry into the cells and can lead to early cell death (85). Testing multiple glycolysis inhibitors revealed that 2DG and HA can also inhibit cytokine production during IAV infection, but my results and the results of others, suggest the mechanisms are different. 2DG likely inhibits virus infection and may also affect NF- κ B activation. HA may affect NF- κ B activation and also affect virus infection, but the most notable effect was the dramatic cell death it caused. Although cell death would stop production of some cytokines, dead cells can also further activate the immune system (86). On the other

hand, NaPyr did not cause cell death and had little effect on NF- κ B or transcription. Instead, NaPyr inhibited mitochondrial damage and production of ROS. This caused me to reject my initial hypothesis that NaPyr was affecting glycolysis and conclude that NaPyr is an antioxidant.

The question then is how an antioxidant would inhibit the immune response. Multiple research papers, including some from Dr. Lupfer, have shown that antioxidants that can prevent mitochondrial damage can also prevent inflammasome activation and release of IL-1 β from infected cells (87). On the other hand, more ROS can enhance inflammasome activation (88). Another research paper showed that NaPyr can prevent cell death independent of ATP production (89) suggesting this was not based on metabolism. More researchers have shown that NaPyr can help decrease inflammation by its antioxidant properties (90). Although NaPyr is an antioxidant, I wanted to know if it has the same effect in all infections, or if this was specific to IAV infection. When I examined cytokine production and caspase-1 activation during infection with *E. coli*, I did not see a difference with any treatment except HA; however, the effect of HA was probably still due to cell death. It is possible that NaPyr did not affect the immune response to *E. coli* because it is not an intracellular infection. *E. coli* also activates a slightly different inflammasome called the non-canonical NLRP3 inflammasome that needs caspase-11 (91, 92). When I tested the effects of NaPyr or glycolysis inhibitors on treatment with LPS+ATP, only HA affected cytokine production in macrophages. This could be due to the fact that LPS+ATP is a much shorter treatment of only 4 hours instead of 24 hours.

To sum up, NaPyr affects cytokine production by inhibiting inflammasome activation because of its antioxidant properties and not by affecting the virus growth or causing cell death. NaPyr does not affect NF- κ B, but it does affect caspase-1 activation. NaPyr and the glycolysis inhibitors 2DG and HA can all impair the immune response to Influenza A virus infections in macrophages, but the mechanisms are different. The glycolysis inhibitor HA causes mitochondrial damage, but not sodium pyruvate. Sodium Pyruvate is an antioxidant. On the other hand, 2DG, and HA are glycolysis inhibitors. In the future, it will be important to verify the role of glycolysis and examine energy production (NAD⁺, ATP) and ROS generation (H₂O₂, O₂⁻) during treatment of cells with sodium pyruvate or glycolysis inhibitor pathways. I also need to see if we cause mitochondrial damage another way, such as an electron transport chain uncoupling reagent like rotenone, to see if this reverses the effects of NaPyr.

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Table 1. MHN Buffer.

Ingredients	Amount
1M MgSO ₄	24.65 g
50 mM HEPES	11,915 g
150 mM NaCl	0.876 g
DD water	QS to 100 ml

Table 2. 1x Plaque Assay Medium.

Ingredients For (20 ml)	Amount
2x MEM medium	10 ml
Molecular water	10 ml

Table 3. 2x Plaque Assay Medium.

Ingredients For (50 ml)	Amount
H ₂ O	31 ml
10x MEM	10 ml
100x glutamine	1 ml
BSA 7,5%	4 ml
Penicillin streptomycin (10000 U/ml each)	1 ml
Sodium bicarbonate	3 ml

Table 4. Wash Buffer: 1x Tris Buffer Saline With 0.05% Tween 20 (TBST).

Ingredients For (1L)	Amount
10x Tris buffer saline (see table 5)	100 ml
H ₂ O	900 ml
Tween	0.5 ml

Table 5. 10x Tris Buffer Saline.

Ingredients For (1L)	Amount
Tris base	24.5 g
Sodium chloride	87.65 g
H ₂ O	900 ml

Table 6. 1xTris/Glycine/SDS Buffer (Running Buffer).

Ingredients	Amount
Tris base	30 g
Glycine	144 g
SDS	10 g
H ₂ O	1L

Table 7. 1xTris/Glycine Buffer (Transfer Buffer).

