การใช้วิตามิน ซี ในความเข้มข้นสูงเพื่อเสริมฤทธิ์การฆ่าเชื้อแบคทีเรียในสัตว์ทดลอง

นางสาวจุลมาตฤ วิรภากร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560

ANTIMICROBIAL EFFECT OF HIGH DOSE ASCORBATE IN MOUSE MODEL WITH BACTERIAL INFECTION

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Medical Microbiology

(Interdisciplinary Program)

Graduate School

Chulalongkorn University

Academic Year 2017

Thesis Title	ANTIMICROBIAL EFFECT OF HIGH DOSE ASCORBATE IN MOUSE MODEL WITH BACTERIAL INFECTION
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จุลมาตฤ วิรภากร : การใช้วิตามิน ซี ในความเข้มข้นสูงเพื่อเสริมฤทธิ์การฆ่าเชื้อแบคทีเรียในสัตว์ทดลอง (ANTIMICROBIAL EFFECT OF HIGH DOSE ASCORBATE IN MOUSE MODEL WITH BACTERIAL INFECTION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. นพ. ดร. อัษฎาศ์ ลีฬหวนิชกุล, 68 หน้า.

แอสคอเบต หรือ วิตามินซี เป็นวิตามินที่มีความสำคัญต่อร่างกาย มีส่วนช่วยเสริมสร้างภูมิต้านทาน, เกี่ยวข้องกับการสร้างคอลลาเจน, เป็นสารต้านอนุมูลอิสระ, มีความสามารถในการทำลายเซลล์มะเร็ง รวมถึงมีฤทธิ์ในการฆ่าเชื้อต่างๆ โดยความสามารถทั้งหลายเหล่านี้ขึ้นกับความเข้มข้นของแอสคอเบตด้วย คุณสมบัติในการฆ่าเชื้อของแอสคอเบตมีความน่าสนใจ โดยเฉพาะการใช้แอสคอเบตที่ความเข้มข้นสูง รวมถึงการใช้คู่กับยาปฏิชีวนะ ในประเทศไทย *Staphylococcus aureus, Pseudomonas aeruginosa* และ *Acinetobacter baumannii* เป็นแบคทีเรียที่พบได้บ่อยในการก่อโรคติดเชื้อแบคทีเรียในโรงพยาบาล แบคทีเรียเหล่านี้มีการพัฒนากลไกให้ดื้อต่อยาปฏิชีวนะเพิ่มมากขึ้น และส่งผลทำให้มีความรุนแรงในการก่อโรคเพิ่มมากขึ้นด้วย ดังนั้นวัตถุประสงค์ของการศึกษานี้ เพื่อศึกษาการทำงานร่วมกันของแอสคอเบตและเซฟไตรอะโซนต่อการฆ่าเชื้อแบคทีเรียโดยวิธี time-killing จากผลการศึกษาแสดงให้เห็นว่าแอสคอเบตที่ความเข้มข้น 10 และ 40 มิลลิโมลาร์ ร่วมกับเซฟไตรอะโซน (1XMIC) มีฤทธิ์ต่อเชื้อ *S. aureus* (ATCC strain) แต่ไม่มีผลต่อ *P.aeruginosa, A. baumannii* และ *E.coli* ทั้งในหลอดทดลอง และในสัตว์ทดลองที่เกิดกล้ามเนื้ออักเสบจากเชื้อ *S. aureus* นอกจากนี้แอสคอเบตที่ความเข้มข้น 80 และ 250 มิลลิโมลาร์ มีผลโดยตรงในการลดการเจริญเติบโตของเชื้อ *S. aureus* ลงที่ 12ชม. และ 3ชม. ตามลำดับ และที่ความเข้มข้น 250 มิลลิโมลาร์ ที่24 ชม. ต่อเชื้อ *P. aeruginosa* มากกว่านั้น แอสคอเบตยังมีผลในการเพิ่มการทำงานของเซลล์แมคโครเฟจในทำลายเชื้อ *S. aureus* และ *P. aeruginosa* ด้วย โดยในทางเดียวกันนี้ฤทธิ์ในการฆ่าเชื้อของแอสคอเบตและเซฟไตรอะโซนให้ผลเช่นเดียวกับใน *S. aureus* MRSA strain จากผลสรุปได้ว่าวิตามินซีในความเข้มข้นที่สูงขึ้นมีผลช่วยลดหรือชะลอการเจริญของเชื้อลงได้ทั้งนี้ขึ้นกับชนิดของเชื้อแบคทีเรีย และอาจมีการพัฒนาเพื่อนำไปประยุกต์ใช้บรรเทาการติดเชื้อแบคทีเรียรวมถึงใช้เสริมการรักษาการติดเชื้อ *S .aureus, P. aeruginosa* และ *A. baumannii* ต่อไป

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5887119620 : MAJOR MEDICAL MICROBIOLOGY

KEYWORDS: ASCORBATE, VITAMIN C, GRAM-POSITIVE, GRAM-NEGATIVE

CHULAMARTD WIRAPAKORN: ANTIMICROBIAL EFFECT OF HIGH DOSE ASCORBATE IN MOUSE MODEL WITH BACTERIAL INFECTION. ADVISOR: ASST. PROF. ASADA LEELAHAVANICHKUL, M.D.,Ph.D., 68 pp.

Ascorbate or Vitamin C is a vitamin that is important to the body. It contributes to the immunity, involves the collagen formation, acts as an antioxidant and has the ability to destroy cancer cells and bacteria. These abilities depend on the concentration of ascorbate. Bactericidal properties of ascorbate were interesting, especially in the high concentrations. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Escherichia coli* are a common etiologies of nosocomial infection in Thailand and worldwide. These bacteria have developed mechanisms of antibiotics resistance and cause more violence in the disease. Hence, the time-killing assay was used to explore the bactericidal synergy of ascorbate in the administration with ceftriaxone, a common antibiotic. The results showed that ascorbate concentrations of 10 and 40 millimolar (mM) with ceftriaxone at the concentration of minimal inhibitory concentration (1X MIC) could inhibit the growth of *S. aureus* (ATCC strain) both in *in vitro* and *in vivo* but not effective against *P.aeruginosa*, *A. baumannii* and *E.coli*. In addition, ascorbate alone at 250mM could inhibit the growth of *S. aureus* and *P. aeruginosa*. Moreover, ascorbate 10 and 40 millimolar can increase macrophage killing activity to *S. aureus* and *P. aeruginosa*. In parallel, MRSA clinical strains were tested with the time-kill assay *in vitro* for the prediction of the *in vivo* clinical responses in *S. aureus*-induced myositis mouse model. Indeed, the synergy in the time-kill assay of ascorbate plus ceftriaxone associate with the favorable outcome in myositis model. In conclusion, ascorbate antibiotic synergy against *S. aureus*-induced myositis was predictable by the time kill assay. This method might be valuable to select the patients potentially benefit from Ascorbate-adjuvant therapy, especially, against the antibiotic-resistant bacteria.

Field of Study: Medical Microbiology Student's Signature .....

Academic Year: 2017 Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and deep appreciation to my advisor, Assistant Professor Asada Leelahavanichkul, M.D., Ph.D., Department of Microbiology, Faculty of Medicine, Chulalongkorn University for his kindness, helpful, guidance, suggestion and all supporting throughout the period of this study.

I wish sincere thanks to Uthaibhorn Singkham-in and all members in Interdisciplinary Program of Medical Microbiology for their experiment assistance.

Finally, I would like to express deeply grateful to my mother, Ms. Punsarika Wirapakorn for encouragement, understanding, and all supporting.

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LIST OF ABBREVIATIONS

Asc	Ascorbate
ATCC	American Type Culture Collection
Cef	Ceftriaxone
°C	Degree Celsius
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
Fe^{3+}	Ferric ion
Fe^{2+}	Ferrous ion
FITC	Fluorescein isothiocyanate
G	Gram
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2	Hydrogen peroxide
IM	Intramuscular
IP	Intraperitoneal injection
LPS	Lipopolysaccharide
Mg	Milligram
MIC	Minimal inhibitory concentration
ml	Milliliter
mM	Millimolar
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
NaOH	Sodium hydroxide

NSS	Normal saline solution
PBS	Phosphate buffer saline
SC	Subcutaneous
TSA	Tryptic soy agar
TSB	Tryptic soy broth
ROS	Reactive oxygen species
μM	Micromolar
μg	Microgram
O_2^-	Superoxide anion

CHAPTER I
INTRODUCTION

Ascorbate, a form of vitamin C with sodium, is a water soluble vitamin that can be obtained from food, fruit or supplements. Ascorbate involves in the production and the repair in most of the organs due to the association with the collagen synthesis process. Function of ascorbate is also associated with free radical control, collagen synthesis, basement membrane construction in epithelial and endothelial cells, bone calcium absorption and immune functions. Patients with severe scurvy, a syndrome of vitamin C deficiency, could be death by infection and bleeding (especially bleeding per gum) and the abnormal wound-healing process(1). Ascorbate could demonstrate either antioxidant or prooxidant properties depend on doses. In addition, several reported have been mentioned about the usefulness of high dose ascorbate as an adjunctive therapy in several diseases; including cancer chemotherapy, infections and immune modulation. In cancer treatment, ascorbate inhibits cell invasion, induces cancer cell apoptosis and effective cancer control when combination with anticancer drugs (2-5). It was explained that ascorbate, in a very high dose, induces the more hydrogen peroxide (H_2O_2) production (pro-oxidant) which has cytotoxic effect against the cancer cells (6). In contrast, bactericidal mechanism of ascorbate is still controversial but the previous studies shows the beneficial effect by inhibition of the growth *in vitro*, improves bacteria clearance *in vivo* and synergy with antibiotics (7-9).

Infection is the important cause of death of patients in the intensive care and is an important health care problem, especially in Thailand. Moreover, drug resistance is a growing problem in Thailand and in other several developing countries. In contrast, the rate of antibiotic discovery is more limited. Hence, the proper adjuvant therapies that improve bactericidal effect might be helpful. The combination of low price drug with the

conventional antibiotics might reduce cost of the treatment which is more suitable to the Thai economy. As mentioned previously, ascorbate in a high dose has been demonstrated beneficial effect through H2O2 production against cancer cells, we hypothesize that ascorbate in a high dose should also synergize with antibacterial drug. Then for a proof of concept, we explored the synergy of ascorbate with ceftriaxone which is the beta-lactam antibiotic that has the effect on most gram negative and some gram positive bacteria. Moreover, macrophage is one of the major phagocytic cells that important in response to foreign bodies, tissue damage or infections (10). Macrophages migrate from the bloodstream and enter into the affected tissue to recognize the foreign molecules, phagocytose them and produce immune effector molecules. Hence, we selected bacteria; *Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumanii* and *Escherichia coli* from ATCC strain to test with ascorbate-antibiotic synergy, *in vitro*, and *in vivo*, and also explore the effect of ascorbate to macrophage functions. Moreover, methicillin resistant S. aereus (MRSA) strains were used to test the effect of ascorbate against the antibiotic resistant bacteria.

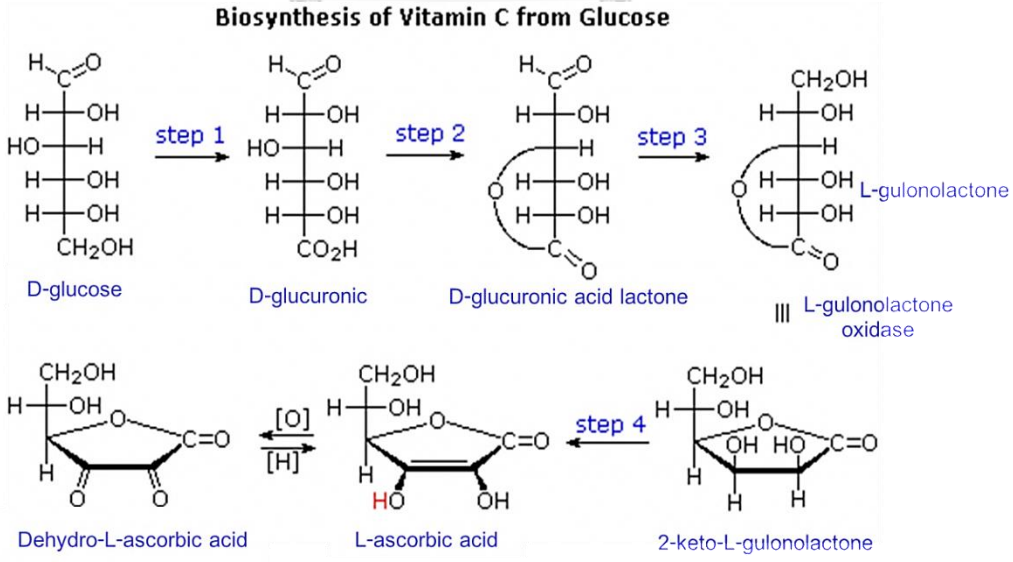
CHAPTER II
OBJECTIVE

1. To determine if ascorbate alone or in synergy with an antimicrobial drug has a bactericidal effect.
2. To determine the effect of ascorbate to improve macrophage function in term of phagocytosis and killing activity.
3. To determine if ascorbate synergized with an antimicrobial drug can attenuate bacterial infection *in vivo*.

CHAPTER III
LITERATURE REVIEW

1. ASCORBATE

Ascorbate is a sodium salt of vitamin c refer to as ascorbic acid. The molecular structure of ascorbate consists of a dibasic acid with 2, 3-enediol hydroxyl group form into a five member of lactone ring and conjugated with sodium ($C_6H_7NaO_6$). Sodium in ascorbate used as a buffer is to reduce the acidity effect of vitamin c against gastrointestinal mucosa. Ascorbate is water-soluble vitamin, unstable, easily oxidized by light, pH, temperature, enzyme and oxygen. Ascorbate is an essential nutrient for human and animals. Most animals synthesis ascorbate from D-glucose via D-glucuronic acid, L-gulonic acid, L-gulonolactone and converted to 2-keto-L-gulonolactone by L-gulonolactone oxidase. Unfortunately, human body cannot produce ascorbate on its own due to the absence of L-gulonolactone oxidase enzyme. Hence, ascorbate is one of the essential vitamin receiving from fruits and many vegetables(11, 12).



https://www2.chemistry.msu.edu/faculty/reusch/virttxtjml/carbhyd2.html

Figure 1. Biosynthesis and structure of ascorbate from glucose in animals.

Vitamin C deficiency is the cause of scurvy as demonstrated by gum swollen, bleeding per gum, muscle weakness or joint pain(13). because ascorbate has an important role in the body for the maintenance of collagen synthesis in connective tissue, bone and blood vessels. Ascorbate also important for the wound healing, an antioxidant protection and the booster of immune defenses both in innate and adaptive immunity (Fig 2). Indeed, ascorbate support several neutrophil functions including chemotaxis (by improve the migration toward the infected area), phagocytosis, killing activity (through the oxidative burst) and cell apoptosis. Typically, intracellular level of ascorbate in immune cell was 10mM by , the level that safety for the cells after oxidative burst activation (14). The *in vitro* studies showed that ascorbate associated with modulation macrophage function and several processes of T-lymphocyte and B-lymphocyte including proliferation, differentiation and maturation of immature T-cells and increase antibody production from B cell (15-17). Moreover, ascorbate reinforcement of epithelial barrier by increase differentiation and function of keratinocyte through modulation signaling and biosynthetic pathway (18).

