Evaluation of Hypochlorous Acid Fogging: An Alternative Disinfection Method

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Abstract
The COVID-19 pandemic has led to increased demand for disinfectants to help reduce transmission of the novel virus. Hypochlorous acid (HOCl) has been studied as a liquid disinfectant in the past, but little research exists on its fogged or aerosolized application, which is the primary focus of this work. Three solutions were tested: one purchased commercial solution (EcoLogic Solutions) and two produced using home units (EcoloxTech Eco One and RIPPO Sprayer). Solution was fogged into a desiccator using a Contronics HU-45 Humidifier. Aluminum squares were coated with either 50 µL of *Enterococcus faecalis* (*E. faecalis*) or H1N1 influenza virus solution and oriented vertically in the desiccator. These pathogens, and how they responded to the fogging treatment when compared to a control group, which was left untreated (not fogged), formed the basis for the results and gave evidence as to whether or not the method was effective at disinfecting the surface. The research found that fogging continuously with the EcoLogic solution yielded log reductions in *E. faecalis* and H1N1 of 6.59 and 4.90, respectively, after 5 minutes. Fogging under the same conditions with solutions produced by the Eco One and RIPPO yielded log reductions in *E. faecalis* of 4.21 and 0.91, respectively. The Eco One solution required more time to yield a ≥3-log reduction (≥99.9% reduction) in H1N1 compared to the EcoLogic solution (7.5 min vs 5 min). The RIPPO solution was not effective in all scenarios. These results suggest that hypochlorous acid (purchased or homemade), when applied as a fog, is effective against certain bacterial and viral pathogens, namely *E. faecalis* and H1N1. However, several factors such as the time fogged and pH of the starting solution, which ultimately determines whether chlorine is present as HOCl or OCl^-, play a significant role in the level of effectiveness observed.

Keywords: COVID-19, disinfection, hypochlorous acid, fogging

1. Introduction

With the emergence of Coronavirus Disease 2019 (COVID-19), the demand for disinfectants has been on the rise, causing shortages in many places such as schools, hospitals, and businesses. This has sparked new research on the use of alternative disinfectants. Not only is it important for these disinfectants to be safe and effective, but it is also important for them to be inexpensive and easy to produce. Hypochlorous acid (HOCl) has gained much attention in this regard as it is a green, non-toxic, antibacterial, and antiviral disinfectant that can be produced cheaply from common household materials.

As early as March 2020, HOCl was approved by the Environmental Protection Agency (EPA) as a viable disinfectant in its liquid form on non-porous surfaces against COVID-19 [1]. HOCl has also been proven to be an effective disinfectant against a variety of other pathogens including those

that are known to cause foodborne illnesses like *Escherichia coli*, *Salmonella*, and *Listeria*, as well as viruses such as the human norovirus and avian influenza virus [2-4]. Being that COVID-19 is categorized as a highly infectious and Biosafety Level 3 (BSL-3) disease, access to it is limited and requires a high level of security clearance, which led the authors to instead study the notoriously hard-to-kill strain of bacteria *Enterococcus faecalis* (*E. faecalis*), and the H1N1 influenza virus. These pathogens were chosen because they do not require BSL-3 clearance yet are still very infectious and relevant today.

E. faecalis is a gram-positive bacterium that is resistant to many antibiotics and is one of the leading causes of urinary tract infections (UTIs) and infections following root canal procedures [5]. H1N1 influenza virus, on the other hand, is an airborne human respiratory virus and was responsible for the swine flu pandemic in 2009 [6]. The H1N1 influenza virus has a lipid envelope similar to SARS-CoV-2, which gives it some additional relevance to the current pandemic. These viruses both cause respiratory illnesses and transmit in similar ways. The main method of transmission is through exposure to respiratory fluids carrying infectious viral particles. There are three principal pathways in which this transmission can occur: inhalation of the virus, deposition of the virus on exposed mucous membranes such as the eyes, nose, and mouth, and touching mucous membranes with soiled hands contaminated with the virus [7,8]. The goal here is to prevent and help reduce the transmission of the virus via the hand-to-mucous membrane pathway by killing the virus at the source: a frequently-touched surface.