Ingredients For (1L)	Amount
H ₂ O	700ml
10x Transfer buffer	100ml
Methanol	200ml

Table 8. Antibodies.

Antibodies	Catalog Number
Rabbit anti-mouse β -Actin	8457S
Rabbit anti-mouse IL-1 β	12507S
Rabbit anti-mouse I κ B- α	9242S
Rabbit anti-mouse Phospho-I κ B- α	2859S
Mouse anti-mouse Caspase-1	661228
Anti-Rabbit-HRP secondary	111-035-144
Anti-mouse-HRP secondary	HAF007

Table 9. Primer Sequences.

Primers	Sequence
Actin forward	GGCTGTATTCCCCTCCATCG
Actin reverse	CCAGTTGGTAACAATGCCATGT
IL-1 β forward	GACCTTCCAGGATGAGGACA
IL-1 β reverse	AGCTCATATGGGTCCGACAG
TNF- α forward	CATCTTCTCAAAATTCGAGTGACAA
TNF- α reverse	TGGGAGTAGACAAGGTACAACCC
IL-6 forward	TCCAGTTGCCTTCTTGGGAC
IL-6 reverse	GTACTCCAGAAGACCAGAGG
M1forward	TGAGTCTTCTAACCGAGGTC
M1 reverse	GGTCTTGTCTTTAGCCATTCC
NP forward	CTCGTCGCTTATGACAAAGAAG
NP reverse	AGATCATCATGTGAGTCAGAC