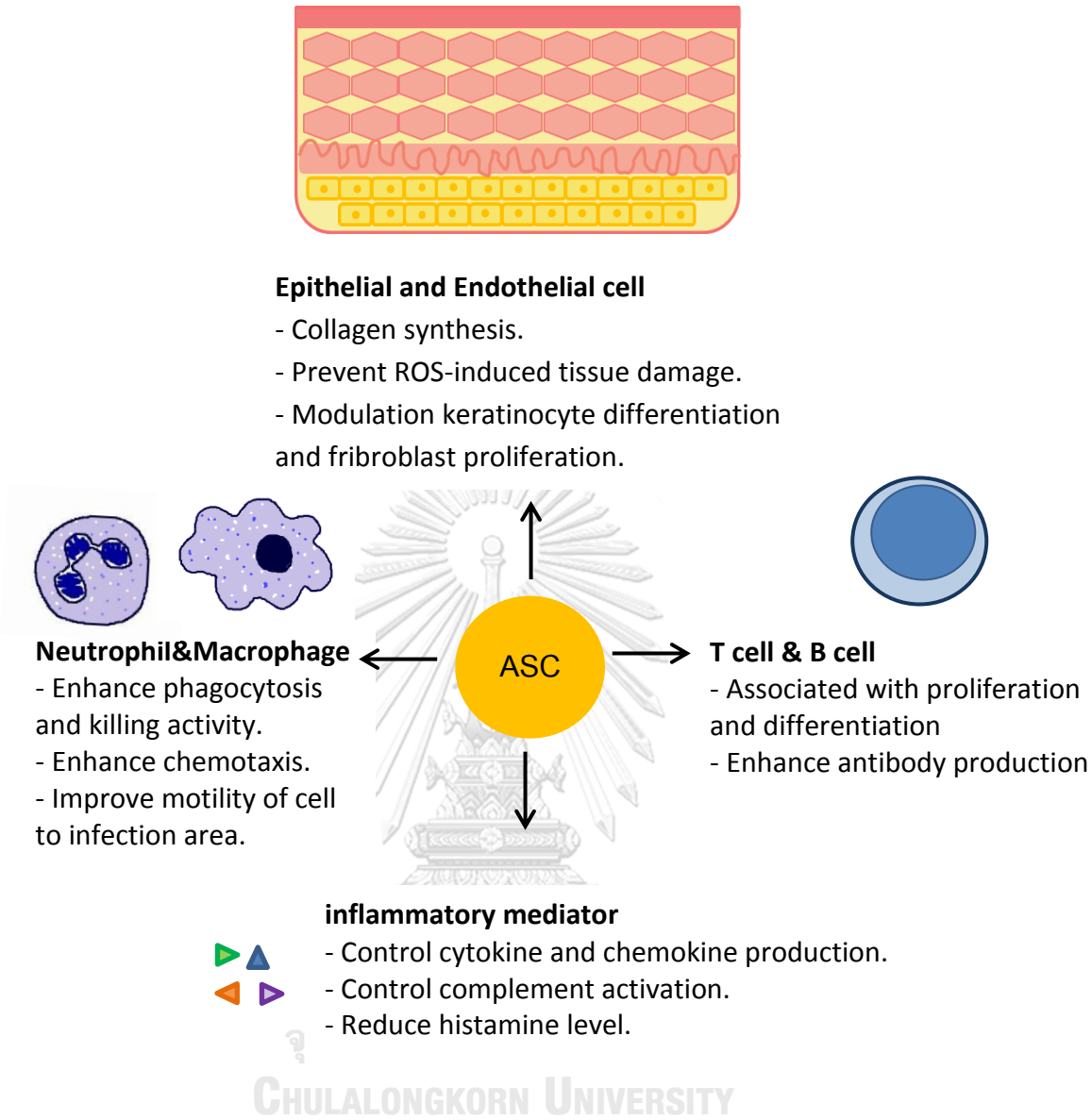


Figure 2. Function of ascorbate to immune system(19)

In general, route of ascorbate administration can be obtained through oral or intravenous. The recommended amount of oral ascorbate is 75-100 mg per day. This range is enough for the maintenance of immune function (neutrophil, monocyte, and leukocytes) and tissue restoration (13). Moreover, oral ascorbate 500mg per day might improve the function of endothelial, vasodilation and reduce blood pressure in patients with hypertension (13, 20, 21). In contrast, high dose oral ascorbate (>1g/day) required

for the treatment of scurvy could not be totally absorbed. Then scurvy treatment need the intravenous administration which leads to the ascorbate plasma concentrations of higher than 20mM after 1-2 hour of the administration (22). The ascorbate absorption from an oral administration is limited by the tight control mechanisms, reduced intestinal absorption and increased excretion from urine, that responsible for the maintenance of the ascorbate plasma level less than 100μm in the normal situation (23).

Interestingly, ascorbate demonstrated the dose-related anti-oxidant or pro-oxidant. Ascorbate in the oral doses works as an antioxidant due to the limited intestinal absorption and the urinary excretion. In antioxidant property, ascorbate is a scavenger of the free radical or reactive oxygen species (ROS). The ROS has an impact on DNA mutation, cell damage in several diseases that related with aging, heart attack, stroke and arthritis. Previous studies report that ascorbate decreases lipid peroxidation in human blood plasma that cause of diabetes or heart disease (24). In contrast, the pro-oxidant property from high doses ascorbate with intravenous administration act as the oxidizing agent that induces hydrogen peroxide (H_2O_2) production through the reduction of metal ion from Ferric ion (Fe^{3+}) to Ferrous (Fe^{2+}). The switching of Fe^{3+} into Fe^{2+} donates an electron to oxygen which form into superoxide anion (O_2^-) and dismutation to H_2O_2. H_2O_2 is a potent oxidant that possesses the killing activity against several organisms and has the cytotoxic effect on cancer cells (6, 11).

The high dose ascorbate (pharmacological ascorbate; >1mM) but not the physiological ascorbate (<0.7mM), is necessary for the anti-cancer adjuvant. The previous researches compare ascorbate concentrations between the low dose at 0.1-0.7mM and the high dose at 1-6mM to test percentage viability of neuroblastoma cell. They found that low dose ascorbate are not effective against cancer cell viability but the high dose ascorbate could significantly decrease cell viability in the dose dependent

manner(25). In addition, high dose ascorbate at 3 to 20mM also effect to increase cytotoxicity of lymphoma, breast, lung, kidney, colon and melanoma cancer by decrease percent survival of cancer cell more than 50%. There was an explanation that high dose ascorbate induce cancer cell death via enhanced apoptosis.(2, 3)

Moreover, the anti-cancer synergy of ascorbate is demonstrated against several other cancer cells (5, 26, 27). Ascorbate enhances anti-cancer effect by the direct induction of cancer cell apoptosis as demonstrated in breast and colon cancer. The ovarian cancer xenografted-mice treated with ascorbate plus chemotherapy show the reduced tumor weight. Ascorbate also reduces toxicity of the chemotherapy in patient in term of the complications on neuron, bone marrow, heart, pancreas, kidneys, gastrointestinal systems and skin (4). The major mechanism of this effect is the high dose ascorbate-induced H_2O_2 production against cancer cells. Interestingly, high dose of ascorbate with the continuously intravenous injection show a good effect, safe and low toxicity in the clinical trial of cancer treatment in patients (25, 27).

The bactericidal effect of ascorbate against bacterial infection is also previously demonstrated against both gram-positive and gram-negative bacteria. The growth of *Helicobacter pylori*, a risk factor for gastric carcinoma was 90% inhibited when treated with high dose ascorbate *in vitro*. On the other hand, in the animal models of *H.pyroli* infection is also attenuated by the high dose ascorbate. They found that high dose ascorbate decreas bacterial number of *H.pyroli* in stomach(7). Besides, the previous report also demonstrates that ascorbate enhances bacterial clearance from lung and bronco alveolar lavage fluid (BALF) in a mouse model of *Pseudomonas* pneumonia(26). Moreover, Ascorbate synergizes with penicillin and tetracyclin against *S.aureus*. Ascorbate also reduces the minimal inhibitory concentration (MIC) of penicillin and tetracycline through the elimination of the resistant plasmids. The combination of high

dose ascorbate with antimicrobial drug increased antibacterial activity by reducing the burdens in ATCC strain and clinical strain of *Mycobacterium tuberculosis* (8, 9).

In addition, we are also interesting in the function of ascorbate toward the immune cell. Macrophage is one of the major phagocytic cells involving in innate and adaptive immunity that is important in the response to foreign organisms, tissue damage and chronic inflammation (10). Macrophages migrate from bloodstream and enter into the affected tissue to recognize the foreign molecules, phagocytose them and produce immune effector molecules to recruite other inflammatory cells and mediators. Previous reported that ascorbate was improve function of peritoneal macrophage in septic mouse induced by *Escherichia coli* (17). Combination of ascorbate with antibacterial increase engulfment and killing activity of macrophage via increased H_2O_2 and O_2^- production (28). The studies on ascorbate combination with antibiotic against bacterial infection are still limited.

Bacterial infections are an important cause of death especially in the patients in the intensive care unit (ICU), mostly is the nosocomial infection with antibiotic resistant bacteria. The nosocomial infection caused by several factors; infection at the site of surgery, from medical equipment such as urinary catheters, venous catheter, endotracheal tube and the long hospital stay. In Thailand, the prevalence of hospital-acquired bacteremia caused by several bacteria as following; *Staphylococcus aureus*-50%, *Pseudomonas aeruginosa*-25%, *Acinetobacter baumannii*-75%, *Klebsilla pneumonia*-66%, *Escherichia coli*-63% and *Enterococcus spp,*-62% (29). These bacteria causing skin infection by transmitting through an open wound, respiratory tract, urinary system, intestinal mucosa and directly into blood circulation. Hence, *S. aureus*, *P. aeruginosa*, *A. baumanii* and *E.coli* are the interesting bacterial target to test the bactericidal effect with ascorbate (10).

In addition, the complication of ascorbate has been reported that the over dose administration of ascorbate resulting in the deposition of oxalate crystal in the urinary system. Oxalate is produced by ascorbate metabolize leading to increasing oxalate excretion in urine. Ascorbate is converted into dehydroascorbic acid (DHA), an oxidized form of ascorbate and then absorbed via sodium-dependent vitamin c transporter (SVTC1) and glucose transport (GLUT) for the storing form in several tissue and cells. The degradation of ascorbate, DHA hydrolysed to diketogulonic acid chain (DKG), is an unstable molecule which produces oxalate as a final product which is eliminated into urine. High excretion of oxalate is a result of the increased precipitation in kidney over other organs. Moreover, oxalate can combine with calcium that is absorbed through intestine. This process causes calcium oxalate stone formation that accumulated in the renal tubular and leading to obstructive nephropathy (30). Oxalate crystal information has been founded in people who take with ascorbate in long term (more than consecutive 1 month) with high dose ascorbate administration (>1g / per day). The increased calcium oxalate in urine include be used as a biomarker demonstrating calcium oxalate crystal in renal tubule (30-33). However, calcium oxalate stone is very common among patients and could be found from other causes (34-36). Thus, these reports of ascorbate induced-calcium oxalate are still debated. It is possible that such patients might have several other factors that prone to generate calcium oxalate stone and precipitate by ascorbate administration. Interestingly, there is no report of calcium oxalate in patients with malignancy treated with high intravenous pharmacologic dose short course of ascorbate. Moreover, the high dose ascorbate in the treatment of infection is very short course of the administration which calcium oxalate crystal could not be found in kidney histology in the mouse model (37).

2. BACTERIOLOGY

Staphylococcus aureus

Staphylococcus aureus is a gram-positive, spherical shape like a grape cluster. It produces white-yellow colonies on nutrient agar and produces hemolysin for lysis red blood that caused beta-hemolysis on blood agar. Catalase positive can differentiate *S. aureus* from *Staphylococcus spp*. *S.aureus* were found as the normal flora in the skin, upper respiratory tract and mucosa. In addition, *S.aureus* is a pathogen of a wide range of infectious diseases such as skin and soft tissue infections, sepsis, endocarditis, pneumonia, osteomyelitis and food poisoning. Skin lesion is the related factors for the infections. Most patients have been infected by surgery, hospital-acquired conditions or the medical appliances. The pathogenic strain can produce virulence factors for the adhesion and the evasion host immune response with various mechanisms. Methicillin-resistant *S. aureus* (MRSA) a strain with the resistance to methicillin is a cause high morbidity and mortality rate. The pathogenesis of *S.aureus* related to virulence factors such as i) alpha-toxin, ii) enterotoxin B and iii) surface protein or microbial surface components recognizing adhesive matrix molecules (MSCRAMM such as collagen-binding protein, fibronectin-binding protein or protein A). These factors involve in the adherence and attachment of bacteria to colonize and invade to host cells and secrete exotoxin/ enzyme for the further tissue invasion and immune evasion (Table 2). Several new and expensive antibiotics such as glycopeptide drug, tigecycline, daptomycin are used with for MRSA treatment. (38, 39).

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a gram-negative, rod-shaped bacteria with blue-green color (pyocyanin pigment). It expresses the large colonies with undulate margin in Tryptic soy agar, beta-hemolysis on blood agar and produce fluorescence detected under ultraviolet light. Normally *P. aeruginosa* growth in 25-37°C and can grow at 42°C which is used for the differentiation from other *Pseudomonas spp*. They are found in the environment, soil and water, plants, animals, including normal flora in gastrointestinal of humans. *P.aeruginosa* is one of the highest rate antibiotic resistant and a most common cause of nosocomial infections in patients staying in hospital more than one week. *P. aeruginosa* used alginate such as slime layers, or biofilm for adhering to host cell, attach epithelial cell in respiratory by pili and use elastase enzyme to destroy elastic fiber of blood vessel to penetrate the host cell. Phospholipase C helps to degrade fat, lecithin to release phosphorylcholine for tissue destruction and leukocidin inhibit neutrophil and lymphocyte to evade cell immunity of host cells (Table 3). Only polymyxin and doripenem are available for multi-drug resistant *P. aeruginosa*. And it is naturally resistant against tigecycline (40, 41).

Virulence factor	Function
Microbial surface component recognizing adhesive matrix molecule (MSCRAMM)	Associated woth attachment to host tissue and immune evasion.
Protease, hyaluronatelyase, elastase and staphylokinase	Enzyme causes of tissue damage that helping in penetrate to host cell.
Protein A	As a superantigen that inhibit opsonization.
Polysaccharide microcapsule	Protect phagocytosis and killing.
Alpha-toxin	Induce cell damage and cell death by pore forming.
Toxic shock syndrome toxin-1 (TSST-1)	Superantigen causes toxic shock syndrome

Table 1. Mechanism of *Staphylococcus aureus* for adherence with host tissues and evasion immune system (38)

Virulence factor	Mechanism
Lipopolysaccharide (LPS)	Stimulation of cytokine production.
Pili	Adhesion to respiratory epithelial cells.
Flagelle	Adherence to mucous membrane and mobility.
Alginate	- Tracheal cell adhesion. - Inhibition of phagocytosis. - Inhibit the action of antibiotics and immune response.
Pyocyanin	- Impairs cillliary function. - Stimulates inflammatory response mediates tissue damage.
Lectines solubles	Inhibit cilliary function.
Exotoxine A	Inhibition of protein synthesis; produce tissue damage.
Exoenzyme S	Inhibition of protein synthesis; immunosuppressive.
Exoenzyme U	Inhibition of phagocytosis.
Rhamnolipide	Heat-labile hemolysin; disrupts lecithin-containing tissue.
Elastases (LasA+LasB)	Degradation of elastin, fibrin, interferon, complement and collagen.
Alkaline protease	Proteolysis.
Phospholipase C	Heat-labile hemolysin; mediates tissue damage.

Table 2. Mechanism of virulence factor in *Pseudomonas aeruginosa*

Acinetobacter baumanii

Acinetobacter baumanii is Gram-negative bacteria, rod-shaped coccobacilli produce the yellow white colonies on nutrient agar and tryptic soy agar with smooth, mucoid, pale color. It causes infection in several organs; skin, soft tissue, respiratory tract, urinary tract and bloodstream in immunocompromised host and critically ill patient. It could be spreading in the environment and could be contagious between patients. *A.baumanii* was a high rate in antibiotic resistance, which resisting to all kinds of antimicrobial drug since 2008. At 2017, WHO adjudge that *A.baumanii* resistant should be closely observed. The virulence factors of *A.baumanii* including; OmpA is a part of porin in outer membrane protein, Capsular polysaccharide, LPS and Phospholipase enzyme these important for the adherence and invasion in human epithelial cell. The interaction with fibronectin on epithelial cell enhance biofilm production with factor H o avoid complement-mediated killing in host serum, It also induces host cell apoptosis by releasing proapoptotic molecules, such as cytochrome c and apoptosis inducing factor into human epithelial cells (42, 43). In addition, penicillin-binding protein 7/8 (PBP7/8) and beta-lactamase PER-1 is important in the resistance against beta-lactam antibiotics. Efflux pump is one of important virulence factors that associated with resistance to almost all classes of antibiotics including aminoglycoside, tigecycline and non-fluoroquinolone antibiotics(44). The antibiotic can be used treat *A.baumanii* is only beta-lactam/beta-lactamase or imipenem with at least one of polymyxin B, colistin or tigecycline. Multidrug administration cause the expensive treatment in this infection.

Escherichia coli

Escherichia coli is a gram-negative bacteria rod shaped, classified in the coliform group, commonly founded as normal flora in intestine. It can produce vitamin k , which associated with blood clotting factor that benefit to host (45). Normally, *E. coli* are harmless with human, but some type of *E.coli* can cause intestinal infection, diarrhea, dysentery, enteritis, urinary tract infection, meningitis and sepsis. Different strains with different virulent factors including enterotoxigenic *E.coli* (ETEC), enteroinvasive *E.coli* (EIEC), enterohemorrhagic *E.coli* (EHEC), enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC) could be found. The pathogenesis are different among strains but geneally involve in capsule, flagella, LPS, fimbriae, outer membrane proteins (OMPs) for the adherance to host cell and several cell lysis toxin (hemolysin, cytolysins) for tissue invasion and immune evasion (46). The treatment depends on the strains of *E.coli*, and beta-lactam usually used as the initial management (47).

3. ANTIBIOTIC SYNERGY TEST

Synergy test is the technique to test the antimicrobial effect of antibiotic combination from two or more than two antibiotics in comparison with the single antibiotic. The result can be interpreted with i) synergy (greater effect with the combination) ii) indifference (the value of combined effect equal to each drug) iii) antagonistic effect (no benefit on the combination). Among several methods of antibiotic synergy test, only “Time-kill assay” and “Checkerboard method” could be used to determine the combination with the different concentration of each antibiotic(48).

Time-kill assay

Time-kill assay is a gold standard test for antibiotics synergy. This method is either use to test efficacy of antibiotic to kill microbe or use to test synergistic bactericidal activity of several antibiotics at the different time-points. Antibiotics combination are “synergistic effect” if the number of bacteria reduced at least 2log cycle (100CFU/ml) in comparison with the single drug incubation. If bacterial burdens decreased between 1-2 log, the interpretation will be “additive effect”. Otherwise, it will be determined as “no correlation effect” if bacteria decreased less than 1 log or increased less than 2 log. On contrary, it will be interpreted as “antagonistic effect” if the number of bacteria increase at least 2log cycle (100 CFU/ml) compared with a single antibiotic (49). The advantage of time-kill method is the simplicity of the procedure. And the limitations are time consuming and labor intensive.