HOCl is a weak acid that is formed by adding chlorine to water. Due to chlorine's considerable solubility in water (7,300 ppm at 20°C and 1 atm), it dissolves easily when administered in controlled amounts [9]. Chlorine is a notorious oxidizing agent in chemical reactions due to its electronic structure. Elemental chlorine, like all halogens, has the tendency to acquire an extra electron from its surroundings to completely fill and stabilize its outer shell with eight electrons. When chlorine gas (Cl_2) is dissolved in water, it undergoes the following hydrolysis reaction:

$$Cl_2 + H_2O \rightleftharpoons HOCl + H^+ + Cl^- \quad (1)$$

This reaction has a very high ionization constant of $K=3.3 \times 10^{-8}$ at 20°C [9], and Cl_2 essentially fully hydrolyzes in a matter of seconds under standard conditions. Only if the pH of the water is below 3, or if chlorine concentration reaches very high levels (greater than 1,000 ppm), will there be any measurable quantity of Cl_2 present in solution [9]. Therefore, it is incorrect to simply refer to disinfection by chlorine, when it is the oxidizing capacity of the chlorine in the hydrolysis product HOCl that provides the major disinfecting action of chlorine solutions [9].

HOCl will undergo a further dissociation reaction in solution with water:

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

This process occurs essentially instantaneously and is reversible. One can describe the equilibrium relationship by deriving an expression for the ionization constant:

$$K=[H^+][OCl^-]/[HOCl] \quad (3)$$

which can be rearranged as follows to show that the relative amounts of HOCl and hypochlorite ion (OCl^-) present in a solution of "free chlorine" are a function of the hydrogen ion activity, or the pH of the solution [9]:

$$K/[H^+]=[OCl^-]/[HOCl] \quad (4)$$

It was these relations that were used to create and plot the curves shown in Figure 1.

Free available chlorine (FAC) refers to the chlorine present as either undissociated HOCl or OCl^-. Despite OCl^- contributing to the total FAC

of a solution, it is a misrepresentation of the solution's overall disinfecting capabilities. Unlike the chlorine atom in a HOCl molecule, the chlorine atom in an OCl^- molecule is tightly bound to the oxygen atom and does not dissociate easily, which is essential for disinfection. HOCl dominates at lower values of pH, while OCl^- dominates at higher values of pH as shown in Figure 1. The level of FAC is highest in pH 5 solutions [10].

HOCl is a powerful oxidizing agent and is estimated to be 80 times more effective than bleach in surface disinfection [11]. It is important to point out that OCl^- is the predominant chlorine species present in bleach. While bleach is a common household cleaner, it can be toxic to humans and has the potential to damage surfaces [12]. For this reason, when hypochlorous acid is made, it is crucial that its pH is in the slightly acidic range, between pH 4 and 6, where the solution is most stable and will best maintain its chlorine concentration and pH, and thus its disinfecting capabilities, over time [13].

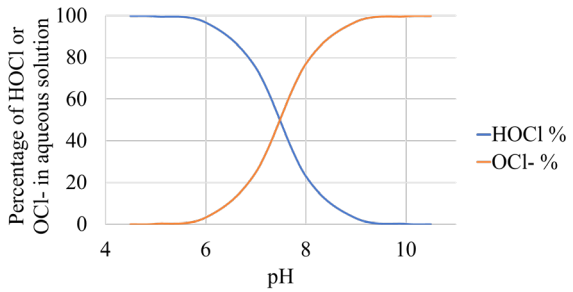

Figure 1. Effect of pH on the predominant chlorine species in aqueous solution. $pK_a = 7.5$.