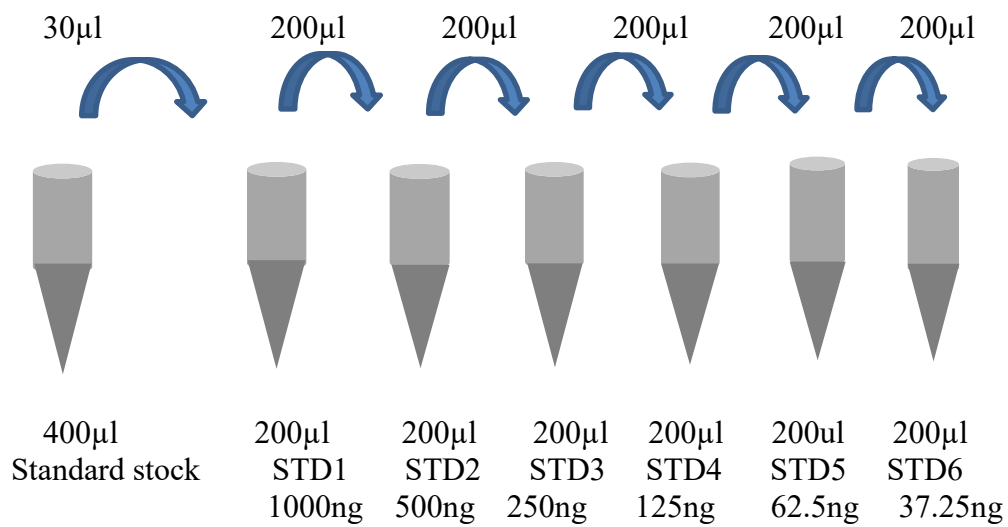


Figure 1. Generating the Standard Curve for ELISA Test

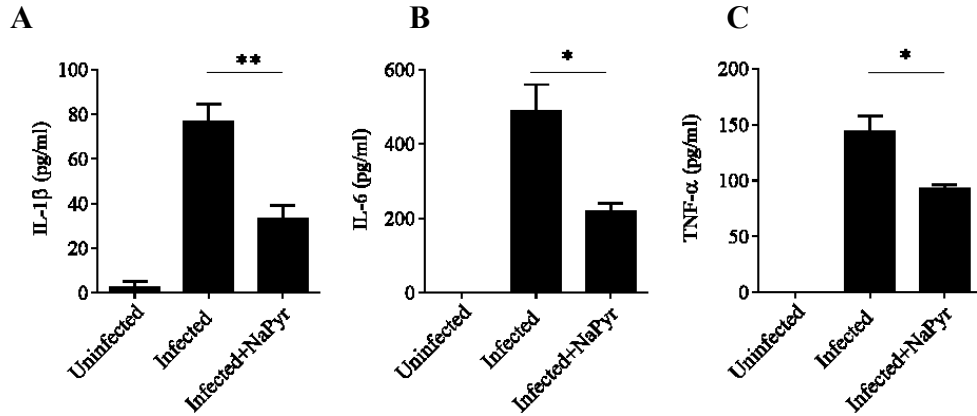


Figure 2. Sodium Pyruvate Affects Cytokine Production During IAV Infection. Samples that treated with sodium pyruvate recorded a low level of cytokines production in figure (A) IL-1 β = 30<80 pg/ml comparing with samples infected with flu virus without NaPyr. In figure (B) samples treated with NaPyr recorded 200 pg/ml IL-6 cytokines < 500 control samples. Figure (C) showed that samples treated with NaPyr recorded 90pg/ml TNF- α <150 pg/ml for control samples.

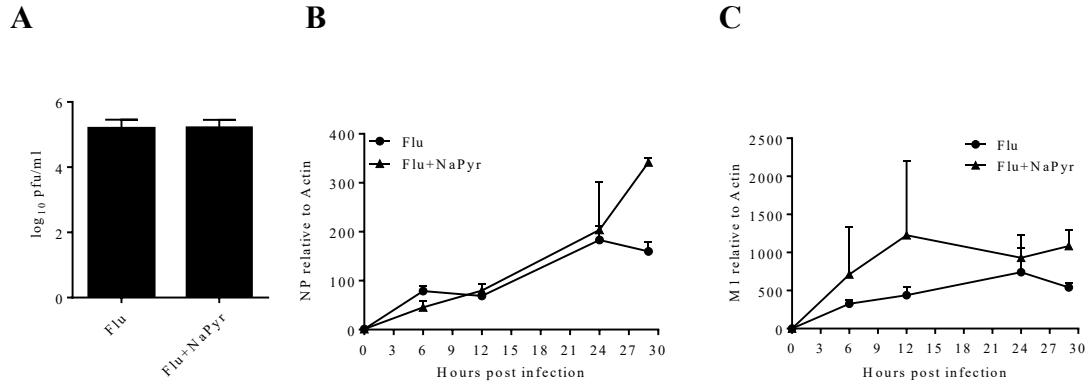


Figure 3. Sodium Pyruvate Does Not Affect Virus Replication. **(A)** both bars show there is no difference in the virus titer in both infected macrophages with flu virus as a control and infected macrophages with flu treated with NaPyr. In **(B)** infected samples with Flu virus and treated with NaPyr recorded the highest point. **(C)** Treated samples recorded highest M1 of the virus.

