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Effects of Angiotensin-converting Enzyme Inhibitors and Angiotensin II Receptor Blockers on the Immune System of *Pseudomonas aeruginosa* Challenged Hamsters

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Abstract

The severe cases of coronavirus disease 2019 observed in patients with hypertension and diabetes mellitus disease have created controversy as to why this was the case and the role of angiotensin receptor blockers (ARBs) and angiotensin enzyme inhibitors was not ruled out as a contributing factor. The objective of this study was to investigate the effects of angiotensin converting enzyme inhibitors (ACEIs) and ARBs on respiratory immunity. The study used a Syrian hamster as an animal model to study the effects of ARBs and ACEIs on the immune system of *Pseudomonas aeruginosa*-challenged hamsters; fever was a response variable. The hamsters were randomly separated into three groups of six. One group was administered enalapril, another with losartan, and the last group was administered water for 30 days. After 30 days, the hamsters were inoculated with 0.2 mL of 1.5×10^8 cfu/mL of *P. aeruginosa*. After 18 h from the inoculation time, the temperature was taken every 2 h for 36 h. The temperature in all groups ranged from 35.5°C to 37.2°C with an average of 36.1°C and there was no mortality recorded at the end of 14 days. The results were analyzed using Dunnett multiple comparisons analysis of variance using Statistical Package for the Social Sciences version 27. There were no significant differences in temperature between hamsters treated with enalapril or losartan and those treated with water (control). In addition, no hamster developed fever. In conclusion, enalapril and losartan may not negatively affect the respiratory immunity. Effects of ACEIs and ARBs on the immune system of *P. aeruginosa* challenged hamsters.

Keywords: Angiotensin converting enzyme inhibitors, Angiotensin receptor blockers, *Pseudomonas aeruginosa,* Syrian hamster

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Introduction

The advent of coronavirus disease 2019 (COVID-19) has brought with it new challenges, especially for people with existing respiratory and cardiac diseases related to the use of angiotensin-converting enzyme (ACE) inhibitors and angiotensin II (Ang II) receptor blockers (ARBs).[1] ARBs and ACE inhibitors are widely used in patients with hypertension and other cardiovascular diseases to improve the quality of life of affected persons.[2] Studies have shown that severe cases of COVID-19 were observed in patients with hypertension, renal disease, and diabetes mellitus.[1] There are controversies as to why this is the case, and the role of Ang II receptor blockers (ARBs) and angiotensin enzyme inhibitors (ACEIs) is not being ruled out as a contributing factor.[3] Angiotensin converting enzyme (ACE) is crucial in the renin-angiotensin-aldosterone system but is also involved in immune regulation.[4] Immune regulation of ACE is through Ang II dependent effects and Ang II independent effects. Ang II independent effects are those in which ACE itself is involved in immune regulation; for example, functional ACE is expressed in antigen-presenting cells, where ACE cleaves peptides and alters the repertoire of Class I major histocompatibility complex peptides.[5] This repertoire is critical to CD8(+) T cell-mediated adaptive immune responses. In addition, ACE is also involved in C3 cleavage, which is a complement protein.[6] C3 is important in initiating complement system activation and plays a critical role in innate immune surveillance.[7] In the same vein, increased expression of ACE by macrophages or neutrophils improves the ability of these cells to respond to immune challenges such as infection; however, administration of ACE inhibitors may reduce the ability of neutrophils and macrophages to kill bacteria.[8] Finally, ACE inhibition can affect T lymphocyte activation and proliferation by altering the immune response through a change in cell surface signals.[9] Ang II dependent effects are those in which Ang II exerts immune regulation: For example, Ang II promotes pro-inflammatory responses and macrophage activation *through* the AT_1 receptor (AT_1R). Furthermore, Ang II induces cytokine expression, including monocytic chemotactic protein, interleukin-8 (IL-8), and IL-18, which are involved in macrophage recruitment and promote monocyte differentiation and polarization, thus directly improving phagocytosis. Drugs that affect Ang II production or its actions have a negative effect on macrophage recruitment and monocyte differentiation.