Checkerboard method

Checkerboard is method to test efficacy of two antibiotics in combination in the different concentrations by serial 2-fold dilution in the axis x and y (Fig 3). MIC of each antibiotic was calculated in fractional inhibitory concentration index (FIC) (Fig 3). Synergy represents by FIC less than or equal to 0.5 (FIC $\leq$0.5). If FIC range 1 to 4 and >4 are interpreted as antagonistic effect. Checkerboard method is an easy test of several concentrations in one test. The limitation of this method are fixed time (mostly 24h) and cannot test more than two antibiotics(49).

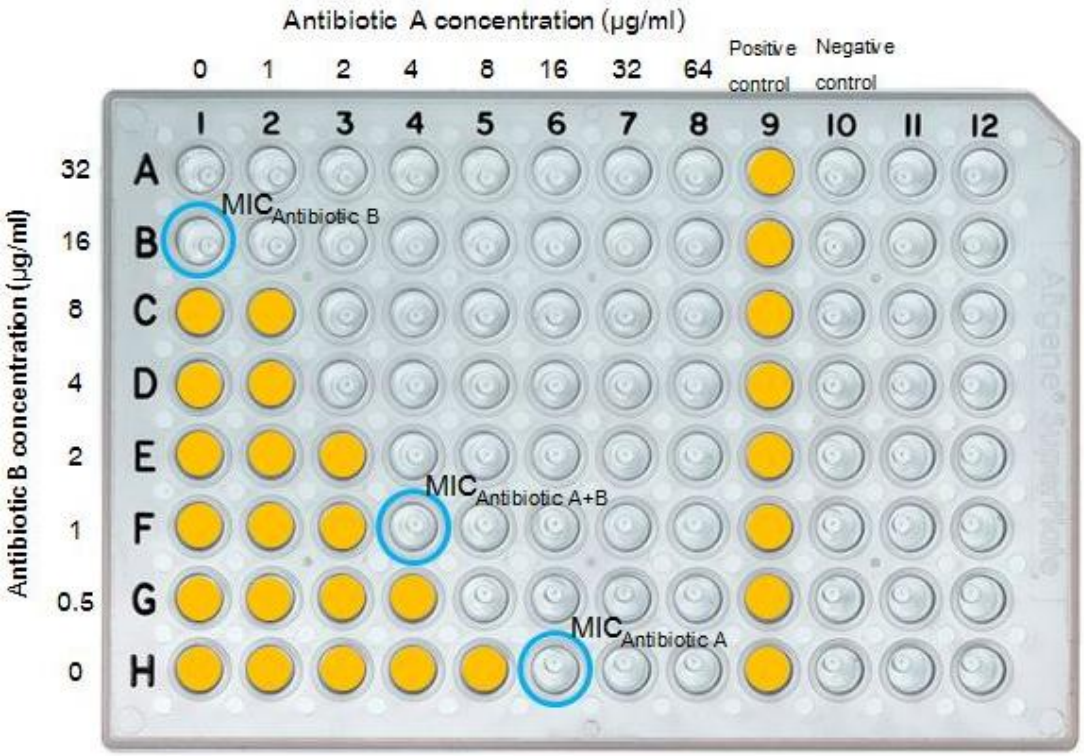


Figure 3. Choosing the MIC value of the checkerboard method

Because we would like to see the detail of the synergy in the different time point and the result of time-kill assay can detect the bactericidal activity of combined antibiotics but checkerboard can be detected only inhibition bacteria growth of antibiotics. Thus, time-kill assay was selected to use in the experiment (Table 3).

Time killing	Checkerboard
Detect the bactericidal activity by culture on agar plate	Detect inhibition bacteria growth from bacterial clear in well plate
One concentration in one test	Vary concentration in one test
Vary time point	Fixed time at 24h.
Time consuming	Time saving

Table 3. Conclusion: Time killing assay compare with Checkerboard

4. β-LACTAM ANTIBIOTIC

β-lactam, antibiotics with β-lactam ring, is the broad-spectrum antibiotics for treatment both gram-positive and gram-negative bacteria. β-lactam antibiotics include penicillins, cephalosporins, monobactams and carbapenems. The mechanism of all β-lactam group action are involve in cell wall synthesis of bacteria by inhibiting peptidoglycan that is major structure of bacteria cell wall (50, 51). Cephalosporins are β-lactam antibiotics that are divided into various generations from first generation to fifth generation. Third generation cephalosporin is effective against both gram-positive and gram-negative bacteria such as; *Staphylococci, Streptococci, E. coli, Pseudomonas aeruginosa, Enterobactericeae.* Indeed, ceftriaxone is third generation of cephalosporin that frequently used as an initial antibiotic for several hospital-acquired infections (skin infection, endocarditis, meningitis, pneumonia, urinary tract infection and sepsis) and also used for the surgical prophylaxis. Ceftriaxone could be administered via intravenous or intramuscular. Ceftriaxone inhibited cell wall synthesis by inactivate penicillin binding protein in inner membrane by prohibit chain-linkage of peptidoglycan include prevent cell wall assembly and division and inhibit transpeptidase enzyme which affected to bacteria death (52). Because of the common use of this antibiotic, we choose to test ascorbate synergy with ceftriaxone. In addition, ceftriaxone is also used in bacterial myositis in patients (53, 54).

5. THE MOUSE MODELS OF INFECTION

Infection models are divided into the local and systemic infection. The systemic infection, also known as “sepsis”, is the local infection in any organs that spread into the circulation. Thus, the model with the severe local infection will progress into the systemic

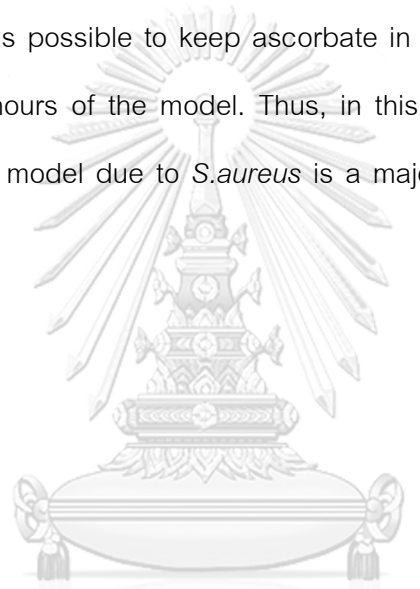
infection. However, the model representative to system infection is mentioned in the literatures as “sepsis model”. Sepsis is a condition that the body responses to infection causes tissue or organ injuries cooperated with symptoms of SIRS (Systemic inflammatory response syndrome). SIRS is characterize by High temperature(>38^{o}C), heart rate >90 beats/min, respiratory rate >20/min and white blood cell >12,000/mm^{3}. The common mouse models of sepsis consist of three models 1. LPS injection 2. cecal ligation and puncture model (CLP) and 3. bacterial instillation model (Table 4) (55). However, the disadvantage of these models in ascorbate is the limitation on the increased level of ascorbate after the intravenous injection. This because the renal funtions of these models are still intact or in mild to moderate impairment results in the rapidly decrease in ascorbate blood level after the injection.

Model	Procedure
LPS induce sepsis	Intraperitoneal injection with LPS that component of Gram-negative bacteria
Cecal ligation and puncture(CLP)	Ligate the cecum and puncture which cause diffusion of bacteria that instead of polymicrobial sepsis
Bacterial instillation	Injection with specific pathogen into the blood that instead of single pathogen

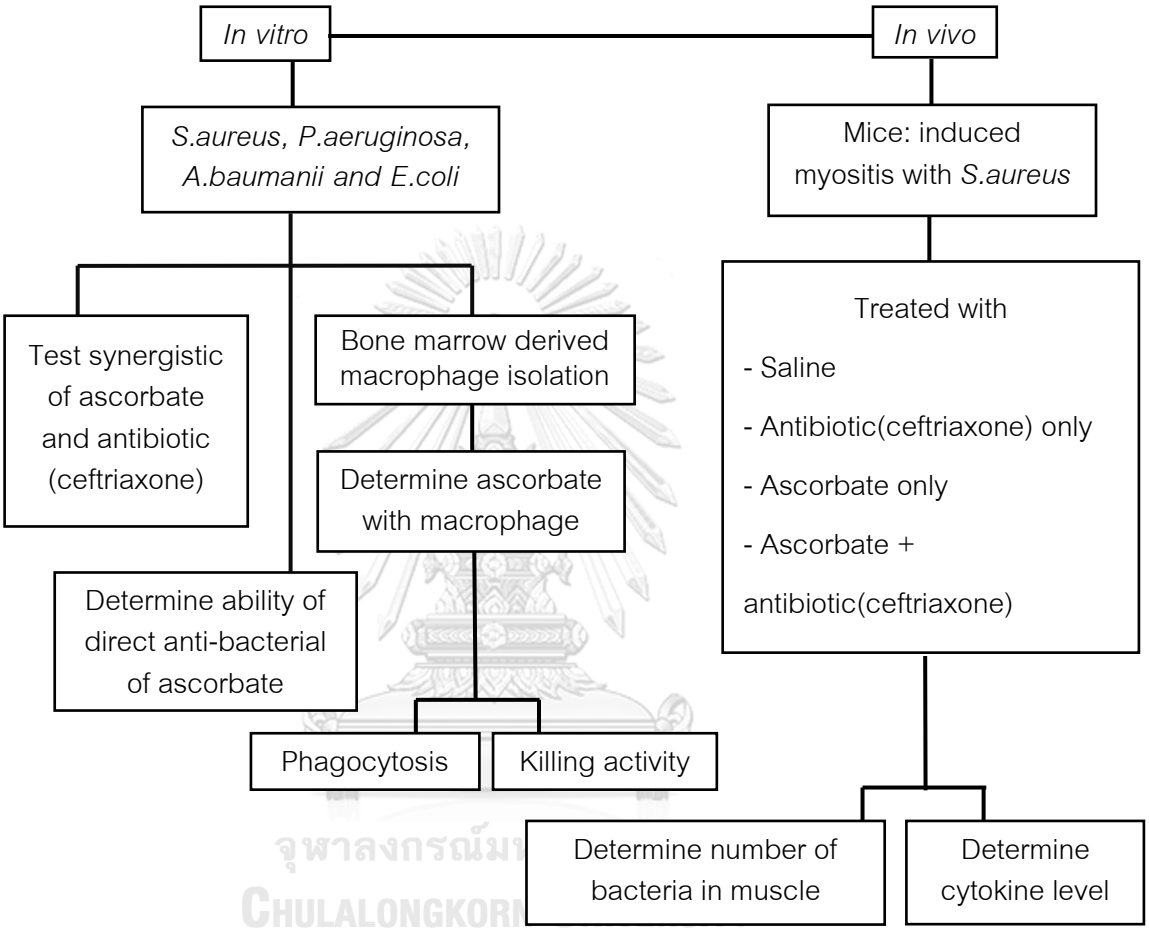
Table 4. Animal model of sepsis

On contrary, the local infection is a localized infection only in one (or some) part (s) of the body. Myositis model is one of the models of local infection. Bacterial myositis is generated by the intramuscular injection of pathogens. In patients, the etiology of

myositis originated from muscle trauma, skin infections/ injury, insect bites, contaminated needle injection, connective tissue disorders and autoimmune disease. Usually, The model is made by injection of pathogen into the leg muscle and the symptoms as characterized by rash, pain, swelling, abscess, gangrenous will be found at the infection site. The advantage of this model in the ascorbate study is the local ascorbate concentrations could be easier increased far beyond the limitation in blood concentration than the sepsis models. With the repeated injection of the very high dose of ascorbate locally, it is possible to keep ascorbate in the concentrations of the high dose effect within 12 hours of the model. Thus, in this study, we used *S. aureus* to induce mouse myositis model due to *S.aureus* is a major cause of myositis in patient (53, 54).



CHAPTER IV
METHODOLOGY



CHAPTER V
MATERIALS AND METHODS

1. BACTERIA

The strains of bacteria were derived from the American Type Culture Collection (*ATCC*) including; *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Acinetobacter baumanii* ATCC 19606. Moreover, Methicillin resistant (MRSA) strains from from the clinical isolation were used to evaluate the possibility of clinical translation. MRSA selected by biochemical test and produce the inhibition zone ≤ 21 mm in disc susceptibility test to cefoxitin (30 µg).

2. BROTH MICRODILUTION SUSCEPTIBILITY TESTING

Broth microdilution susceptibility testing was used to test the "Minimum Inhibitory Concentrations (MIC)" of ceftriaxone that performed followed standard guideline of Clinical and Laboratory Standards Institute (CLSI)(56). In short, bacteria was incubated in Mueller-Hinton broth with ceftriaxone perform 2-fold serial dilution (ceftriaxone; 0, 2, 4, 8, 16, 32, 64, 128, 256, 512µg/ml) and incubate at 37°C, overnight. Collect the MIC value of each bacteria from the lowest concentration of ceftriaxone that inhibit growth of bacteria (Table 4).

Bacteria	1XMIC of Ceftriaxone (μg/ml)
S.aureus	4μg/ml
P.aeruginosa	16μg/ml
A.baumanii	128μg/ml
E.coli	4μg/ml
S.aureus MRSA #1	512μg/ml
S.aureus MRSA #2	512μg/ml

Table 5. Minimum Inhibitory Concentrations (MIC) of ceftriaxone

3. ASCORBATE PREPARATION

Ascorbate (Sigma-Aldrich, St. Louis, MO, USA) 1M (1.98 g in 10 ml sterile water), adjusted to pH 7.3 with sodium hydroxide (NaOH) and diluted to a concentration of 10, 40, 80 and 250mM were used for the experiments. Ascorbate was prepared freshly before use in each experiment and the light and high temperature were avoid to keep ascorbate from degradation.

4. TIME KILLING ASSAY

Time killing assay was performed followed a previous study (57). As such, time killing assay was used to investigate the synergistic effect between ascorbate and Ceftriaxone (Sigma-Aldrich, St. Louis, MO, USA). Each bacterial strain (*S. aureus, P. aeruginosa, E. coli, A. baumanii*) (1×10^6 CFU/ml) was incubated with various conditions,

including control (Phosphate saline buffer (PBS)), ascorbate alone, ceftriaxone alone and ceftriaxone plus ascorbate. In ceftriaxone alone and ceftriaxone plus ascorbate, ceftriaxone at 25% of the minimal inhibitory concentration (MIC) of Ceftriaxone (0.25x) or at the MIC concentration (1x), with ascorbate (at 10, 40mM) were tested. Of note, the concentration of Ceftriaxone varies depends on the MIC of each bacterial strain. The incubation was performed at 37^{o}C in incubator shaking and the supernatant was collected at 0, 2, 4, 6, 12, 24h. Bacterial counts were determined by plating of the serial 10-fold dilutions of supernatant on the appropriate agar plates for each bacterium. Then, the plates were incubated at 37^{o}C, 24 h before the enumeration of CFUs. The procedure was repeated at least three times per bacterial strain and dose. The control of macrophages with bacteria but no ascorbate and ceftriaxone was used also.

5. ANTIBACTERIAL TEST

Antibacterial test was used to test antibacterial efficacy of ascorbate. The bactericidal activity was performed followed as previous studies (58). Bacteria was sub-cultured in Tryptic soy medium (TSB) at 37°C. Then, centrifugation at 3500 rpm, 10 min and adjust the bacteria concentration to 1x10^{6} cells/ml by measure OD600nm. Bacteria were added to TSB medium or 10, 40, 80 and 250mM ascorbate with different concentrations incubated at 37°C for 0, 2, 4, 6, 12, 24h. Then the supernatant in serial dilution was added in TSA agar plates at 37°C overnight for colony enumeration. The procedure was repeated at least three times per bacterial strain and dose. Control groups was the same dose of bacteria in TSB medium alone.

6. BONE MARROWS DERIVED MACROPHAGES

Bone marrows (BM)-derived macrophages was performed as previously protocol (59). Mice was sacrificed, collected the femurs and bone marrow cell was isolated. Cells were incubated in Dulbecco's Modified Eagle Medium (DMEM) media (high glucose DMEM supplement with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% sodium pyruvate, 5% horse serum (HyClone™ donor horse serum, Thermo Scientific, Waltham, MA, USA) and 20% L929-conditional media) in 5% CO_2 incubator at 37°C for 7 days. Then, cells were harvested by using cold Phosphate buffer saline (PBS). Confirmation of macrophage phenotype with anti-F4/80 and anti-CD11b antibodies (BioLegend, CA, USA) by flow cytometry was performed.

7. FLUORESCENT LABELED BACTERIA AND PHAGOCYTOSIS

Fluorescent labeled bacteria and phagocytosis was performed as previously described(60). After bacteria growth Tryptic soy broth (TSB), bacteria was harvested by centrifuge, washed the pellet with PBS and bacteria were heated killed at 56°C for 60 min in water bath. Then, adjust the bacteria concentration to $1x10^9$cell/ml by measure OD600nm. Bacteria was labeled with 500µg of fluorescein isothiocyanate (FITC) (Sigma-Aldrich, St. Louis, MO, USA) for 60 min. After incubation time, the solution was centrifuged and washed with PBS until no yellow color of FITC.

For phagocytosis, BM-derived macrophages $1x10^5$ cell/well will co-cultured with ascorbate concentrations (10 and 40mM) and heat-killed bacteria (final ratios macrophage to bacteria 1:500, 1:1000) in final volume 200 µl and incubated for various time points. Then, add 100µl of 0.2% trypan blue in PBS to quench extracellular

bacteria-FITC labeled for 1 min. At the end, read phagocytosis fluorescent intensity was measured at the excitation and emission wavelengths of 493nm and 520nm.