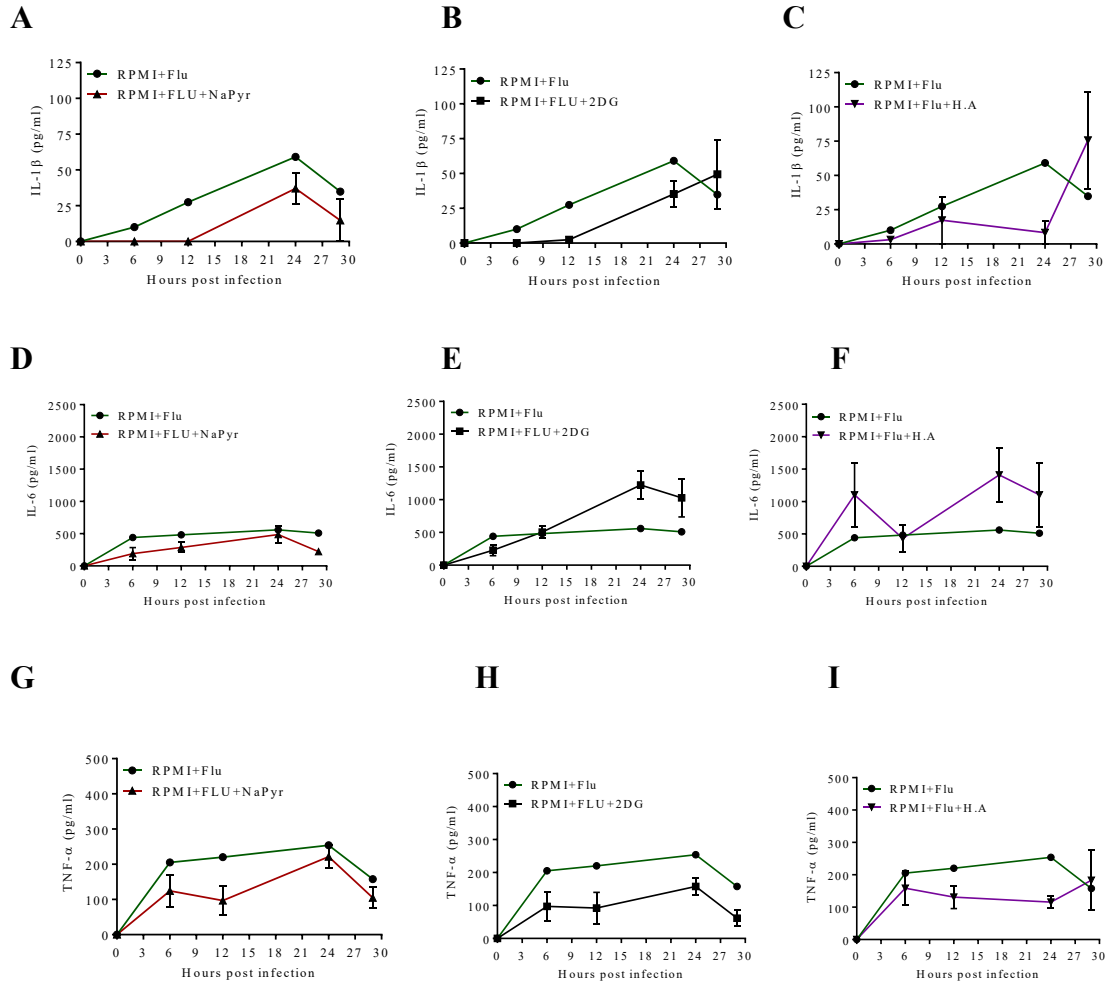


Figure 4. Inhibitors of Glycolysis Also Affect Cytokine Production. In figure (A-C) the IL-1 β production is less in infected sample treated with 2DG, NaPyr, and HA comparing to the control while IL-1 β showed increase in the cytokine production in sample treated with HA at 29 h. (D-F) showed that all treated samples have decreased the cytokines production IL-6 but not HA. (G-I). NaPyr and 2DG showed decrease in the TNF- α production < the control RPMI+Flu 200pg/ml at 6, 12, 24, and 29h. H.A showed increase cytokines > 190 at 29 h.

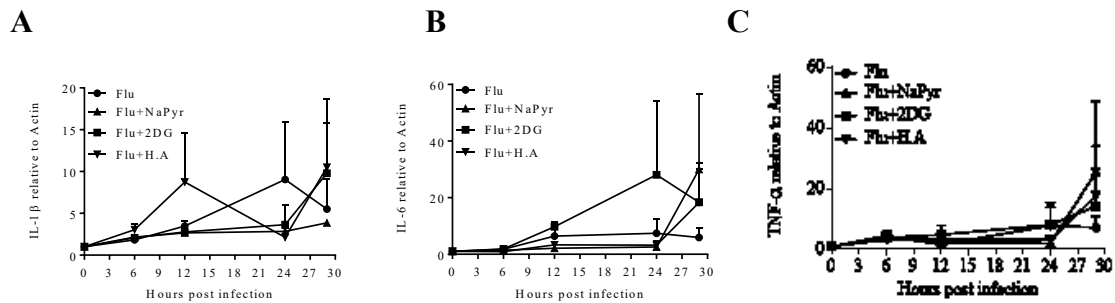
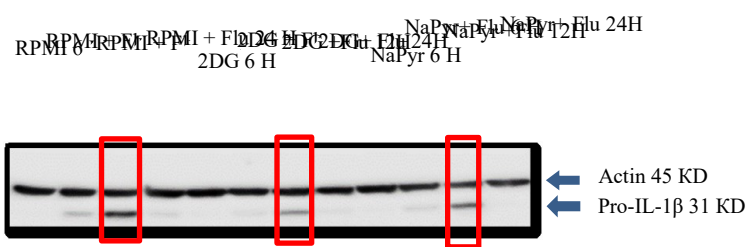
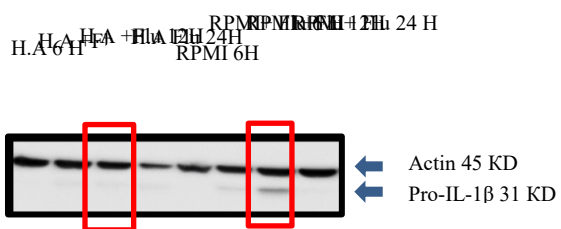