ACEIs competitively inhibit the angiotensin converting enzyme to prevent the conversion of angiotensin I to Ang II,[10] were, as ARBs block the effects of Ang II in producing their blood

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pressure lowering effects.[11] The action of both ARBs and ACEIs on angiotensin converting enzyme has the potential to negatively affect the immune system. ACEIs and angiotensin receptor blockers (ARBs) were at the center of the debate about their effect during COVID-19 infection.[1] This study used a Syrian hamster (*Mesocricetus auratus*) as an animal model to study the effects of ARBs and ACEIs in relation to respiratory colonization and infection with *Pseudomonas aeruginosa;* further Enalapril and Losartan were used to represent ACEIs and ARBs, respectively. *P. aeruginosa* is a Gram-negative, rod-shaped, aerobic, non-spore-forming bacteria that is ubiquitous in the environment where it can cause disease in a variety of hosts including plants, nematodes, insects, and mammals.[12,13] It is an opportunistic pathogen that sets on the host when the normal immune defenses are disabled.[14] The bacteria are also a major problem in patients with cystic fibrosis, burn wounds, chronic wounds, chronic obstructive pulmonary disorder (COPD), and nosocomial infections such as ventilator-associated pneumonia, catheter-associated urinary tract infections, and asthma.[15] Furthermore, studies have shown that *P. aeruginosa* can colonize and cause infection in mice[17,18] and in Hamsters.[19,20] *P. aeruginosa* was selected for this study because it is an opportunistic pathogen and can cause a wide range of severe opportunistic infections in patients with underlying medical conditions[16] but may not cause serious infection in immune-competent hosts. *P. aeruginosa* PA1 used in this study is a virulent strain that was first isolated from a patient with respiratory tract infection in China.[21] The objective of the study was to answer the question does the oral administration of ARBs and angiotensin converting enzyme inhibitors (ACEIs) have a negative effect on the respiratory immunity? The development of fever in hamsters was studied as a response variable in this study. Furthermore, If the hamsters that were treated with enalapril or losartan developed fever and control hamsters did not develop fever, it would mean that enalapril and Losartan have a negative effect on the respiratory immune system of hamsters since *P. aeruginosa* should not normally cause a disease in immune-competent hosts.[16,22,23]

Materials and Methods

This was a post-test only control group study design that was conducted in the Microbiology Laboratory of the School of Veterinary Medicine of the University of Zambia. The sample population was calculated using the "resource equation" method.[24] Before infecting the hamsters with *P. aeruginosa*, the strain of *P. aeruginosa* was reconfirmed by sequencing; then antibiotic resistance was carried out to check the resistance pattern of the strain since resistance is associated with virulence.[25] The Ethics Clearance was granted by the University of Zambia Biomedical Research Ethics Committee (UNZABREC) IRB00001131 of IORG0000774. The approval number for the study is REF. No. 2504-2022.

Animal Treatment and Control Groups

For this study, 18 male hamsters were used. Male hamsters were used to reduce confounders that may arise due to the estrous cycle in female hamsters.[26] Furthermore, male hamsters were used to reduce the chances of using a pregnant hamster during the study. The hamsters were randomly separated into three groups of six. A set of hamsters was equal to six hamsters for the purposes of this study. The drugs used in this study were Enalapril maleate, which is an ACEI, and Losartan potassium, which is an Ang II receptor blocker. A set of six hamsters was treated with Enalapril 12 mg/L in drinking water for 30 days, and another set of six was treated with Losartan 28 mg/L in drinking water for 30 days. The last set of six (06) of hamsters was given distilled water; this was the negative control group. The dose for the drugs was calculated from the maximum recommended dose in the treatment of humans. For Enalapril, the maximum recommended dose is 40 mg/day,[27] while that for Losartan is 100 mg/day.[28] During the study period, the hamsters were kept in groups of six. The hamsters had free access to food and water that contained drugs for the experimental hamsters and distilled water for the control hamsters. The hamsters were fed Nutrisure commercial pellets manufactured by NUTRIFeeds Zambia Limited, with a formulation of moisture content of 120 g/kg-crude protein maximum, 13 g/kg lysine maximum, 6.5 g/kg-crude fat, 30/40 g/kg-min/max crude fiber, 120 g/kg-calcium, 10/12 g/kg min/max and phosphorus-6 g/kg-minimum. The hamsters were kept in cages with paper litter, and the litter was changed twice a week during the study.