8. MACROPHAGE KILLING ACTIVITY

Macrophage killing activity was performed followed as previous studies (61). BM-derived macrophages at $1x10^3$ cells were added in 96-well plates in DMEM media and incubated overnight, old media was removed. Then, macrophage cell culture with ascorbate concentrations (10 and 40mM) and live bacteria (final ratios macrophage to bacteria 1:100000) at 37°C for 15 minutes, and then the culture supernatant was removed and washed with PBS. DMEM with 100 µg/ml of gentamicin solution was added to each well incubate in 5% CO2 incubator at 37°C for 1h. to eradicate extracellular bacteria. Subsequently culture supernatant was removed and lysed cells with lysis medium (distilled water containing 0.01% bovine serum albumin and 0.01% Tween-80) at 37°C for 10 minutes. The cells lysate were serial dilutions and plated on Tryptic soy agar (TSA) for the count the colony of bacteria.

9. ANIMALS

ICR mice, aged 12-week old (National Laboratory Animal Center, Nakornpathom, Thailand) were used. The animal protocols were approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (Permit number. 015/2560). All procedures were performed under isoflurane anesthesia.

10. *Staphylococcus aureus* MYOSITIS MODEL

Staphylococcus aureus myositis model was performed as previously protocol (62). The log-phase of *S.aureus* was centrifuged and washed the pellet with normal saline. Myositis induction was done by the intramuscular (IM) injection into the upper thigh with 0.1ml containing $3x10^9$ CFU of *S.aureus* suspensions. Mice was divided into four groups. Total 32 mice (8 mice per group);

- Group 1: No treatment as a control (bacteria + NSS).
- Group 2: Ceftriaxone only (intraperitoneal ,IP), once after *S.aureus* injection.
- Group 3: Ascorbate(50mg/kg) only (subcutaneous, SC), every 2h.
- Group 4: Antibiotic with ascorbate(50mg/kg)

After *S.aureus* injected, Group 1 and 3 intrperitoneal administered with normal saline and group 2 and 4 administered with ceftriaxone. Afterthat, subcutaneous at site of *S.aureus* injection asministered with ascorbate in group 3, 4 and treated every 2h. All groups was observed every 2 hours until 8 hours mice was sacrificed by isoflurane with cardiac puncture to collect blood. Blood was centrifuged (13,000 g for 5 min) and serum was stored on -80°C until use. Excise kidney to determine kidney damage from ascorbate treatment and muscle tissues from the inoculation site for bacterial counting (size; 100 mg) by homogenized, serial diluted with PBS and plated on Tryptic soy agar (TSA) for determining colony forming unit. The experiment made under biosafety cabinet class II with personel protective equipment (PPE). The waste put in red bag for autoclave, the sharp instrument put in the special provided box at the operative field and the sacrificed or death mice put in the bag and keep in provided 4°C refrigerator at room 301 of animal unit.

Intra-facility contaminate protection of *Staphylococcus aureus* MRSA. We followed by universal precaution principle by preparation *S. aureus* MRSA in biosafety

cabinet II at Aor Por Ror building, 17 floor and transpot by keep in closed container. Then, Mice were injection with S.aureus MRSA there are keep in individual ventillation cage (IVC) cabinet which decrease the contamination to other mice in the facility. In addition, two normal mice was keep in IVC cabinet for the test of the infection contamination.

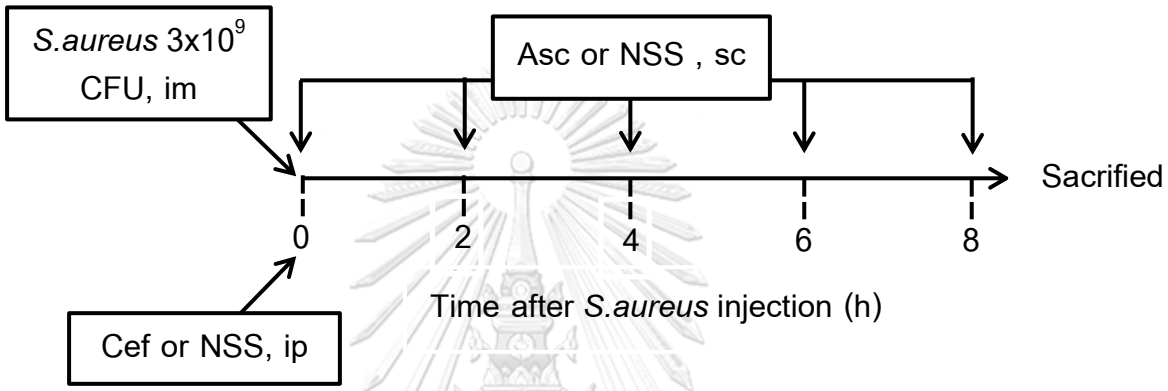


Figure 4. Plan of *Staphylococcus aureus* myositis mouse model. *Staphylococcus aureus* was upper intramuscular injected and then ceftriaxone or normal saline was administered in intraperitoneal immediately. Following, the subcutaneous (at site of *S.aureus* injection) administered with ascorbate (Asc) or normal saline (NSS). At 8 h, mice were sacrificed and collected for muscle tissue, blood, and kidney.

11. ORGAN HISTOLOGY

Organ histology was performed follow previous protocol(37). Fixed kidney tissue with 10% formalin before embedded in paraffin and 4um sections were stained with periodic acid-Schiff reagent. Kidney damage was identify by tubular epithelial swelling, loss of brush border, and vacuolar degeneration. The range of kidney damage was estimated at x200 magnification using 10 randomly selected fields, according to the following criteria: 0, normal; 1, area of damage 25% of tubules; 2, damage involving 25–

50% of tubules; 3, damage involving 50–75% of tubules; and 4, 75–100% of the area being affected.

12. SERUM CYTOKINE

Serum cytokine (TNF-α, IL-6 and IL-10) was measured by sandwich ELISAs assays (eBioscience, USA) followed the manufacturer's instructions. Coat plate with 100µl of Capture antibody and incubate on 4°C overnight. Then wash with 250µl Wash buffer (PBS and 0.05% tween20). Plate was block with 200µl of 1XELISA diluent, incubate 1h. at room temperature and wash at least one. Add 100µl top standard of antibody TNF-α, IL-6 and IL-10 and top standard perform 2-fold serial dilution until 8 point. Following add serum 100µl and then incubate at room temperature for 2h or 4 °C overnight. After wash with wash buffer, Add 100µl of detection antibody incubate 1h at room temperature. Wash and add 100µl of Avidin-HRP 30 minutes at room temperature. In the end, wash 5 time and add 100µl of substrate 15 minutes. After incubation add 50µl of stop solution. Finally, read fluorescent intensity was measured at the excitation and emission wavelengths of 450nm and 570nm.

13. STATISTICS ANALYSIS

The differences between the data or group was determined by unpaired student *t*-test or one-way analysis of variance (ANOVA) with Turkey's comparison test in the comparison of 2 and 3 groups, respectively. The differences at the $p < 0.05$ level were considered as statistically significance (SPSS 11.5 software) (SPSS Inc., Chicago, IL, USA).

CHAPTER VI
RESULT

Bactericidal synergy between ascorbate and ceftriaxone demonstrated by time-kill assay

We tested the synergy with antibacteria due to the synergy of ascorbate with anticancer and antifungal are mentioned previously (37, 63). Synergistic effect against *S.aureus, P. aeruginosa, A. baumanii* and *E. Coli* at each time-point was demonstrated by time-kill assay between different concentrations of ascorbate (10 and 40 mM) and ceftriaxone at 0.25 and 1-fold of MIC (0.25x MIC and 1x MIC), respectively. We test the synergy with time-kill because we would like to see the detail of the synergy in the different time point and summarize of the synergistic effect at the result at 24h time-point. The positive synergy was reported when the number of bacteria reduced at least 2log cycle (100 CFU/ml) compared with ceftriaxone alone followed guideline of National Committee for Clinical Laboratory Standards. Control groups of PBS and ascorbate alone was not effective for all bacteria (fig 5, 6, 7, and 8). Ceftriaxone 0.25x MIC concentration was lower than MIC so ceftriaxone 0.25x MIC alone does not affect to *S.aureus, P. aeruginosa, A. baumanii* and *E. Coli*. The result of ascorbate with ceftriaxone was not effective against *S. aureus*, *A, baumanii* and *E.coli* (fig 5A, 7A, and 8A). In addition, ascorbate at 10 and 40 mM (plus 0.25x MIC ceftriaxone) reduced bacteria at 3-12 h of incubation only in *P. aeruginosa* (fig 6A) but not at 24h. Hence, we concluded that ascorbate with 0.25x MIC not synergistic effect (fig 6A) because the standard interpretation determined the result at 24h. In contrast, at 1x MIC of ceftriaxone, ascorbate 10 and 40mM was showing significant bactericidal, against *S. aureus,* when synergy with ceftriaxone at 24 h of the incubation (fig 5B) but not effect to the other bacteria (fig 6B, 7B, and 8B).

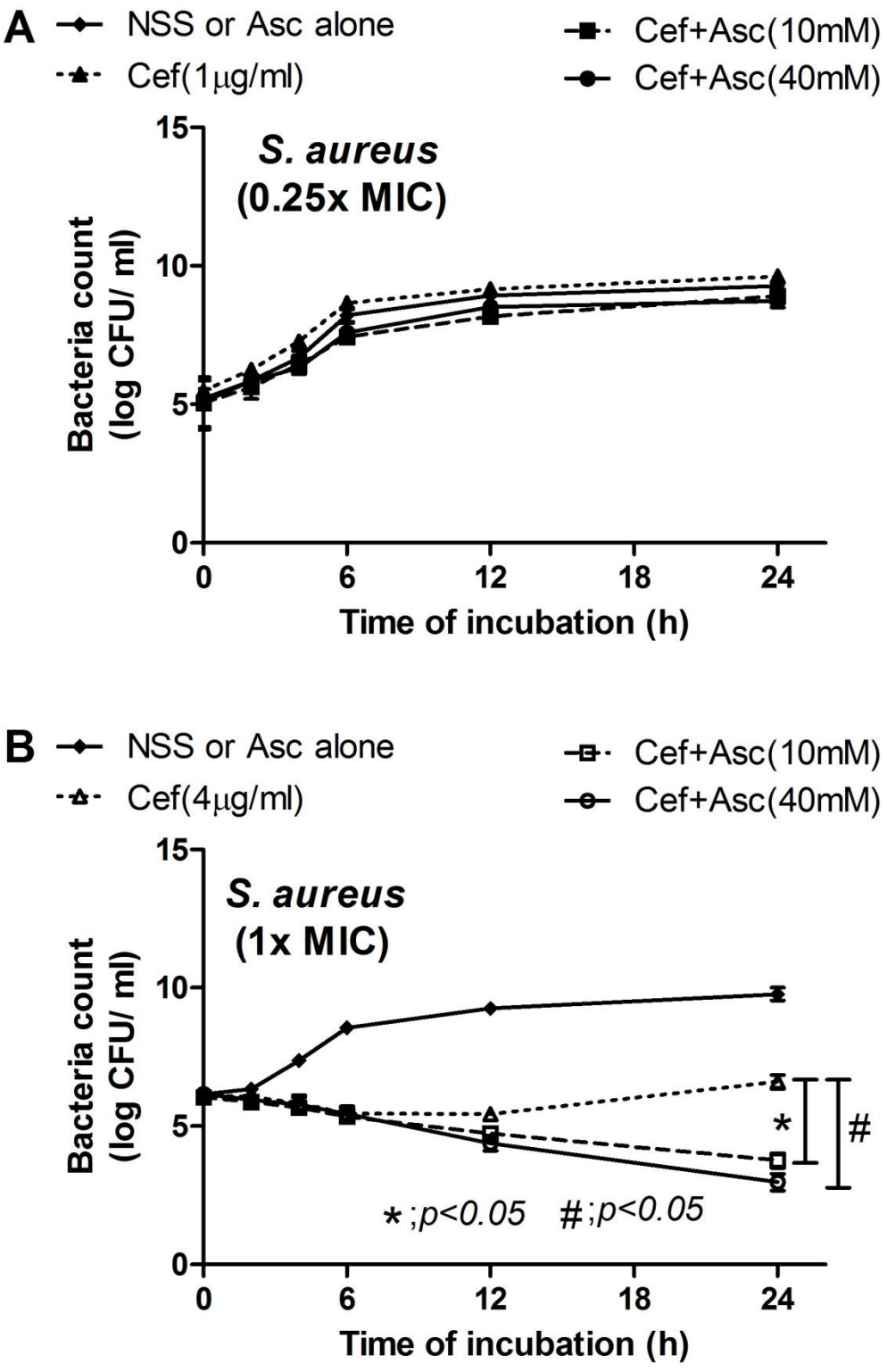


Figure 5. The *in vitro* test for Ascorbate-Ceftriaxone synergy by time kill assay with Ceftriaxone concentration at 0.25 fold of minimal inhibitory concentration (0.25x MIC) (A) and at equal to MIC (1x MIC) (B) against *Staphylococcus aureus*.

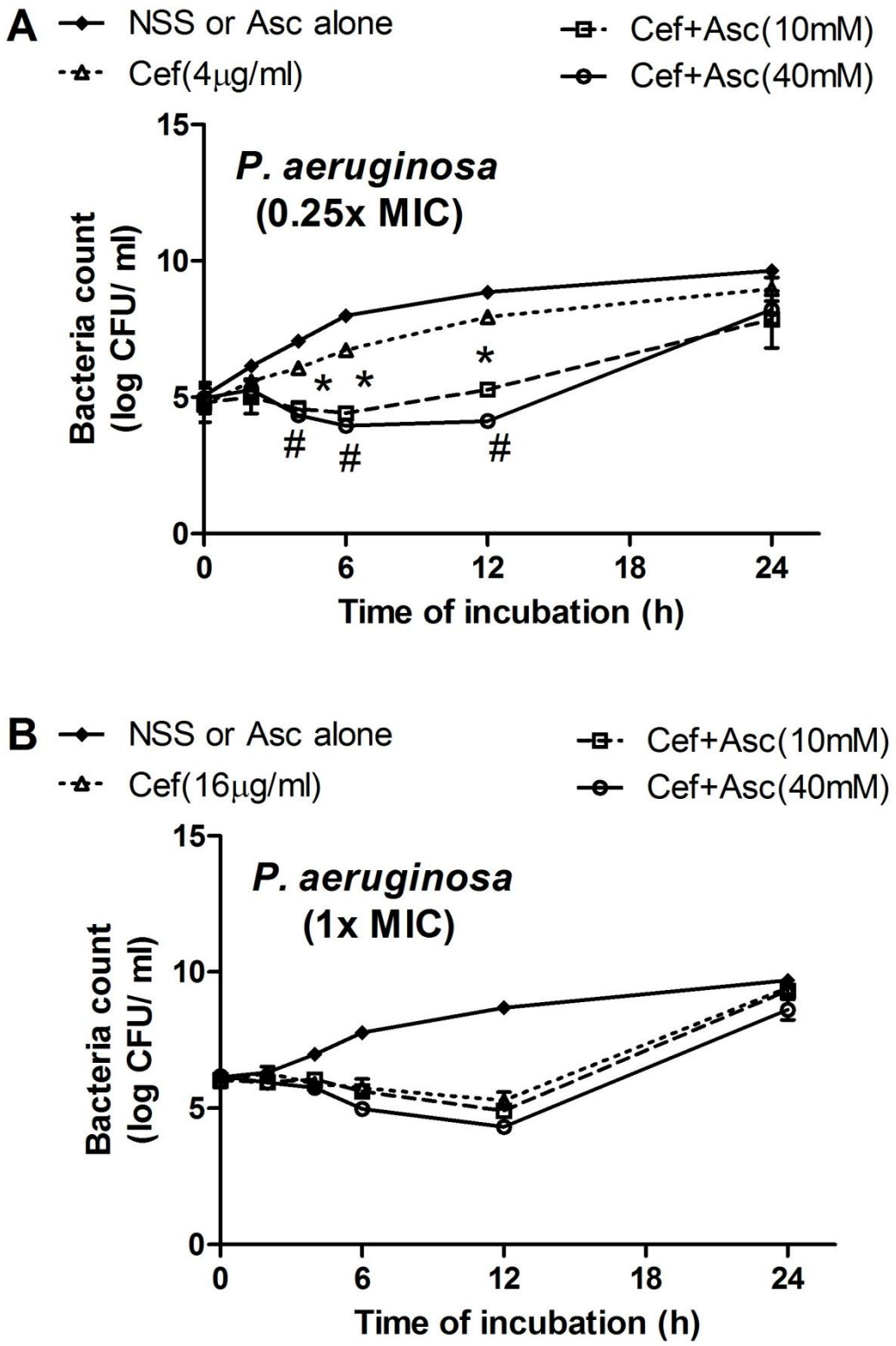


Figure 6. The *in vitro* test for Ascorbate-Ceftriaxone synergy by time kill assay with Ceftriaxone concentration at 0.25 fold of minimal inhibitory concentration (0.25x MIC) (A) and at equal to MIC (1x MIC) (B) against *Pseudomonas aeruginosa*.