Figure 5. RT-PCR of Cytokine Gene Expression. (A-C) RNA was extracted from cells infected and treated as indicated at different times after infection (6, 12, 24, and 29h). RNA was converted to cDNA and used for RT-PCR.

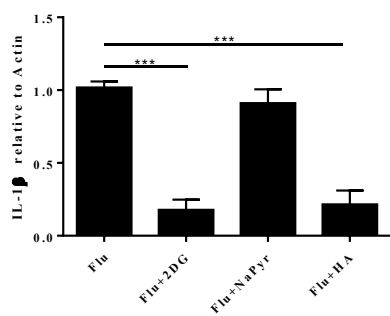
A



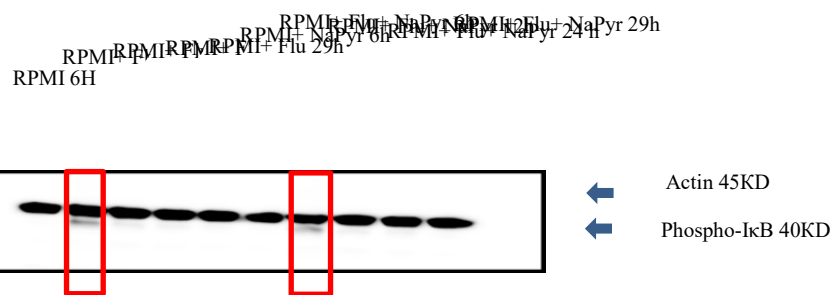
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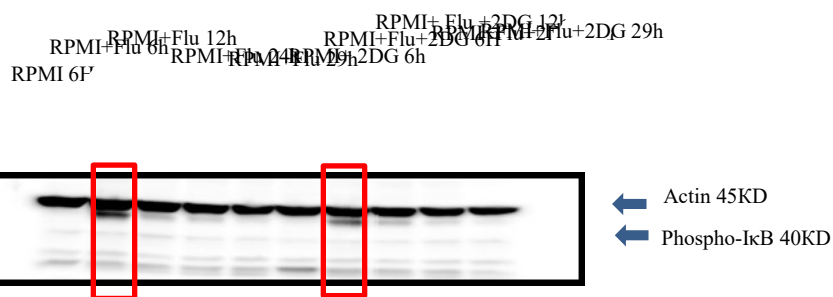
C



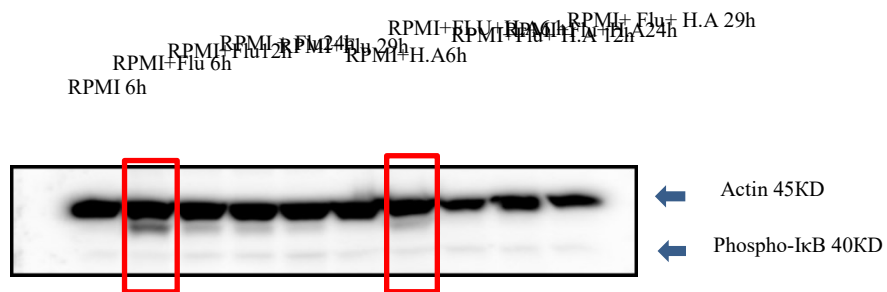
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E



F



G

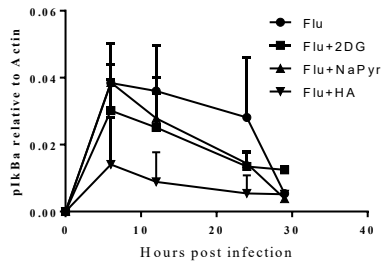


Figure 6. IL-1b Expression and Inflammatory Signaling Pathways. **6A-B** show that the production level of pro-IL-1 β with all samples treated with NaPyr, 2DG, and H.A at 12-hour samples compared to the control RPMI+ Flu at 12 -hours. **C** shows densitometry graph for pro-IL-1 β . **(6D-F)** Measuring (Phospho-I κ B) in samples treated with NaPyr, 2DG, and H.A at different time point compared with control RPMI+Flu. **G** shows Phospho-I κ B densitometry.