Active chlorine species, like HOCl and OCl^-, contribute to the inactivation of microbial cells. However, the ways in which the microbial cells are inactivated are different between HOCl and ionized OCl^-, and this is due to the interaction with the chlorine species and the cell walls and membranes [2]. The microbial cells have a lipid bilayer, or a hydrophobic layer of the plasma membrane, and the interactions between the chlorine species and this bilayer explains the differences in the inactivation of the microbial cells. Due to the negative charge of the lipid bilayer, ionized OCl^- is unable to penetrate the cell membrane of the microbial cells (Figure 2). So, OCl^- is only able to act from outside the cell, by inactivating functional proteins that are in the plasma membrane. On the other hand, HOCl can penetrate the lipid bilayer since it is uncharged and has relatively smaller size, which allows it to inactivate microbial cells from inside the cell, by what is believed to be inhibition of enzymes essential for microbial growth as well as damage to the DNA [2]. A major reason why HOCl has better disinfection power compared to OCl^- is because of the differences in where the inactivation of the microbial cells happens: HOCl can inactivate cells from inside the membrane, while OCl^- is only able to inactivate cells from outside the cell.

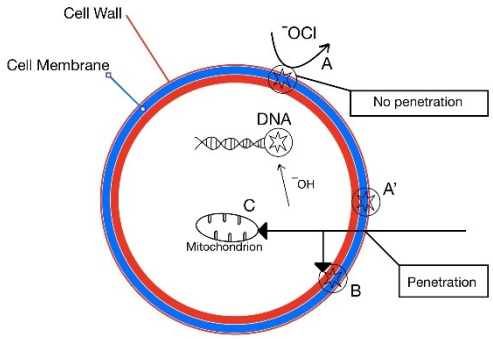

Figure 2. Mechanism of HOCl invasion of negatively-charged pathogen membranes [2].

Much of the previous research on HOCl as a disinfectant has focused on its liquid application, where the bacteria or virus is directly immersed in the solution. This research focuses on the fogging of HOCl solution as a means to disinfect a large space in a short amount of time. Fogging involves aerosolizing the liquid, usually through some

device such as an ultrasonic fogger or humidifier, and dispersing the fog throughout an open space. The exact methods and apparatus used will be discussed in further detail in the following sections.

2. Experimental Methods

2.1. Solution Preparation

Hypochlorous acid was produced using two home units available to consumers to buy and use: the EcoloxTech Eco One and RIPPO Sprayer. 1 L of solution was generated using the Eco One by combining 1 L of tap water with 2 g of non-ionized salt and 1 teaspoon of 5% distilled white vinegar in the unit and running two consecutive 10-minute cycles, as instructed by the manufacturer. 300 mL of solution was generated using the RIPPO by combining 300 mL of tap water with 22.5 g of non-ionized salt and allowing the unit to run for two consecutive 10-minute cycles, as also instructed by the manufacturer. The solution produced from each unit was stored in separate opaque bottles away from sunlight. The third solution was purchased from EcoLogic Solutions located in Brooklyn, NY. The free available chlorine concentration (FAC) of each solution was measured by titration, and pH was measured using a digital pH meter.

(a) (b)

Figure 3. (a) Eco One and (b) RIPPO.

2.2. Preparing Bacterial Cultures

E. faecalis (ATCC® 19433™) was grown in Brain Heart Infusion Agar (BHI, Difco™) for 24 hours at 37°C. Cells were removed from the plates using a sterile inoculating loop and the bacteria were transferred into a test tube containing 10 mL phosphate-buffered saline (PBS). This tube was vortexed for 30 seconds to ensure all cells were in suspension. A sample of the vortexed bacterial solution was diluted 1:100 and the optical density (OD) of this diluted solution was determined at 600 nm using a spectrophotometer. The bacterial suspension in the original tube was adjusted using more PBS or bacteria until a 1:100 dilution yielded an OD of 0.100, which is approximately 1 x 10^{10} cells per mL. Fifty µL of the adjusted concentrated bacterial suspension was spread onto each of three aluminum squares (approximately 1 square inch). The aluminum squares were placed into Petri dishes, without lids, and allowed to dry for approximately 1 hour at 37°C until a dry film was observed on the surfaces of the aluminum squares.