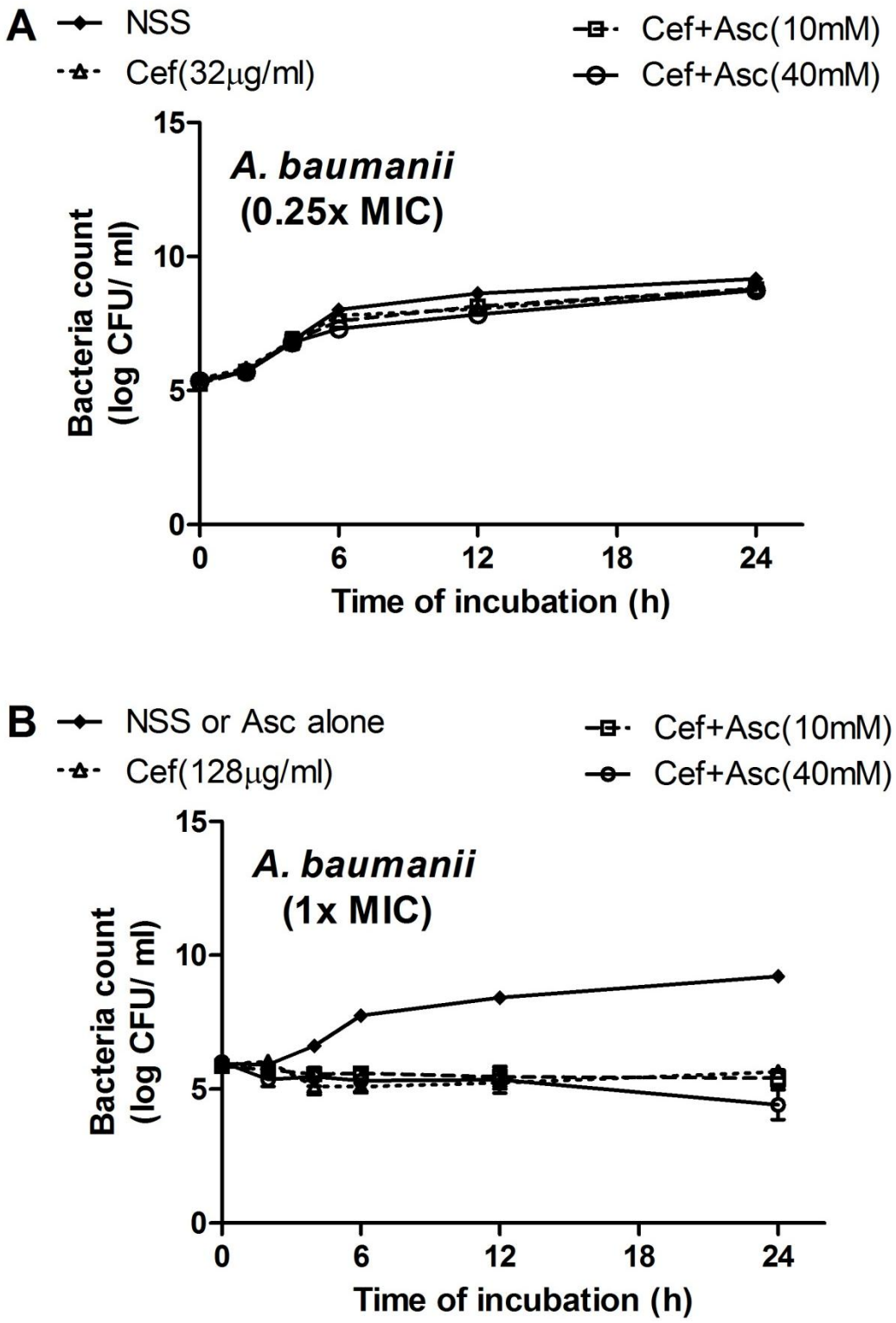


Figure 7. The *in vitro* test for Ascorbate-Ceftriaxone synergy by time kill assay with Ceftriaxone concentration at 0.25 fold of minimal inhibitory concentration (0.25x MIC) (A) and at equal to MIC (1x MIC) (B) against *Acinetobacter baumanii*.

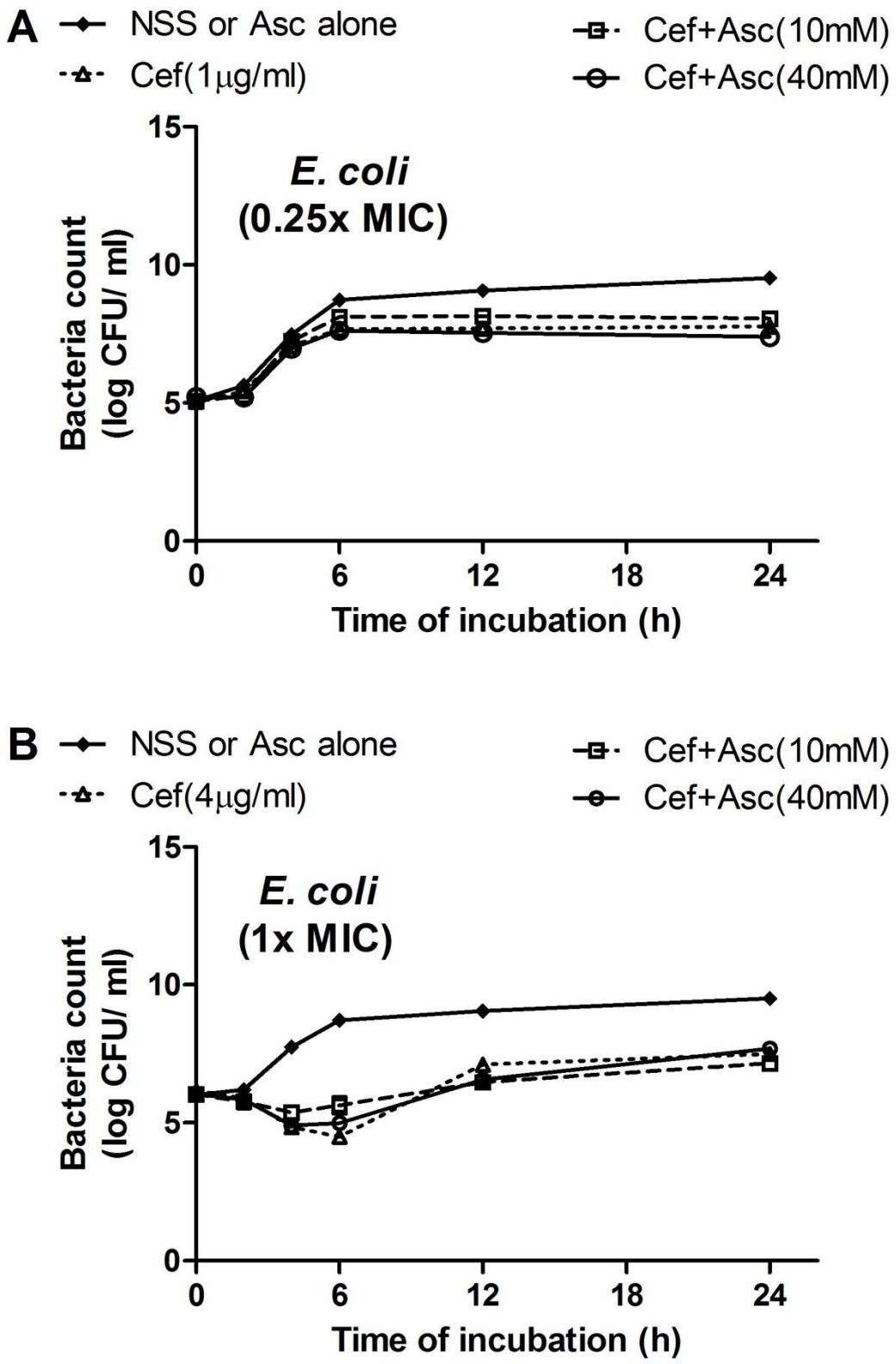


Figure 8. The *in vitro* test for Ascorbate-Ceftriaxone synergy by time kill assay with Ceftriaxone concentration at 0.25 fold of minimal inhibitory concentration (0.25x MIC) (A) and at equal to MIC (1x MIC) (B) against *Escherichia coli*.

Antibacterial efficacy of different concentration of ascorbate

Although antibacterial efficacy of ascorbate alone at 10 and 40mM was not affected to all of bacteria (fig 9A, 9B, 10A and 10B), the higher concentration might show some effects. Thus, we used ascorbate in the concentrations that too high to use in patients in the real situation. Because the highest blood concentration in clinical trial in patients are 10-40 mM, we select the concentration at 80 and 250 mM as a hypothetical concentrations to test. Indeed, bactericidal effect of ascorbate in the high concentrations (80 and 250 mM) was demonstrated only in *S. aureus* at 4h and 6h of incubation, respectively (fig 9A). Ascorbate alone at these doses at 24h incubation also significantly decrease bacteria burden of *P. aeruginosa* (fig 9B), but not in *A. baumanii* and *E. coli* (fig 10). Interestingly, with ascorbate at 250mM, bactericidal effect against *S. aureus* could be demonstrated as early as 3h of the incubation (fig 9A) but the effect against *P. aeruginosa* was detectable only at 24 h (fig 9B). This implies that ascorbate in very high dose has direct bactericidal effect. And the effect of high dose ascorbate is specific to bacteria which might associated with the different structure of the bacterial cell wall.

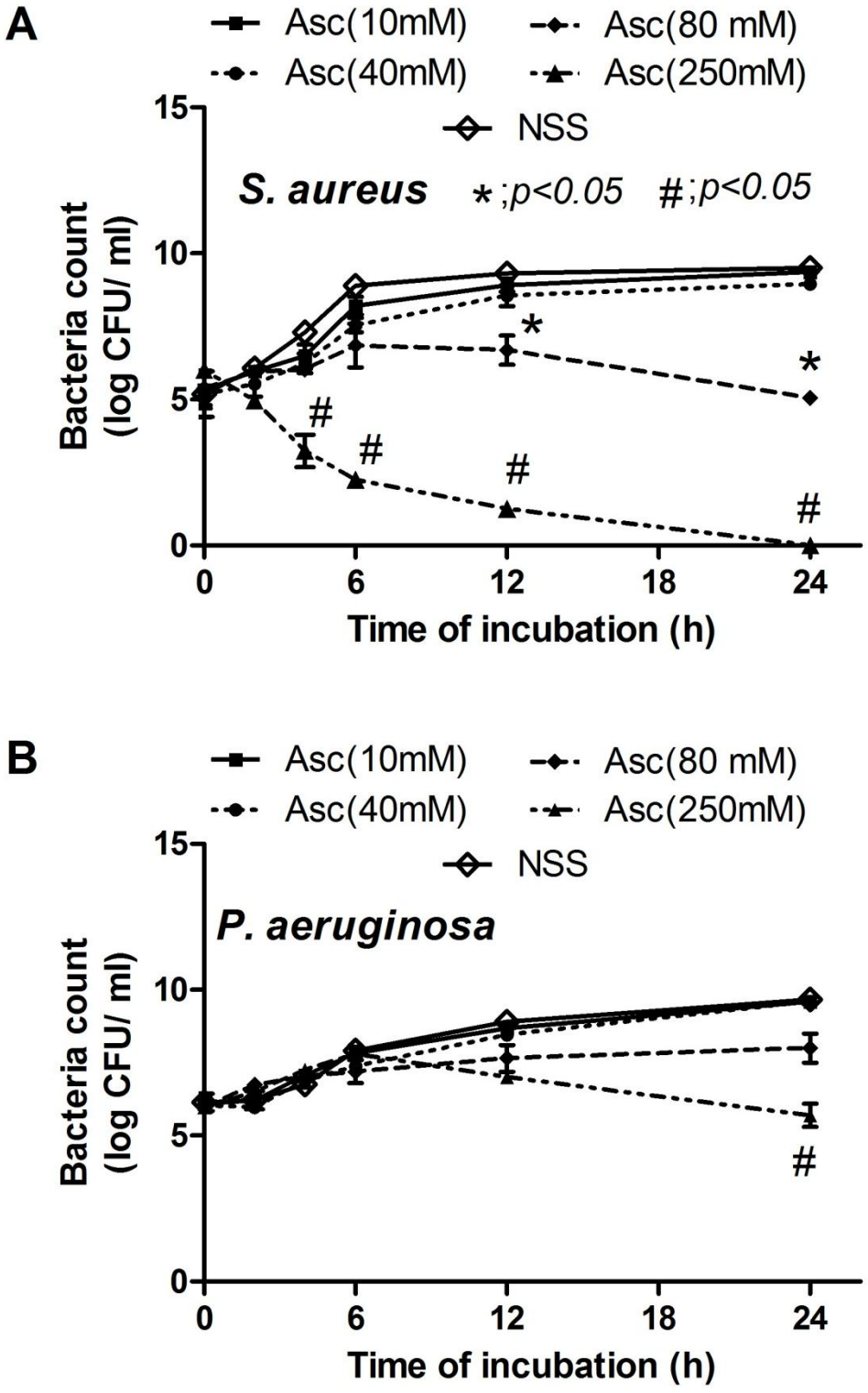


Figure 9. The *in vitro* test for ascorbate bactericidal activity in different concentrations by time kill assay against *Staphylococcus aureus* (A), *Pseudomonas aeruginosa* (B).

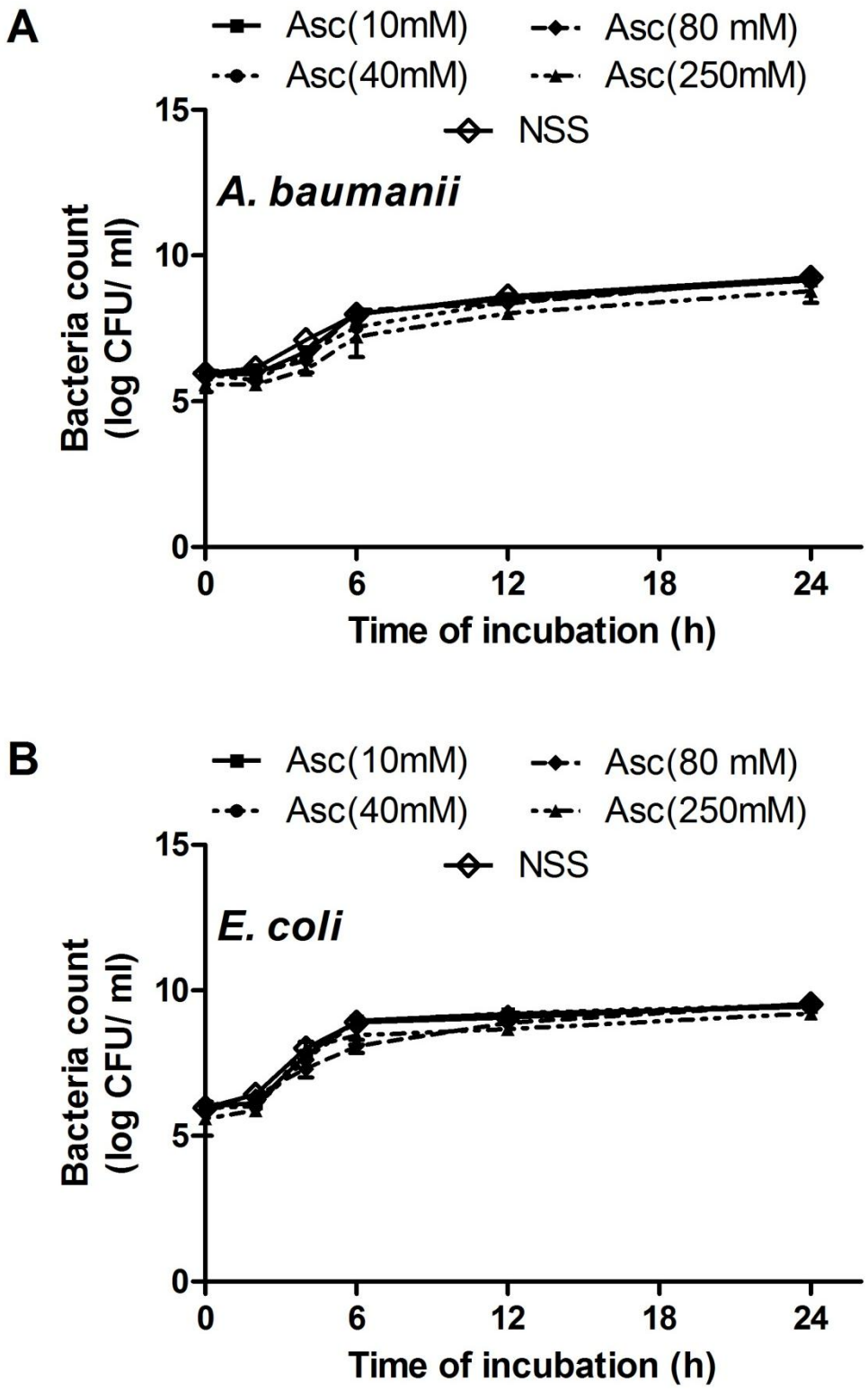


Figure 10. The *in vitro* test for ascorbate bactericidal activity in different concentrations by time kill assay against *Acinetobacter baumanii* (A), *Escherichia coli* (B).