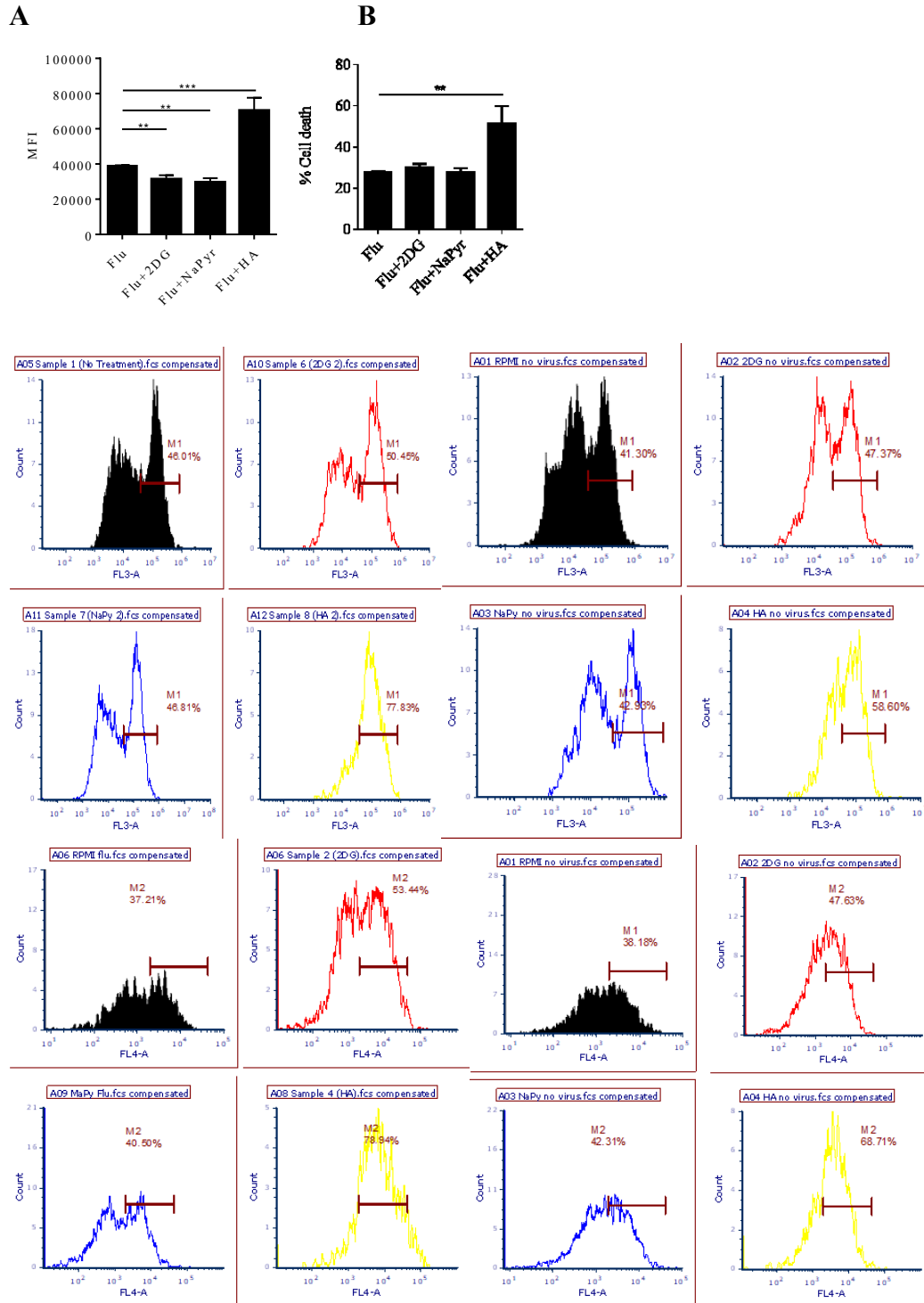
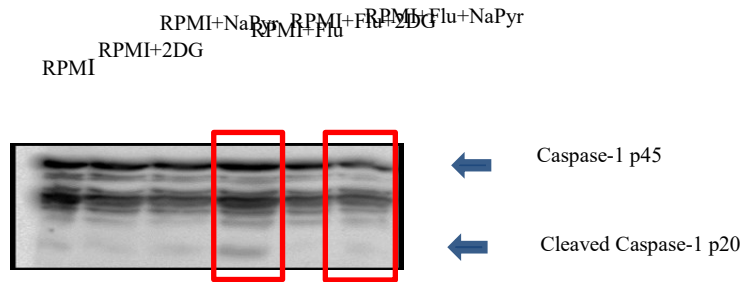