2.3. Fogging Procedure

Two of the three prepared aluminum squares were fogged and compared to a third untreated (not fogged) control. Once fogging began, the control was processed to determine the initial bacteria or virus populations. The samples to be fogged were oriented vertically in a desiccator (VWR Type 250) as shown in Figure 4. Hypochlorous acid was pumped into an ultrasonic fogging machine (Contronics HU-45 Humidifier) and then fogged into the desiccator in continuous streams with an average volumetric flow rate of 0.0556 L/min and particle size between 1 and 3 micron [14].

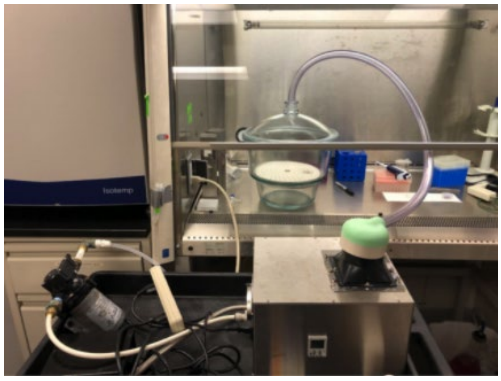

Figure 4. Experimental fogging apparatus.

2.4. Bacteria Quantification (Post-Fogging)

The fogged samples and un-fogged control were assessed for the number of living, or surviving, bacteria cells. Each sample was immersed individually in a 50 mL test tube containing 5 mL of PBS and vortexed for 5 minutes to suspend all the bacterial cells on the aluminum plate in solution. Then, six 1-in-10 serial dilutions, in PBS, were made and 0.1 mL of each dilution was spread, in triplicate, on BHI plates which were then incubated for 24 hours at 37℃. After incubation, only the dilution plates containing 30 – 300 colony forming units (CFUs) were counted. When taking into consideration the inoculum dilution spread onto counted plates, the average +/- standard deviation of bacterial cells on the control and fogged samples was calculated. The number of surviving fogged bacteria was reported, relative to the untreated control, as the log-reduction of cells caused by the fogging treatment.

2.5 Cell Culture

MDCK-2 (ATCC® CRL-2936™) cells were cultured and grown in OptiPRO™ SFM (Gibco) with 1x Glutamax (Gibco) and 100 units/mL penicillin and 100 µg/mL streptomycin. H1N1 PR8 (ATCC® VR-95™) influenza virus were cultured in MEM (Gibco) with 0.2% BSA (Sigma). This step was necessary as viruses need a host cell in order to replicate.

2.6. Virus Growth and Infection

50 µL of virus solution was put on the surface of the aluminum samples and dried for 1 hour at 25℃. The samples were fogged according to the same fogging procedure done for the bacteria tests. Post-fogging, the viruses were collected and vortexed with 5 mL of medium. 250 µL of virus solution was added into each well after serial dilution from 10^{-1} to 10^{-6}. The well plates were placed on the rocker for 30 minutes at room temperature. After rocking, the well plates were placed in the incubator at 37°C and 5% CO_2 for 1 hour. Then virus solution was replaced with MEM medium with 0.3% tragacanth gum (Sigma) and 0.2% BSA. After 4-day incubation at 33°C and 5% CO_2, the samples were stained with 1% crystal violet (Sigma). A plaque assay was used to count the number of infectious viral particles present after fogging compared to the untreated (not fogged) control.

3. Results and Discussions

3.1. Fogging Tests

Previous experiments conducted by Feng et al. have shown that continuous fogging is more efficient than pulse fogging in terms of the time needed to achieve a ≥5-log reduction in bacteria and ≥3-log reduction in virus, which is required by the EPA in order to claim disinfection [15]. The specific EPA guideline is for these log-reduction targets to be met within less than 10 minutes of contact time. Continuous fogging implies that the sample is fogged once for a set period of time in the beginning and then allowed to rest, whereas pulse fogging implies that bursts of fog are introduced into the desiccator according to set intervals. Furthermore, it was found in these studies that when samples were oriented vertically, the bacteria or virus became harder to kill due to the solution not being able to collect on the surface as easily. For these reasons, the procedure followed here involved testing the most efficient fogging method (continuous fogging) against the more difficult orientation (vertical). Aluminum was used because it is a common metal and surface, and also is hydrophilic, which allowed the bacteria and virus solutions to dry relatively quickly. The exact grade of aluminum is unknown as it was provided as scrap metal from the campus machine shop.