Effect of ascorbate on phagocytosis and killing activity of macrophage

The effect of ascorbate on macrophage did not enhance phagocytosis functions against all of the bacteria ATCC strains (fig 11), different from the reports of the improved neutrophil phagocytosis of ascorbate (37, 64-66). On the other hand, ascorbate at 10 and 40mM improved macrophage killing activity against *S. aureus* and *P. aeruginosa* in the concentration dependent manner (fig 12A, C), but there is no effect on *A. baumannii* and *E. coli* (fig 12B, D). It is interesting to note that both *S. aureus* and *P. aeruginosa* were ascorbate sensitive organisms as tested by the incubation with ascorbate alone (fig 9 and 10).

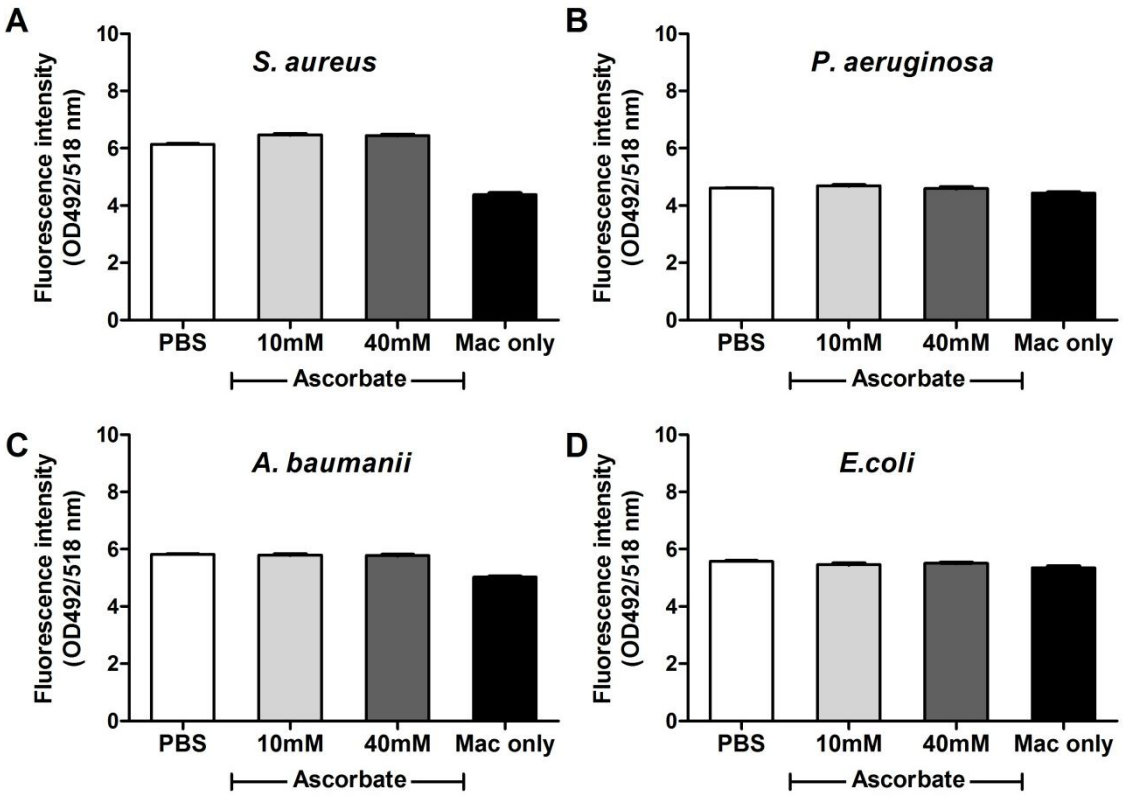


Figure 11. Macrophage phagocytosis function after incubation with PBS, ascorbate at 10 and 40mM against *Staphylococcus aureus* (A), *Pseudomonas aeruginosa* (B), *Acinetobactor baumannii* (C) and *Escherichia coli* (D) were demonstrated.

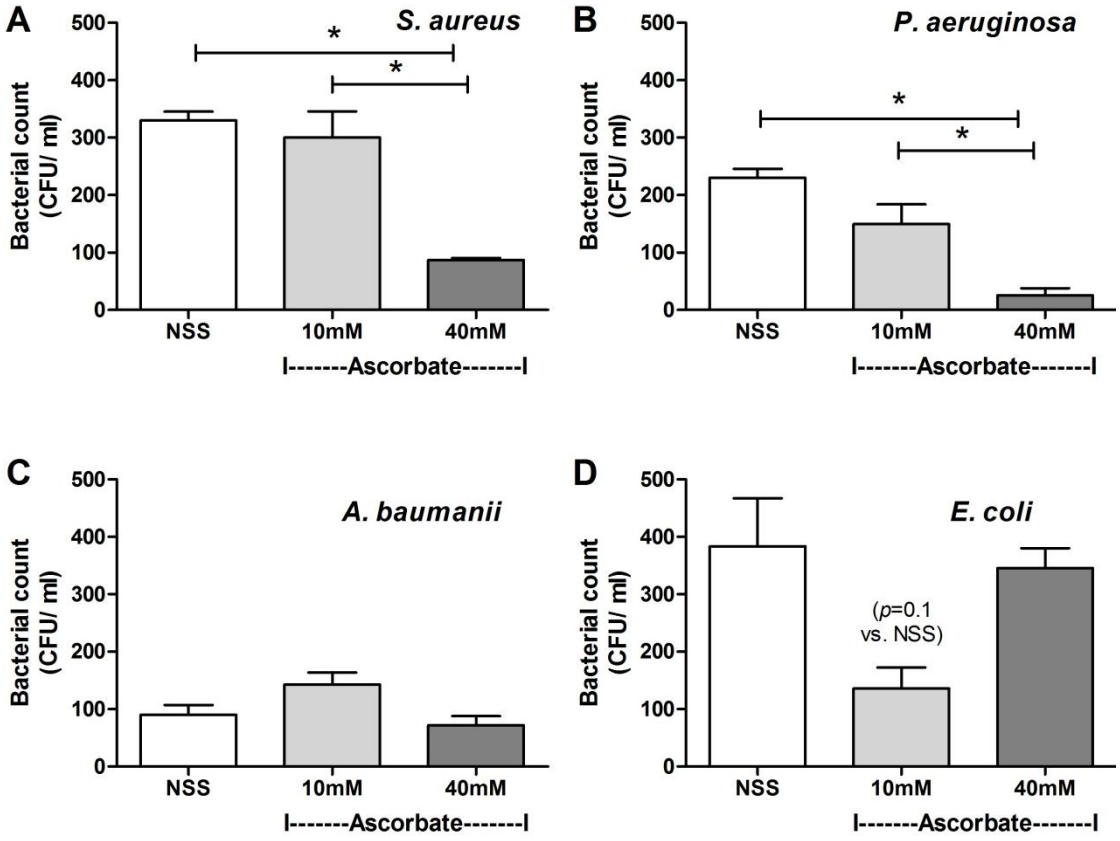


Figure 12. Macrophage killing activity after incubation with PBS, ascorbate at 10 and 40 mM against *Staphylococcus aureus* (A), *Pseudomonas aeruginosa* (B), *Acinetobactor baumannii* (C) and *Escherichia coli* (D) were demonstrated. *, $p < 0.05$

Ascorbate-Ceftriaxone bactericidal synergy against staphylococcal myositis mouse model demonstrated by bacterial count and serum cytokine

The staphylococcal myositis mouse model was performed to test the possibility of clinical translation of the *in vitro* results. Because the adequate ascorbate concentrations (> 10mM) is needed for bacterial neutralization (37), Serum ascorbate after subcutaneous injection between 10 - 40mM was achievable within approximately 1.5h and Ceftriaxone injection did not alter ascorbate level (fig 13). Because of ascorbate level >10mM for 1.5h after a single subcutaneous injection, thus the repeated administration of ascorbate subcutaneously every 2h for totally 4 doses is neccessary to maintain ascorbate level more than 10 mM for at least 8h. The mouse samples were collected at 2h after the last dose of ascorbate injection.

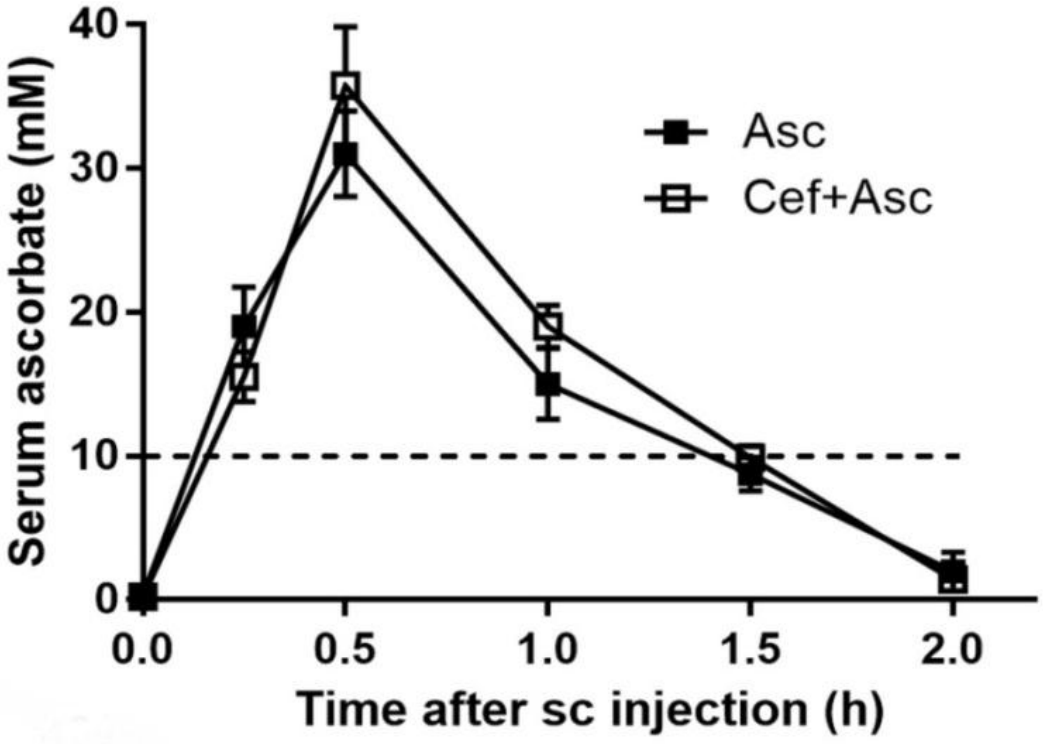


Figure 13. Serum ascorbate after a single subcutaneous injection in mice with ascorbate alone (Asc) and ascorbate with Ceftriaxone (Cef+Asc) was demonstrated.

In vivo test, myositis mice were treated with ceftriaxone or ascorbate alone showed a tendency of the reduction in bacterial number in the muscle but not significant. In contrast, with ceftriaxone plus ascorbate, there was the significant decrease in bacterial number in muscle when compared with NSS, ceftriaxone and ascorbate alone (fig 14). Serum cytokines TNF-α, IL-6 and IL-10 was also decreased with ascorbate plus ceftriaxone but not with each substance alone (fig 15A-C). In addition, high dose ascorbate was not induce renal toxicity and oxalate crystal, an important complication of ascorbate administration, could not be detected in the kidney histology (fig 16, 17 and 18).

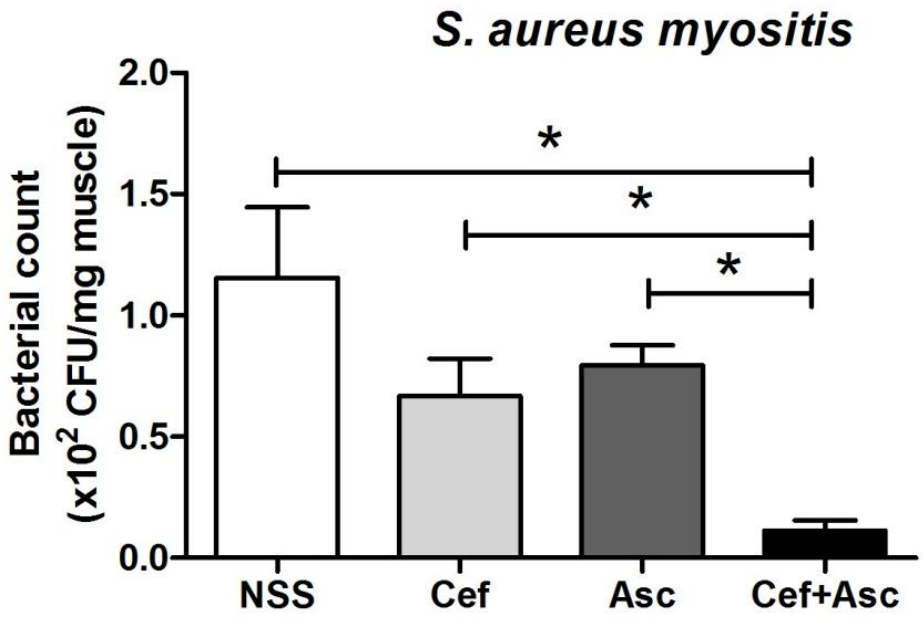


Figure 14. The *in vivo* test in *Staphylococcus* myositis mouse model administered with normal saline (NSS), Ceftriaxone alone (Cef), ascorbate alone (Asc, 50mg/kg) and Ceftriaxone plus ascorbate (Cef+Asc) as demonstrated by intramuscular bacterial count.

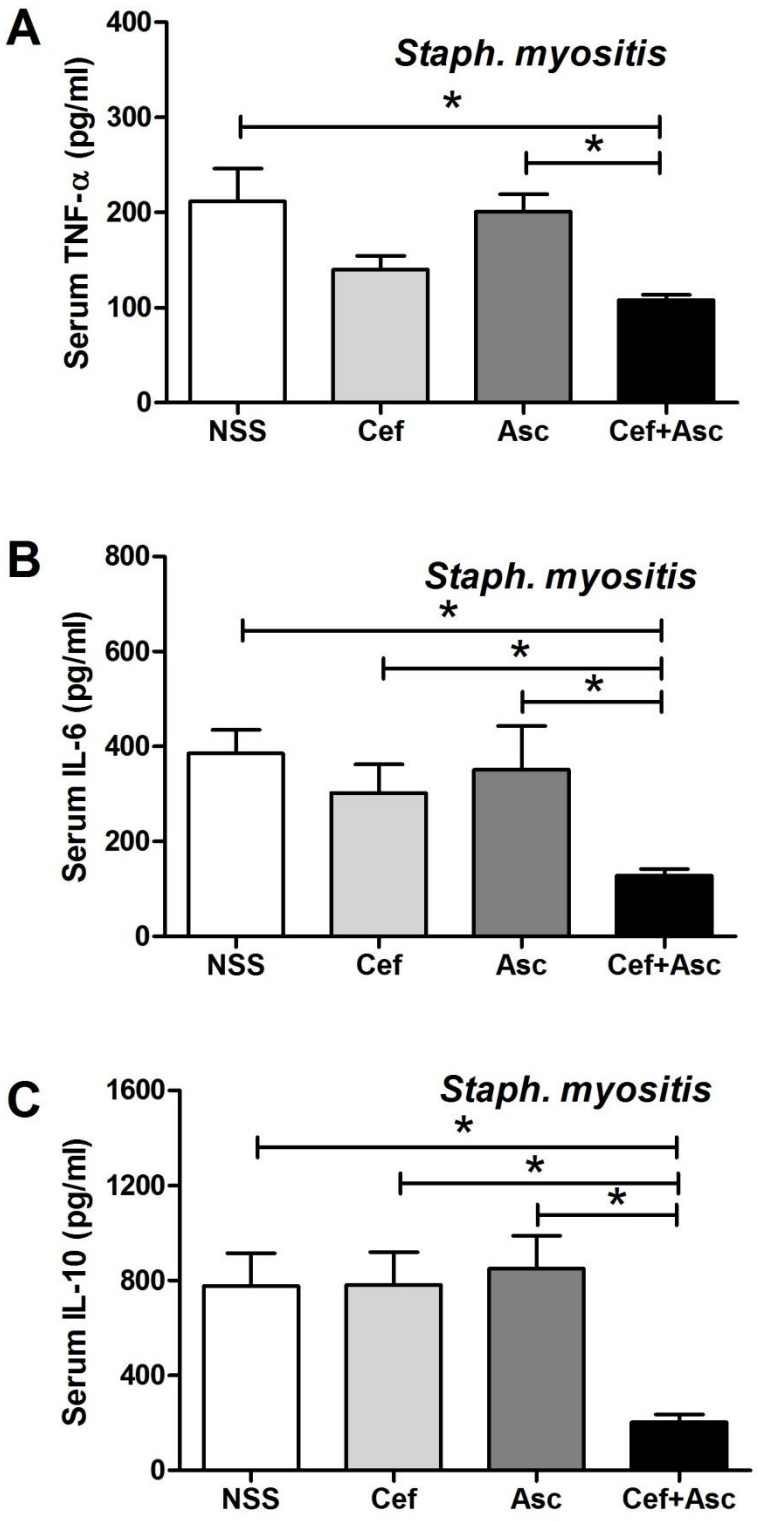


Figure 15. Serum cytokines TNF-α (A), IL-6 (B) and IL-10 (C) in *Staphylococcus* myositis mouse model administered with normal saline (NSS), Ceftriaxone alone (Cef), ascorbate alone (Asc) and Ceftriaxone plus ascorbate (Cef+Asc) were demonstrated