Figure 7. The Effects of NaPyr to Cell Death and Mitochondrial Damage. The graphs above showed the cell death and mitochondrial damage, and that is clearly described the lowest damage during treated infected macrophages with flu virus and treated with sodium pyruvate. While the mitochondrial damage hits to the 80000 MFI in case of using H.A and it hits to 50000 MFI in case of using 2DG.

A



B

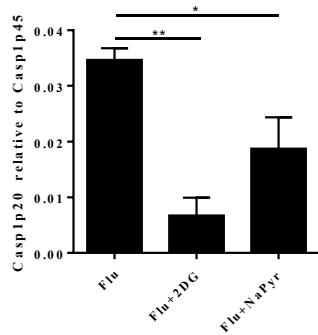
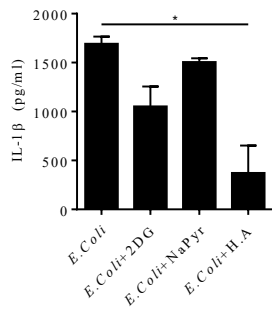
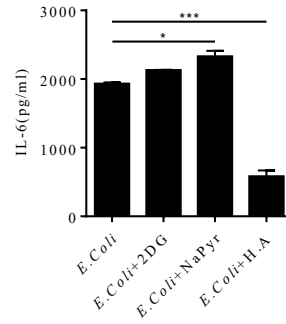


Figure 8. The effect of NaPyr on Caspase -1 Activation in Flu Infection. Shown the activation of caspase-1 in RPMI+Flu+ NaPyr samples compared with RPMI+Flu and other inhibitors. In **(B)** showed the densitometry of caspase P20 relative to caspase P45.

A



B



C

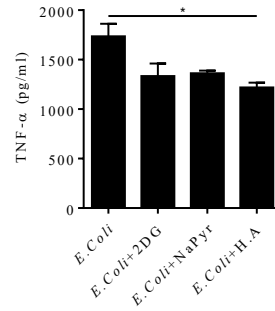


Figure 9. Effects of NaPyr on *E. Coli*. (A) recorded that control samples peak to 1600pg/ml RPMI + *E. Coli*. *E. Coli* treated with NaPyr showed 1500pg/ml. (B) showed 2000pg/ml to treated samples with NaPyr. In (C) treated samples with NaPyr recorded 1400pg/ml.