Source of the Isolated *P. aeruginosa* Used in the Study

A patient isolate of *P. aeruginosa* stored in 20% glycerol at –80°C was obtained from the Tropical Diseases Research Centre (TDRC) of Zambia, under strict microbial containment. The vial containing the bacteria was allowed to thaw at room temperature in the laboratory. The isolate was then sub-cultured on nutrient agar and incubated aerobically at 37°C for 24 h. Before proceeding with infecting the hamsters, the isolate was reconfirmed as *P. aeruginosa* using molecular methods.

Molecular Confirmation of *P. aeruginosa*

DNA was extracted from a 24-h culture of *P. aeruginosa* using the heating method.[29,30] A loop-full colony of bacteria was picked from a plate and placed in 120 μL of DNase/RNase-free water in a sterile Eppendorf tube to obtain a turbid suspension of bacteria. The cell suspension was placed in an IWAKI THERMO ALUMI BATH at 95.0°C for 10 min to lyse the cells and then placed on ice immediately. The Eppendorf tube containing the lysate was then centrifuged for 3 min at 10,000 × g to pellet the cell debris. The supernatant was then transferred to a new Eppendorf tube and used as a template DNA in polymerase chain reaction (PCR). PCR was performed using the KOD Xtreme TM Hot Start DNA Polymerase kit (TOYOBO CO., LTD) and Universal bacterial 16s rRNA primers (Forward-5′ AGAGTTTGATCCTGGCTCAG 3′, Reverse-5′ ACGGCTACCTTGTTACGACTT 3′) following manufacturer instructions. PCR amplification was performed using a Veriti 200 thermal cycler (Applied Biosystems) with an initial denaturation step of 98°C for 2.0 min, followed by 30 cycles at 98°C for 10 s, 52.0°C for 05 s, annealing and a final extension step at 72°C for 2.0 min. The PCR products were visualized on 1.5% agarose gel electrophoresis after ethidium bromide staining.

After PCR amplification, the PCR amplicons were transferred to a DNA miniprep spin column (Promega wizard® SV Gel and PCR clean-up Kit) and purified following the manufacturer's instructions. Thereafter, master mix for the big dye was made as follows: Forward primer – big dye 0.5 μL, sequencing buffer 3.75 μL forward primer 0.5 μL, DNase/RNase free water 12.92 μL, and purified DNA template 2.0 μL. Another master mix was made for the reverse primer as follows: Big dye 0.5 μL, sequence buffer

3.75 µL reverse primer 0.5 µL, DNase/RNase free water 12.92 µL, and purified DNA template 2.0 µL. Then, big dye PCR amplification was performed using a Veriti200 Thermal cycler (Applied Biosystems) with an initial denaturation step of 96°C for 1.0 min, followed by 25 cycles at 96°C for 10 s, 50.0°C for 5s, annealing and a final extension step at 60°C for 2.0 min. After big dye PCR cycles, the DNA precipitated. After BigDye amplification, the amplicons were precipitated using the ethanol precipitation method. Master mix for ethanol precipitation was made as follows: 2.0 µL of 3.0 mM sodium acetate, 2.0 µL of 125.0 mM EDTA and 50.0 µL of absolute ethanol to make a total of 54.0 µL of master mix. Thereafter, 54.0 µL of master mix was added to 20.0 µL of big dye PCR product in a PCR tube and incubated in the dark at room temperature for 15.0 min. After incubation, the mixture was centrifuged at 15000 rpm for 15.0 min. The supernatant was discarded. Then 200 µL of 70% ethanol was added, the mixture was centrifuged again at 15000 rpm for 15.0 min, and the supernatant was discarded. The PCR tubes were then air-dried at room temperature in the dark. After air drying, 20.0 µL of formamide was added to the PCR tubes. The tubes were then placed on the Veriti200 thermal cycler (Applied Biosystems) for denaturation at 96°C for 2.0 min after which the tubes were taken for sequencing.