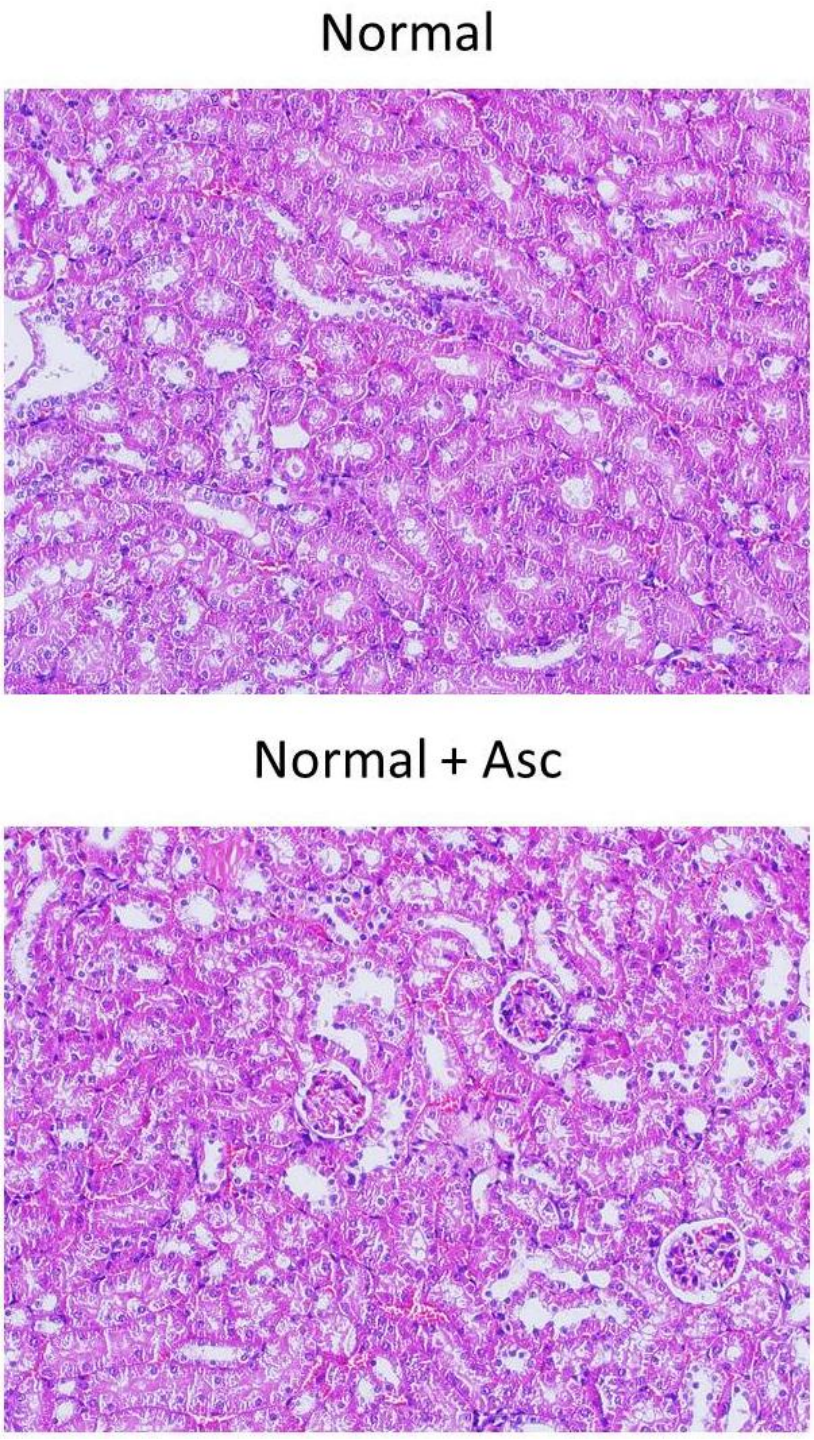


Figure 16. Kidney histology of normal mice treated with or without ascorbate

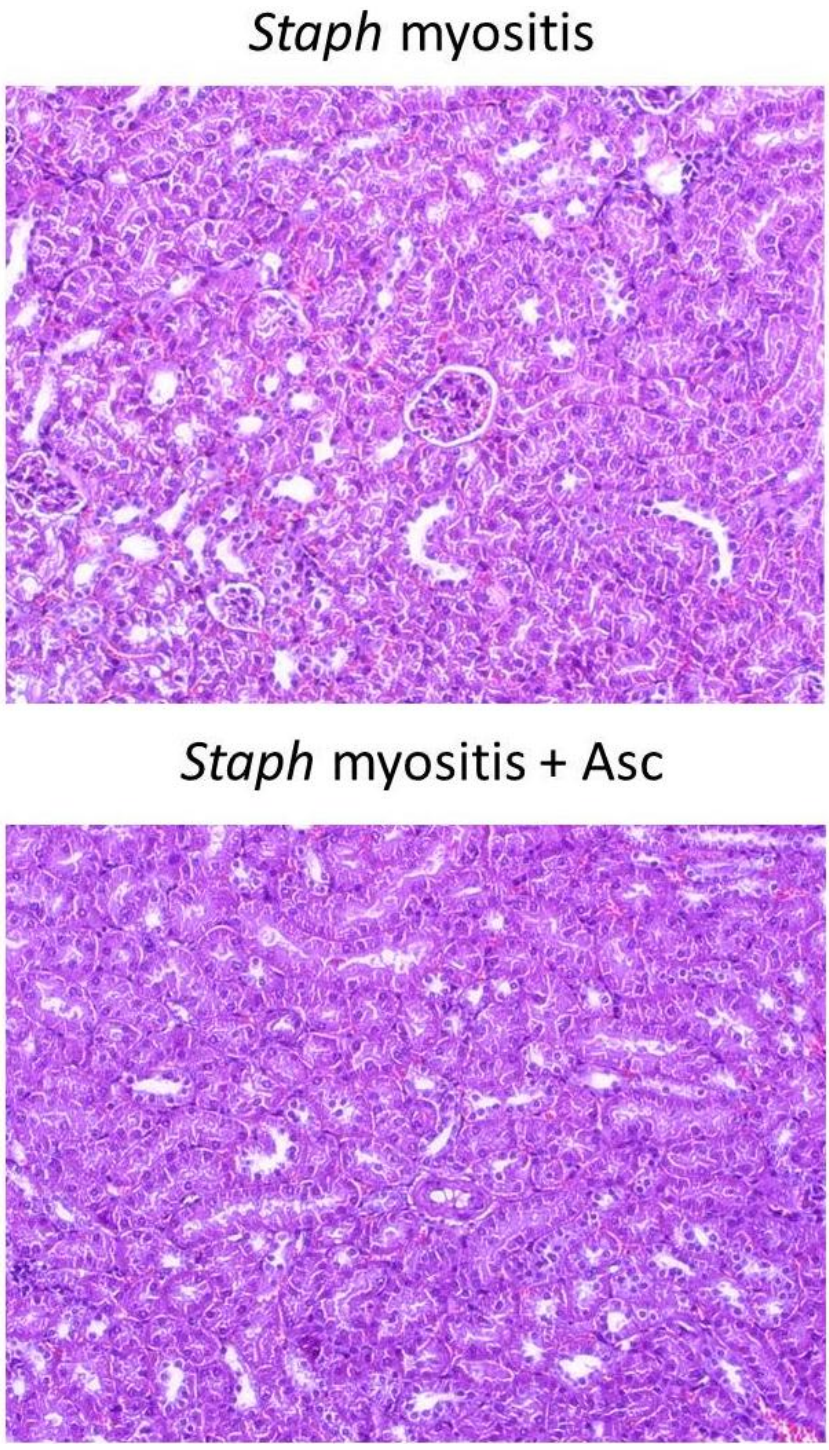


Figure 17. Kidney histology of *Staphylococcus* myositis mouse model treated with or without ascorbate

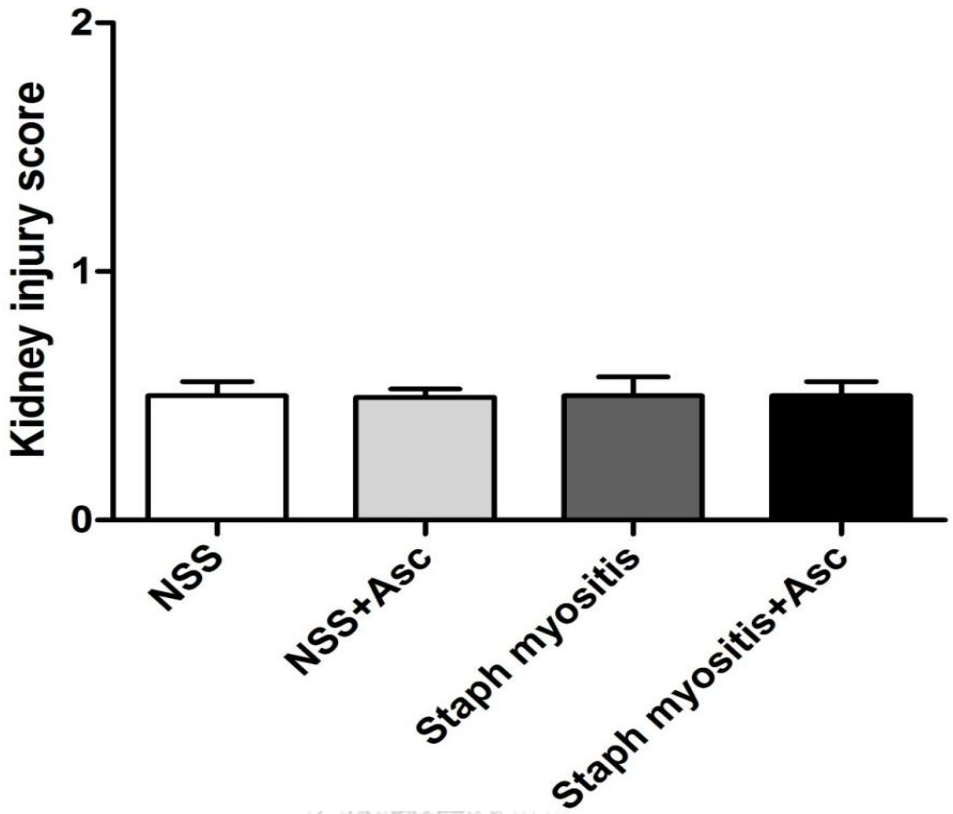


Figure 18. Kidney injury score of normal mice and *Staphylococcus* myositis mouse model with or without ascorbate

Bactericidal synergy between ascorbate and ceftriaxone against *S. aureus* MRSA strain demonstrated by time-kill assay, bacterial count and serum cytokine

Furthermore, for the closer resemble of the clinical situation, 2 clinical isolates of MRSA were tested in the mouse model. Although the MIC of ceftrizone against both strains of MRSA was higher than 512 μg/ml, MRSA#1 seems to be more susceptible to ascorbate than MRSA#2 as demonstrated by the reduction of bacteria as early as 3h of ascorbate plus ceftriaxone incubation (fig 19A). In contrast, in time kill test of MRSA#2, the bacterial burden decreased only at 24h of the incubation (fig 19B). Control groups of PBS and ascorbate alone was not effective against MRSA #1 (fig 19A). But ascorbate 10 and 40mM plus ceftriaxone was significant reduced bacterial burdens at 3-18h and obviously seen significant at 24h of incubation in comparison with ceftriaxone alone (fig 19A). However, in MRSA #2, only the condition of ascorbate 40mM plus ceftriaxone could reduce bacterial burdens significantly at 24h (fig 19B). In parallel, the effective synergy of ascorbate plus ceftriaxone as demonstrated by the reduced intramuscular bacterial burdens and serum IL-6 was demonstrated only in myositis from MRSA #1 (fig 20A, B) but not strain #2 (fig 21A, 21B).

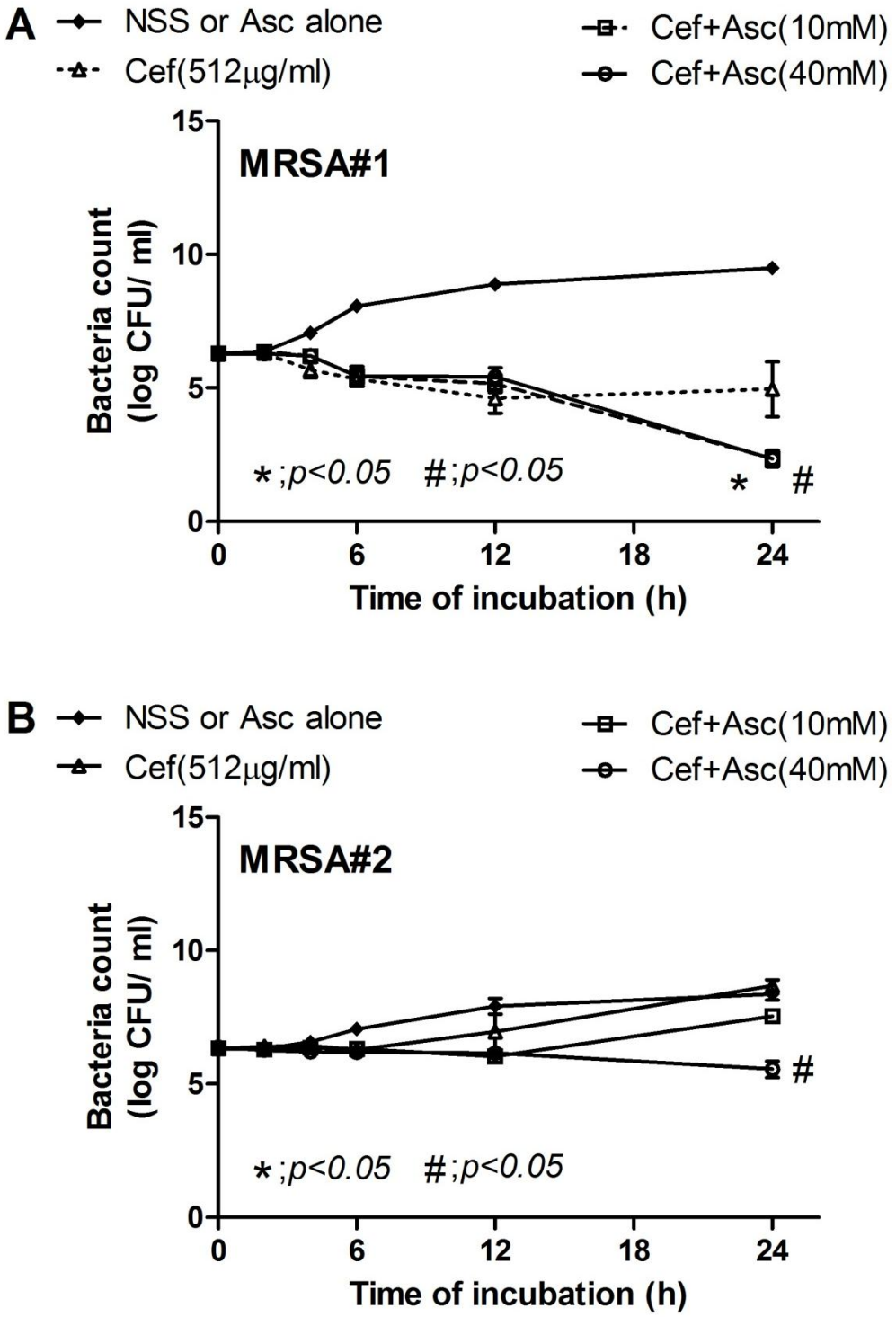


Figure 19. The *in vitro* test for ascorbate-ceftriaxone synergy by time kill assay with Ceftriaxone concentration at 512 μg/ml and ascorbate at 10 and 40mM against clinical isolated Methicillin resistant *S. aureus* (MRSA) strain #1 (A) and strain #2 (B) were demonstrated.