Overall, the commercial solution provided by EcoLogic Solutions was the most effective in killing *E. faecalis* and H1N1 influenza virus, yielding log reductions in these pathogens of 6.59 and 4.90, respectively, after 5 minutes of continuous fogging. Out of the two solutions produced by the home units, the Eco One solution performed better than the RIPPO solution against both pathogens, but still not as well as the EcoLogic solution. However, the Eco One did achieve notable log reductions in *E. faecalis* and H1N1 of 4.21 and 5.36 after 5 and 7.5 minutes of continuous fogging, respectively (Figures 5 and 6). Although the Eco One did not meet the standards set out by the EPA, the log reductions observed were fairly close to the targets of ≥5-log reduction in bacteria and ≥3-log reduction in virus and most likely could have been met with a few more minutes of additional fogging.

Where the EcoLogic and Eco One solutions really differ is in their chlorine concentration. In this pH range of 4-5, HOCl is the predominant chlorine species as shown in Figure 1. Therefore, the difference in efficacy between these two solutions can be attributed to the amount and type of free available chlorine (FAC) that they contain. This is measured through chlorine concentration, and these concentrations are given in the legend of Figure 5. There is almost a 200 ppm difference in chlorine concentration between the EcoLogic and Eco One solutions, which is likely why there is improved performance with the EcoLogic solution. Because the pH of the EcoLogic solution is on the higher end of the 4-5 range, it likely contains some additional FAC in the form of OCl^-, which although is known to be less effective than HOCl, does possess some disinfecting properties.

The RIPPO solution possessed comparatively little disinfecting abilities, achieving no more than a 1-log reduction in any of the performed tests. Its high pH (pH 8-9) and chlorine concentration (~750 ppm) is indicative of the fact that the solution contains OCl^-, the less effective form of free available chlorine. It is important to note that although less effective, the RIPPO was not designed for a fogging application, rather it was designed to be sprayed directly from the bottle (see Figure 3b). It has been previously found by Feng et al. that fogging can reduce the initial chlorine concentrations by up to 50%. Therefore, it is likely that any disinfecting ability that the RIPPO had in the form of OCl^- was eliminated by the fogging procedure. Furthermore, it should be noted that the base bacteria used for testing by the EPA are different and therefore possess different resistances to such treatments (they use *Staphylococcus aureus* and *Pseudomonas aeruginosa* and do not yet possess a standard procedure for fogging) [15].

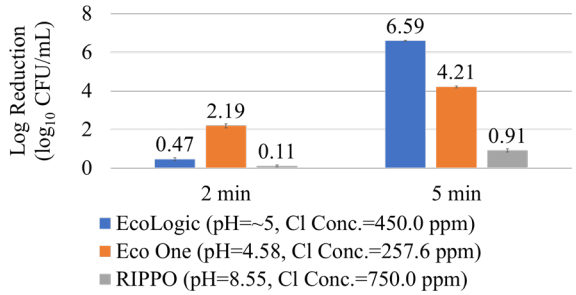

Figure 5. Reductions in *E. faecalis* after 2 and 5 minutes of continuous fogging, followed by 5 minutes resting in the desiccator.

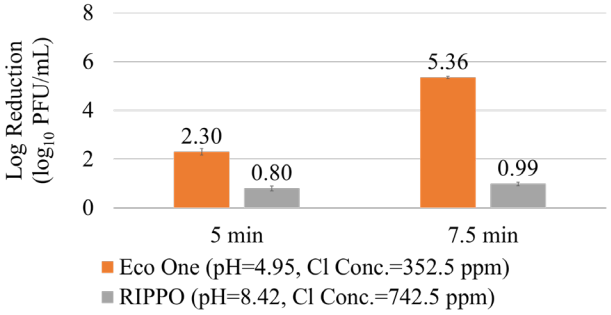

Figure 6. Reductions in H1N1 influenza virus after 5 and 7.5 minutes of continuous fogging, followed by 5 minutes resting in the desiccator.