PCR tubes were transferred to SeqStudioTM TM Genetic Analyzer Applied biosystems by Thermo Fisher Scientific for sequencing according to the manufacturer's instructions. After sequencing, the sequences were cleaned by editing out non-standard DNA bases and aligned using Unipro UGENE version 45.0 software.[31] The non-standard DNA bases were edited out by comparing sequence chromatogram and the expected base in Unipro UGENE version 45.0 software. After alignment, a contig sequence was generated. The contig sequence was analyzed using the Basic Local Alignment Search Tool (BLAST) in the National Centre for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the bacteria species that was sequenced.

Antibiotic Resistance

Antibiotic resistance tests were carried out using the automated VITEK 2S200 version 9.02 system (bioMérieux, Marcy l'Etoile, France) using AST-GN86 cards according to the manufacturer's instructions. Antibiotic minimum inhibitory concentration (Cefazolin, Ceftazidime, Cefepime, Imipenem, Gentamicin, Tobramycin, Ciprofloxacin, and Levofloxacin) data for the isolate were interpreted for susceptibility and resistance according to the VITEK2S200 version 9.02 Advanced Expert System and Clinical and Laboratory Standards Institute guidelines of 2017 (CLSI MI00-S27).

Infecting Hamsters with *P. aeruginosa*

The hamsters were intranasally inoculated with 0.2 ml PBS pH 7.0 containing 1.5×10^8 cfu/mL,[19,32] which translates into an effective dose of 3.0×10^7 cfu/mL. After inoculating the hamsters with *P. aeruginosa*, the animals were re-housed in cages for 18 h and continued drinking water containing drugs (Enalapril or Losartan) for experimental hamsters and distilled water for control hamsters. After 18 h, temperature changes were measured every 2 h for 36 h and then once a day for 3 days using a Bo Hui infrared thermometer model T-168. The temperature was measured in the back of the neck with the thermometer touching the hamster. The hamsters were further observed for 14 days. The temperatures from each treatment were averaged to get one temperature reading. Average temperature = (Temperature for hamsters in treatment group)/6. The average temperature was used to compare the infection results.

Verifying Infection Success

To establish that *P. aeruginosa* infection was a success, three 16-week-old male hamsters were randomly selected and infected with *P. aeruginosa* as before, for a negative control, two randomly 16-week-old hamsters were intranasally administered 0.2 mL of PBS pH 7.0. The hamsters were sacrificed after 24 h of being infected.[19] General anesthesia (xylazine 10 mg/kg body weight[33] was administered to hamsters until no pedal withdrawal reflexes were present. The hamsters were sacrificed, and a nostril swab, trachea, and lung tissue were collected. The lung and trachea were homogenized separately in 1.0 mL of PBS pH 7.0. The homogenate was then cultured in nutrient broth at 42°C for 24 h. Subsequently, a sub-culture was performed on nutrient agar at 37°C for 24 h. Biochemical microbiological studies were conducted to identify the isolated organism.