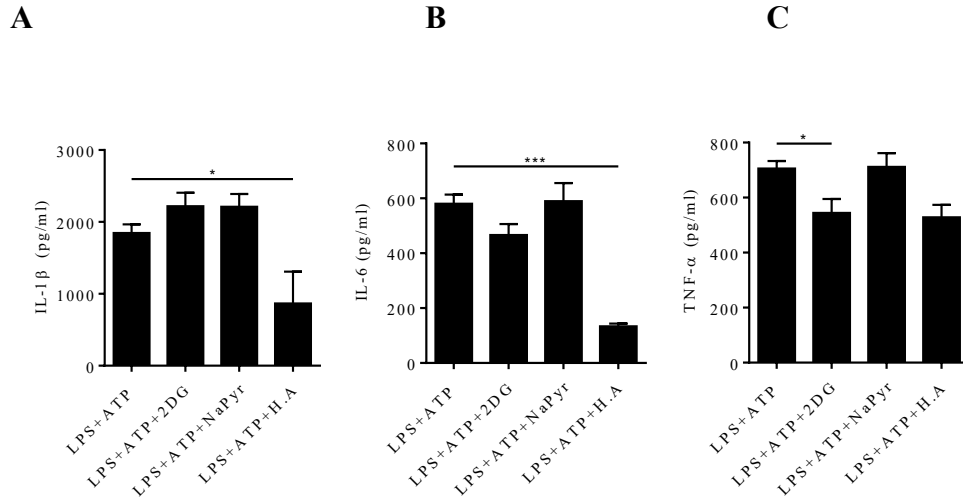


Figure 10. Effects of NaPyr on LPS+ATP. In **(A)** IL-1 β control sample RPMI+LPS+ATP recorded 1900pg/ml. In **(B)** samples treated with RPMI+LPS+ATP+2DG showed 500pg/ml IL-6 cytokines production <590 pg/ml with control samples. **(C)** showed TNF- α production samples treated with RPMI+ LPS+ATP+ NaPyr 700 pg/ml equal to control samples.

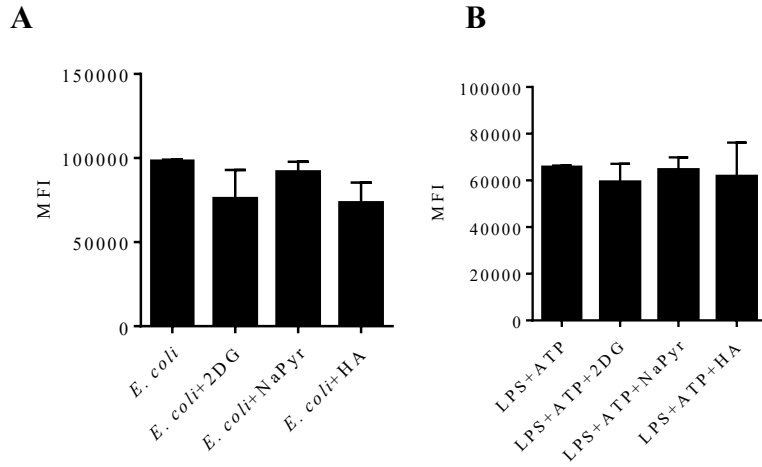
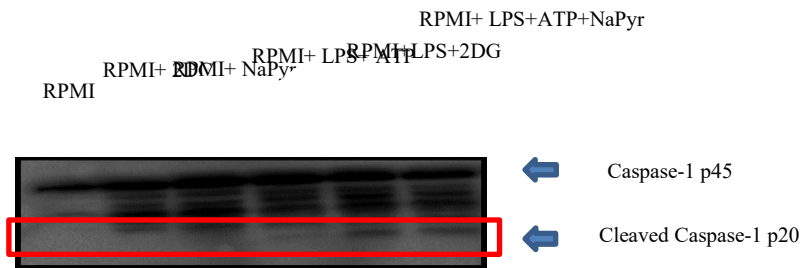
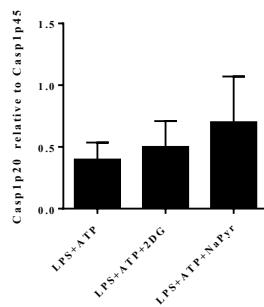


Figure 11. Effects of NaPyr on Mitochondrial Damage for Other Infections. **(A)** showed the mitochondrial damage in the control RPMI and LPS +ATP samples with different treatments. **(B)** showed the mitochondrial damage with *E.Coli* compared to the control.

A



B



C

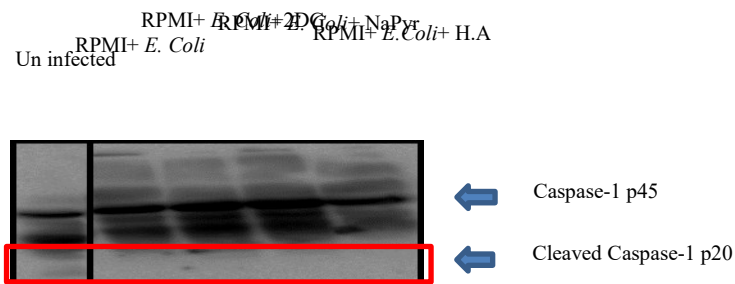


Figure 12. Testing the Effect of NaPyr on Caspase -1 Activation with Other Infection. (A) showed caspase-1 activation with infected samples with LPS. (B) densitometry for the western blot. (C) comparing treated samples with NaPyr, 2DG, and H.A in during *E. Coli* infection.