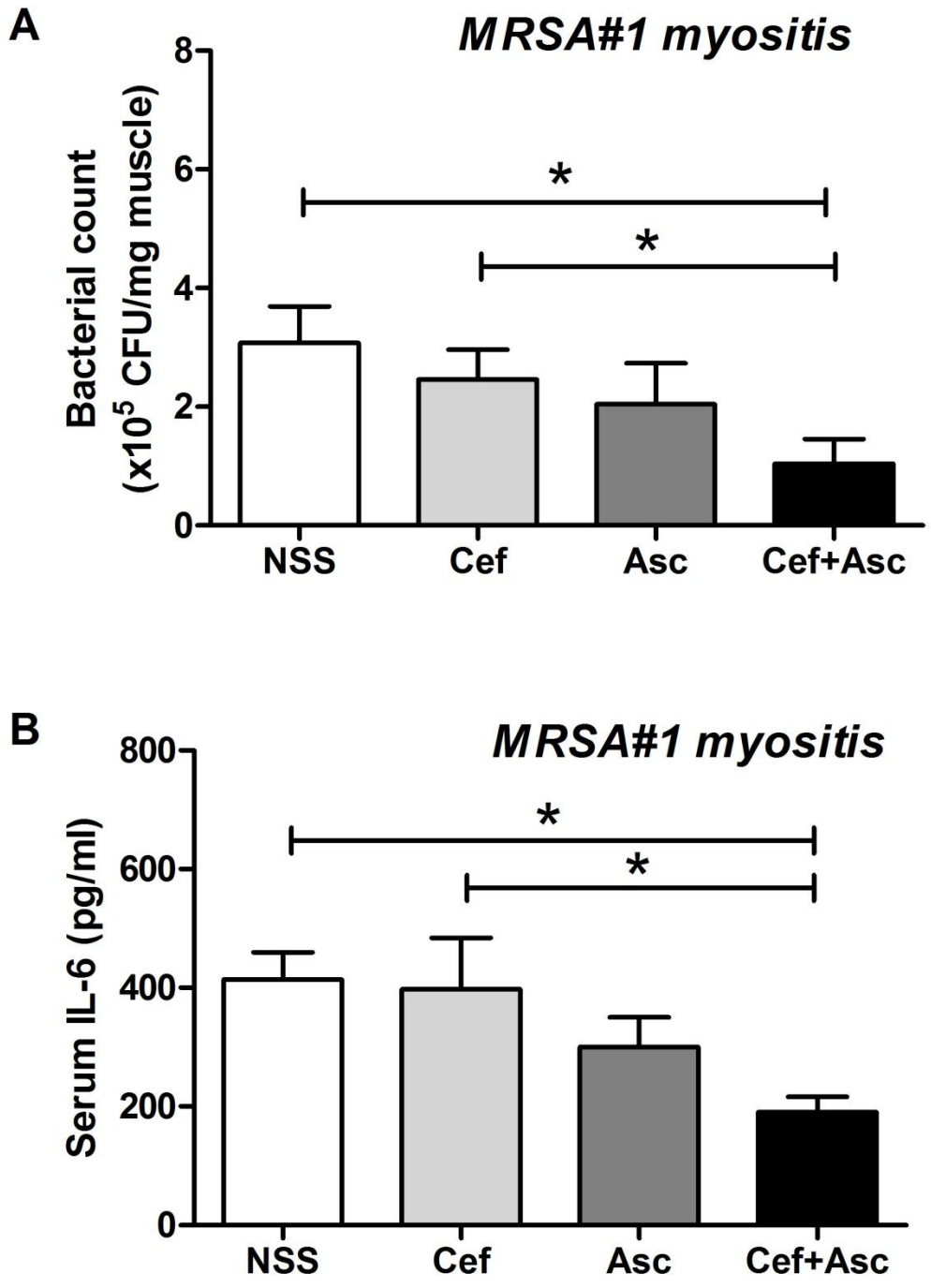


Figure 20. *In vivo* test of ascorbate-ceftriaxone synergy as measured by intramuscular bacterial count (A) and serum IL-6 (B) from MRSA strain #1 are shown.

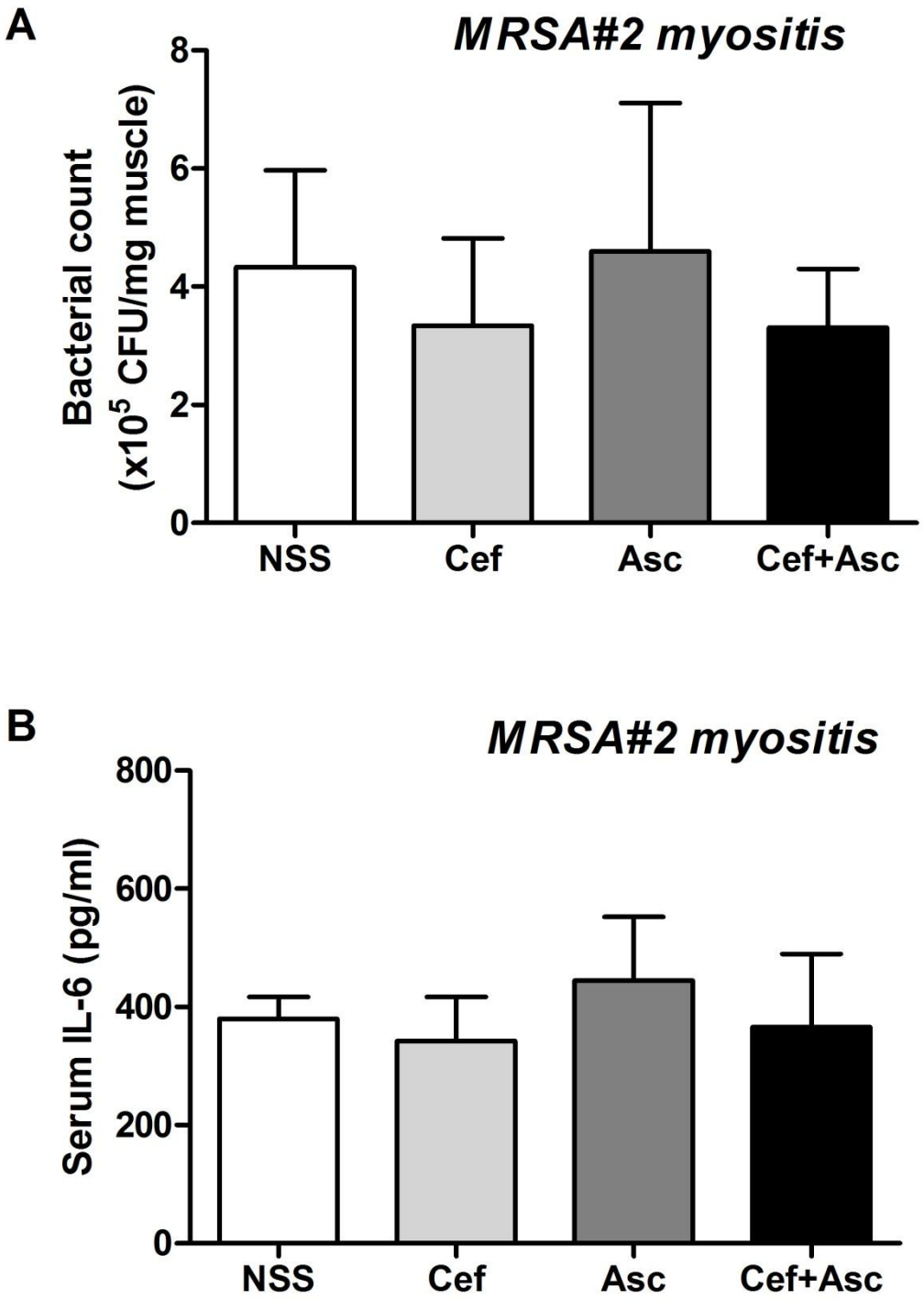


Figure 21. *In vivo* test of ascorbate-ceftriaxone synergy as measured by intramuscular bacterial count (A) and serum IL-6 (B) from MRSA strain #2 are shown.

CHAPTER VII
DISCUSSION

Nosocomial infection are the major problems found worldwide (67). Bacteria that are commonly found in the opportunistic infection are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumonia*, *Escherichia coli* and *Enterococcus spp* (29). Therefore, there are various studies in the attempt to develop the treatments of these infections.

Ascorbate, an orphan bactericidal drug

Ascorbate is an essential vitamin which has been known more than hundreds years. It is interesting that several anecdotal data imply that ascorbate could use against infection. The motto “an apple a day make disease away” is one of the anecdotal idiom that demonstrated the general belief of ascorbate effect againt viral infection. However, the data of ascorbate againt infection is very less. Several studies demonstrated bactericidal effect of ascorbate against several bacteria. Indeed, the effect on *Staphylococci* and *Pseudomonas aeruginosa* is previously mentioned (9, 26). Surprisingly, we could also demonstrate the direct effect of ascorbate against *Staphylococci* and partially effect on *Pseudomonas* spp. with the very high ascorbate concentration *in vitro*. Although the acidity of ascorbate might responsible for the bactericidal effect, we could demonstrated the bacterial effect even with neutral pH. This supported the direct effect of ascorbate on bacteria. Unfortunately, the mechanisms ascorbate bactericidal effect are still not clear. It is possible that ascorbate directly alters bacterial cell wall or it might increased hydrogen peroxide (H_2O_2) in the *in vitro* system or ascorbate might change nucleic acid of organisms (8). Despite the known bactericidal effect against some bacteria, ascorbate does not developed as a

bactericidal agent. This is in part, due to the less intesting in the old drug which is not commercially benefit. Thus, there is no drug company that is willing to invest on ascorbate. Then the development of the new indication of the old drug rely on the non-profit organization such as university and institute. Non surprisingly, the studies on ascorbate in cancer therapy is inititated by the National Institute of Health in the US. Hence, we hope that we will be a small initiator who is trying to make use of the old drug which is very low prize to improve the limited economic situation in our country.

It is very interesting to note that ascorbate could act as the anti- and pro- oxidant by the dose adjustment. Despite low dose ascorbate could attenuate sepsis by the anti-oxidant effect(68, 69) , the bactericidal effect rely on the high dose. As such,a pro-oxidant property of ascorbate could be found with the concentration of ascorbate in blood >0.2 mM. This concentration is also beneficial as the adjuvant treatment with anti-cancer,anti-fungi and anti-bacterial drugs (3, 37, 63, 70) through the induction of H_2O_2 and other unclear mechanisms (7, 71). Thus, combined between ascorbate and antibiotic was interesting to study. We demonstrated bactericidal synergy (ascorbate-ceftriaxone) by time-kill assay, *in vitro* and *in vivo*. And the effect of ascorbate in macrophage function was also explored.

Ascorbate-ceftriaxone *in vitro* synergy against *Staphylococci*

Because it is hypothesized that the effect of ascorbate in anti-cancer is due to the influx of H_2O_2 into the malignant cell after the pore-forming anti-cancer (72). Thus, we hypothesized that this mechanism might also be the same as bactericidal effect. Then we select ceftriaxone and common beta-lactam which could create small pore on the cell wall of bacteria. And we tested ascorbate-beta-lactam effect. Indeed, the synergy against *S. aureus* demonstrated at 24h in 10 and 40mM of ascorbate plus ceftriaxone 1xMIC but no effect in ceftriaxone at 0.25x MIC. This implied that the concentration of

antibiotic is very important for the synergistic effect. It is possible that we need the antibiotic effect that enough to make some pore on the cell wall of bacteria. In addition, the ascorbate-antibiotic synergy could not demonstrated with other selected gram-negative bacteria. Although ascorbate plus ceftriaxone decreased *P. aeruginosa* was demonstrated at 3-12h after of the incubation, but the effect was disappeared at 24h. This data suggested that ascorbate synergistic effect against only the specific bacterial, perhaps shows the effect only against gram positive. However, other gram positive bacteria were not tested in the current study due to the limitation of the culture method. The culture of *Streptococci* required the special culture media which will be another variable factor in these groups of the selected bacteria. Thus, *S. aureus* is the only gram-positive bacteria used in our study. The selectivity of ascorbate effect against gram-positive not gram negative is very interesting. This implied that ascorbate alter some molecule on gram-positive bacterial cell wall, such as lipoteichoic acid, but less effective on gram-negative molecule, perhaps endotoxin. The underlying mechanism of the ascorbate synergy that selectively occurs against gram positive is very interesting but we could not explore this topic due to the limitation in the funding and the studying time of the investigator.

Another interesting data is that ascorbate alone show bactericidal effect against *S. aureus* at 80 and 250mM. Hence, ascorbate possesses a direct bactericidal effect in a very high concentration which is different from our original hypothesis. Thus, it seems that i) it is not necessary to make a pore on cell wall for ascorbate to be effective as a bactericidal agent and ii) the effect of ascorbate alone, *in vitro*, implies that the complex systems of hydrogen peroxide production, *in vivo*, (eg Fe2+ or several enzymes) might be not necessary. So there might be another mechanism responsible for ascorbate bactericidal effect. We hypothesize that ascorbate might generate H_2O_2 directly from

water (H_2O) in the *in vitro* system that enough to make some pores on cell wall allowing ascorbate influx intracellularly through the direct prooxidant effect or the further induction of intracellular H_2O_2. Indeed, there is a report that ascorbate could eliminate the bacterial plasmids intracellularly (8). However, we could not prove this interesting hypothesis. In parallel, the dose of ascorbate could be lower from 80-250 mM to 10-40 mM to demonstrate the bactericidal effect. We hypothesize that the pore-forming action on cell wall of ascorbate alone need the very high ascorbate concentration. But with the present of beta-lactam antibiotic, it is easier to make pores on cell wall that allow intra-organism ascorbate delivery through antibiotic-induced membrane injury. More studies are interesting.

Ascorbate-ceftriaxone *in vivo* synergy against *Staphylococci*

Another explaination of ascorbate effect is the immune modulation. Because ascorbate is necessary for macrophage killing activity (28), we tested ascorbate to phagocytosis and killing activity of macrophage *in vitro*. The results show ascorbate at 10 and 40mM not effect in phagocytosis activity against all bacteria. Likewise, in previous study, ascorbate was enhance macrophage function by increase killing activity against *S. aureus* and *P. aeruginosa* with ascorbate at 40mM through the enhanced H_2O_2 production in lysate of peritoneal macrophage(28). Hence, it is possible that ascorbate enter macrophage and improve the killing activity from increase of superoxide and H_2O_2 of high dose ascorbate intracellularly which associate with reactive oxygen species in macrophage killing activity.

In the same line the *in vivo* results demonstrated decreased bacteria burden and pro-inflammatory cytokine IL-6in muscle of *S. aureus* myositis model treated with ascorbate plus ceftriaxone. Hence, the *in vivo* study implies that ascorbate attenuated

myositis mice through the improved macrophage killing activity with the direct ascorbate-ceftriaxone effect.

More importantly, we demonstrated the usefulness of the time-kill assay in the situation the more resemble to the clinical situation. The positive synergy of time-kill assay against MRSA strain #1 which the synergy started as early as 3h associated with a better outcome than the infection with MRSA strain #2 (positive synergy only at 24h of the assay). This suggests that time-kill assay possibly useful in the screening test in the real clinical situation. The earlier synergy might associate with the better *in vivo* outcomes. The positive synergy of the time-kill assay within a few hours could be a supportive data to use this strategy (ascorbate adjuvant to antibiotics) in the situation with the worst case of the non-drug available in the severe multidrug resistant. Besides, the blood concentration of ascorbate in patients was easier controllable and easier monitoring the continuous intravenous injection in comparison with mouse experiments (73). Thus, there is a hope that ascorbate could be used as bactericidal synergy in the real patient situations. More studies are warrant in this topic.

In addition, it should be mentioned that we could not identify oxalate crystal in all of the mice despite the clear documentation on this complication (30, 32). It is possible that ascorbate induced ascorbate crystal occur in the chronic administration. Then the short term administration might be less effect by this complication. More study is necessary.

In conclusion, we have demonstrated the synergy of high dose ascorbate with antibiotic against *S. aureus*. Thus, the adjunctive administration of ascorbate to antibiotic might be a beneficial strategy against the resistant bacteria, especially in the situation with limited resources. Further studies with other antibiotic against more

bacteria could be explored. Anyway, the proof of concept to use ascorbate in combination with antibacterial drug to improve drug efficacy is feasible from our initial experiments.

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APPENDIX

Reagent preparation

1. **Sodium L-ascorbate**

   Stock solution (1M Asc)

Sodium L-ascorbate powder	1.98	g
Sterile water	10	ml

   Filtered by 0.45 μm and adjust to pH 7.3 with NaOH, Ascorbate should be prepared freshly before using experiment and avoid from light and high temperature.

   Working stock (0.5M)

1M Asc	5	ml
Sterile water	5	ml

2. **Tryptic soy broth (TSB)**

Pancreatic digest of casein	15	g
Enzymatic digest of soy bean	5	g
Sodium chloride	5	g
Distilled water	1000	ml

   The solution was mixed and sterilized by autoclaving at 121°C for 15 minutes.

3. **Tryptic soy agar (TSA)**

Pancreatic digest of casein	15	g
Enzymatic digest of soy bean	5	g
Sodium chloride	5	g
Agar	15	g
Distilled water	1000	ml

   The solution was mixed and sterilized by autoclaving at 121°C for 15 minutes.

4. **Phosphate buffer saline (PBS)**

Stock solution (10X PBS)

NaCl	80	g
KCl	2	g
Na_2HPO_4	11.5	g
KH_2PO_4	2	g
Distilled water	1000	ml

Working solution (1X)

10X PBS	100	ml
Distilled water	900	ml

5. **Dulbecco's Modified Eagle Media complete (DMEM complete media)**

DMEM media	100	ml
(HyClone™, Thermo Scientific, Waltham, MA, USA)		
Fetal bovine serum (FBS)	10	ml
HEPES	1	ml
Sodium pyruvate	1	ml
Penicillin/Streptomycin	1	ml

6. **L929 culture supernatant**

Thaw cell L929 by using DMEM serum free media

Plate cell on TC culture flask

Pass cell by using Trypsin-EDTA

Culture cell in 8ml of DMEM complete media in 5% CO_2, 37°C

Collected of supernatant when cell growth at 70-80%

Filtered by 0.2µm

Freeze cell by using 10%DMSO in DMEM completed media, keep in -80°C overnight before transferring to liquid nitrogen for long storage

7. **Bone marrows-derived macrophages Media (BMM media)**

DMEM complete media	80	ml
L929 culture supernatant	20	ml
Horse serum	5	ml

8. **Lysis buffer**

Bovine serum albumin (BSA)	0.1	g
Tween 80	0.1	ml
Distilled water	1000	ml

9. **Fluorescein isothiocyanate (FITC)**

Stock solution (0.01g/ml)

FITC powder	0.1	g
Acetone	10	ml

Be careful not to use plastic pipette.

Working stock (500µg/ml) 0.5 9.5

0.01g/ml FITC	0.5	ml
1X PBS	9.5	ml

10. **0.2% Trypan blue solution**

0.4% Trypan blue solution	50	ml
1X PBS	50	ml

The solution was filtered by 0.4

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