Data Analysis

The collected data were analyzed using IBM-Statistical Package for the Social Sciences version 27 program. Dunnett's Multiple Comparison analysis of variance (ANOVA) (Dunnett's t-test) was used to analyze the differences in temperature between experimental hamsters and control hamsters. $P < 0.05$ was considered significant for Dunnett's multiple comparisons. In this study, temperature was a response variable and fever was defined as temperature above 37.5°C.[34]

Interpreting the Results

If experimental hamsters developed fever and control hamsters did not develop fever, it would mean that enalapril and Losartan have a negative effect on the respiratory immune system of hamsters since *P. aeruginosa* should not normally cause a disease in immune-competent hosts.[16,22,23]

Results

The study used a Syrian hamster (*M. auratus*) as an animal model to study the effects of ARBs and ACEIs on the respiratory immune system using *P. aeruginosa* as an immunity bacterial challenge pathogen; furthermore, Enalapril and Losartan were used to represent ACEIs and ARBs, respectively. An isolate of *P. aeruginosa* was obtained from TDRC and then sub-cultured on nutrient agar; Figure 1 shows the growth of the isolate on nutrient agar at 37°C after 24 h. Before proceeding with hamster infection with the isolate; the isolate was confirmed using 16s rRNA universal primers PCR as shown in Figure 2.0 and sequencing. The sequence aligned 100% with *P. aeruginosa* PA1 Accession number: MK685346.1. After reconfirming by PCR and sequencing that the isolate was *P. aeruginosa;* the isolate was then subjected to drug sensitivity using VITEK 2S200 automated system Table 1. To establish whether intranasal infection would work; three randomly selected hamsters were intranasally infected with 0.2 mL of PBS containing 1.5×10^8 cfu/mL of *P. aeruginosa* and another 2 hamsters (negative control) received 0.2 mL of PBS intranasally; the hamsters were sacrificed after 24 h and homogenates of the lungs, nostril swab and trachea were cultured at 42°C and then sub-cultured at 37°C. Table 2

Table 1: Antibiotic resistance results

Selected organism	*Pseudomonas aeruginosa PA1*				
Antimicrobial	*MIC*	*Interpretation*	*Antimicrobial*	*MIC*	*Interpretation*
Cefazolin	≥ 64	R	Imipenem	2	S
Ceftazidime	16	I	Gentamicin	≥16	R
Cefepime	32	R	Tobramycin	4	S
			Ciprofloxacin	≤0.25	S
			Levofloxacin	1	S

Key: S: Sensitive, R: Resistant, I: Intermediate, MIC: Minimum inhibitory concentration

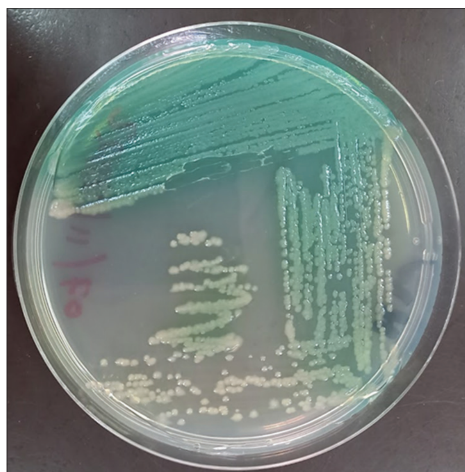

Figure 1: The growth of *Pseudomonas aeruginosa* on nutritional agar after 24 h of culture. The green color is due to the pyocyanin produced by *P. aeruginosa* colonies

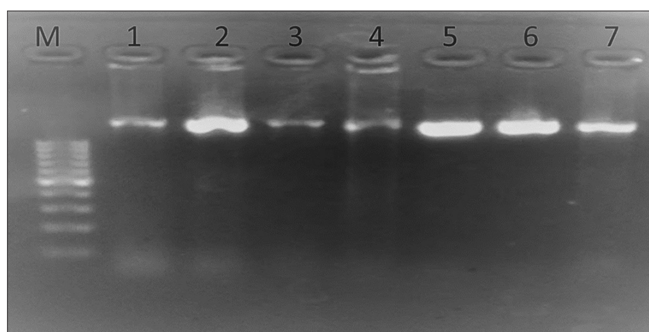

Figure 2: The staining with ethidium bromide of the 16s polymerase chain reaction (PCR) product.
Key: M=Ladder marker, numbers 1–7=16s PCR products

shows that *P. aeruginosa* was isolated from the nostril, trachea, and lungs of the hamsters that were infected with the isolate, while *P. aeruginosa* was not isolated from negative controls. Following the above results, 0.2 mL containing 1.5×10^8 cfu/mL of *P. aeruginosa* was administered to hamsters that had been taking Enalapril, Losartan, or water. After 18-h post-infection, the temperature of the hamsters of each set of treatment (Enalapril, Losartan, and Water) was measured every 2 h for 36 h, there after temperature was measured once a day for 3 days. The hamsters were further observed for 14 days to see if any mortality would be recorded; no mortality was recorded after 14 days from all treatment groups (Enalapril, Losartan, and Water). During the experiment, the mean, minimum, and maximum temperatures of the hamsters treated with Enalapril, Losartan, or water were recorded as shown in Table 3.

Table 2: Establishing infection success of *Pseudomonas aeruginosa* in Hamsters

Treatment	*Nostril*	*Trachea*	*Lung*
0.2ml PA	Positive	Positive	Positive
0.2ml PA	Positive	Positive	Positive
0.2ml PA	Positive	Positive	Positive
0.2ml PBS	Negative	Negative	Negative
0.2ml PBS	Negative	Negative	Negative

Key: 0.2 mL PA: 0.2 mL of 1.5×10^8 cfu/mL of *Pseudomonas aeruginosa*. Positive: *P. aeruginosa* was isolated. Negative: *P. aeruginosa* was not isolated

Table 3: Minimum, Maximum, and Mean temperatures for the hamsters

Treatment	*N*	*Mean*	*Minimum*	*Maximum*
Water	18	36.0378	35.53	37.23
Enalapril	18	36.0222	35.67	36.30
Losartan	18	36.1050	35.83	36.53
Total	54	36.0550	35.53	37.23

Key: N: Number of times the temperature was taken.

The temperature in all groups ranged from 35.5°C to 37.2°C with an average temperature of 36.1°C. To draw inferences from the data, a histogram of mean temperatures with error bars was generated using IBM SPSS version 27 [Figure 3] and there was no significant difference in mean average temperature between hamsters treated with Enalapril or Losartan and hamsters treated with water. The mean temperature measurements were further analyzed using Dunnett's multiple comparison ANOVA to test whether there was a significant difference in temperatures between hamsters treated with Enalapril or Losartan and hamsters treated with water. Table 4 shows that there was no significant difference in mean temperature between enalapril or losartan and water.

Discussion

P. aeruginosa is an opportunistic pathogen that sets on the host when the normal immune defenses are disabled.[14] It is a major problem in patients with cystic fibrosis, burn wounds, chronic wounds, COPD, nosocomial infections such as ventilator-associated pneumonia, catheter-associated urinary tract infections, and asthma.[15] *P. aeruginosa* PA1 is a multidrug resistant strain[30] [Table 1], which was first isolated from a patient with respiratory tract infection in China.[21] Multidrug resistance is positively correlated with the virulence of *P. aeruginosa*.[25] Pyocyanin, a green metabolite of *P. aeruginosa* colonies in culture[16] [Figure 1] is one of the virulent factors that is produced by *P. aeruginosa* PA1.[35] A study by Coalson *et al.*[20] found that hamsters that were infected with an inoculum of 0.5 mL suspension containing 10^6 cfu/mL of *P. aeruginosa* cleared the infection within 120 h, while 100% mortality was observed in hamsters that were inoculated with 0.5 mL of 10^8 cfu/mL. Furthermore, Bartram *et al.*[36] found that in

Table 4: Multiple comparisons ANOVA for temperature changes. *P* value between enalapril and water is 0.98 and *P* value between losartan and water is 0.65. The table shows that there was no significant difference in mean temperature between hamsters treated with enalapril, or losartan and water (control)

Multiple comparisons						
Dependent variable: Temperature						
Dunnett t-test (2-sided)[a]						
(I) Treatment	*(J) Treatment*	*Mean difference (I-J)*	*Standard error*	*Sig.*	*95% confidence interval*	
					Lower bound	*Upper bound*
Enalapril	Water	−0.016	0.086	0.98	−0.21	0.18
Losartan	Water	0.067	0.086	0.65	−0.13	0.26

[a]Dunnett t-tests treat one group as a control and compare all other groups against it

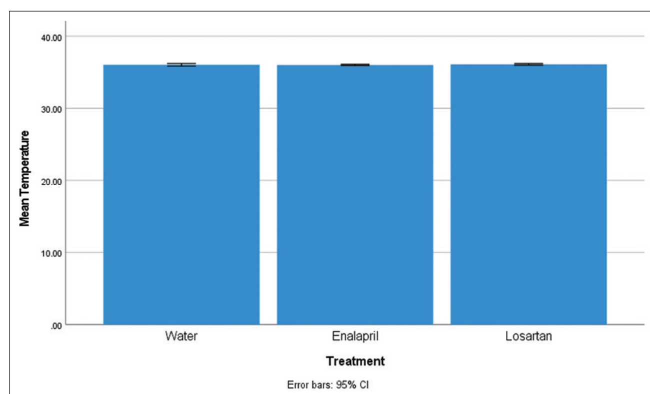

Figure 3: A bar graph of mean temperature changes of hamsters treated with enalapril, losartan, and water. It shows that there is no significant difference between the temperature of the control and hamsters treated with enalapril or losartan at a 95% confidence interval

human volunteer studies, an oral dose of 10^6 cfu/ml of *P. aeruginosa* was required to colonize the intestinal tract. Based on the literature, this study used 0.2 ml PBS pH 7.0 containing 1.5×10^8 cfu/mL[19] as an experimental dose for the hamster bacterial challenge test. The hamsters in this study did not develop fever after inoculation with 0.2 mL of 1.5×10^8 cfu/mL of *P. aeruginosa* PA1. Fever is defined as a temperature above 37.5°C.[30] Furthermore, Harrison's Principles of Internal Medicine, 21st edition defines fever as a temperature above 37.7°C, which represents the 99th percentile for healthy individuals.[37] According to the definition of fever above, no hamster had a temperature above 37.5°C, as can be seen in Table 3. Furthermore, multiple comparisons ANOVA showed that there were no significant differences in the mean temperature for the hamsters treated with enalapril or losartan and control at Dunnett's t-test $P < 0.05$ as shown in Table 4 and Figure 3. A study by Johanson *et al.*[38] found that an infectious dose of 5.0×10^6 cfu/mL of *P. aeruginosa* was lethal to 65% of cystic fibrosis mice within 7.0 days; this lethal dose of *P. aeruginosa* is lower compared to the dose used in this study of 3.0×10^7 cfu/mL; in this study, there was zero mortality after 14 days of observation of hamsters. *P. aeruginosa* has an incubation period of 24–72 h.[39] The implication is that the hamsters were able to overcome the infection[20] and that taking Enalapril or Losartan did not cause the hamsters to develop fever after being infected with an opportunist pathogen. A study by Kurahashi *et al.*[40] concluded that injury to the alveolar epithelium is important in the release of pro-inflammatory mediators into the circulation that are primarily responsible for septic shock. In the case of this study, it is possible that there was no damage to the respiratory epithelium; therefore, no pro-inflammatory mediators were able to flow into the circulatory system; this may explain why none of the hamsters presented with fever. In conclusion, enalapril and Losartan did not negatively affect respiratory immunity.[20] The implication is that enalapril and Losartan may not have a negative effect on the respiratory immune response to an infection.

Limitation of the study

Interpretation of the study result may be limited to *P. aeruginosa* PA1. In addition, these results may not apply to other drugs other than enalapril and Losartan.

Conflicts of Interest

The authors declare no potential conflicts of interest with respect to research, authorship, and/or publication of this article.

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