3.2. Ultraviolet-Visible (UV-Vis) Spectroscopy Characterization Tests

As mentioned previously in this report, HOCl is approximately 80 times more effective than bleach (known chemically as sodium hypochlorite or NaOCl) in surface disinfection [11]. Due to the difference in efficacy between the three solutions, UV-Vis spectra were taken to characterize and compare the different solutions to bleach. Figure 7 shows the UV-Vis spectra of lab-grade bleach and the RIPPO solution. While not identical, the two spectra show distinct similarities, with a gradual rise and fall in absorbance and a maximum peak around 290 nm. Further comparison of the RIPPO spectra with NaOCl standards published online show even stronger similarities [16]. These results again suggest that the RIPPO unit is producing bleach and therefore may not be suitable for disinfection.

Figure 8, on the other hand, shows the UV-Vis spectra of the EcoLogic and Eco One solutions. The range of wavelength in this graph was reduced to 200-350 nm because outside of this range existed no notable spikes or trends in absorbance. However, the fact that the two spectra follow somewhat similar trends (from right to left) and are clearly different from the spectra in Figure 7, provides evidence that the EcoLogic and Eco One solutions are characterized by something other than bleach, likely hypochlorous acid.

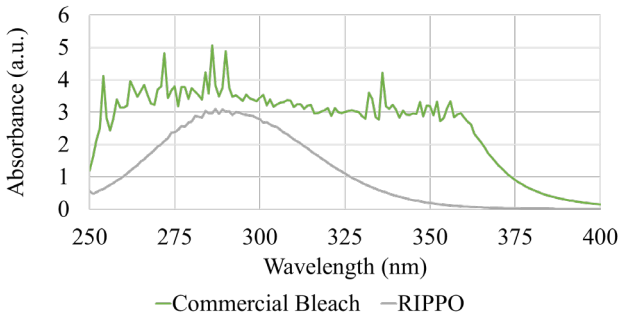

Figure 7. Ultraviolet absorption spectra of lab-grade commercial bleach and the RIPPO solution.

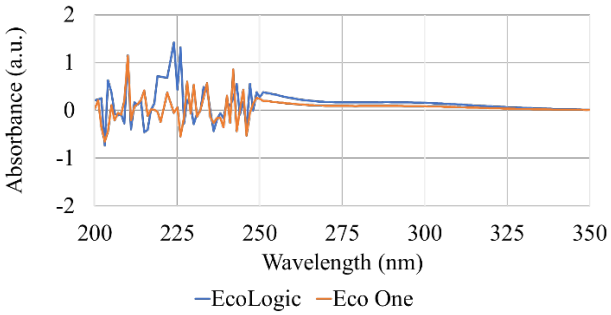

Figure 8. Ultraviolet absorption spectra of the EcoLogic and Eco One solutions.

3.3 Solution Stability Tests

The stability of the Eco One solution in terms of pH and chlorine concentration was tested to see how it compared with the stability of the EcoLogic solution, which has previously been found by Feng et al. to be very stable (it took a total of 50 days to start seeing significant, defined as >10%, changes in these numbers). Table 1 shows the pH and chlorine concentration (in ppm) of the Eco One solution over a three-week period. The solution was found to be stable, showing only a ~2.4% and ~1.4% decrease in pH and chlorine concentration, respectively, over the 3-week period. These decreases are within reasonable experimental error and likely resulted from the inherent inaccuracies in the pH and titration methods used. This result is important because if this solution is to be used commercially, it should have a long-shelf life.

Table 1. Stability of the Eco One solution over a three-week period.

Time (days)	pH	Cl Conc. (ppm)
0	4.95	352.5
7	4.84	350.0
14	-	-
21	4.83	347.5

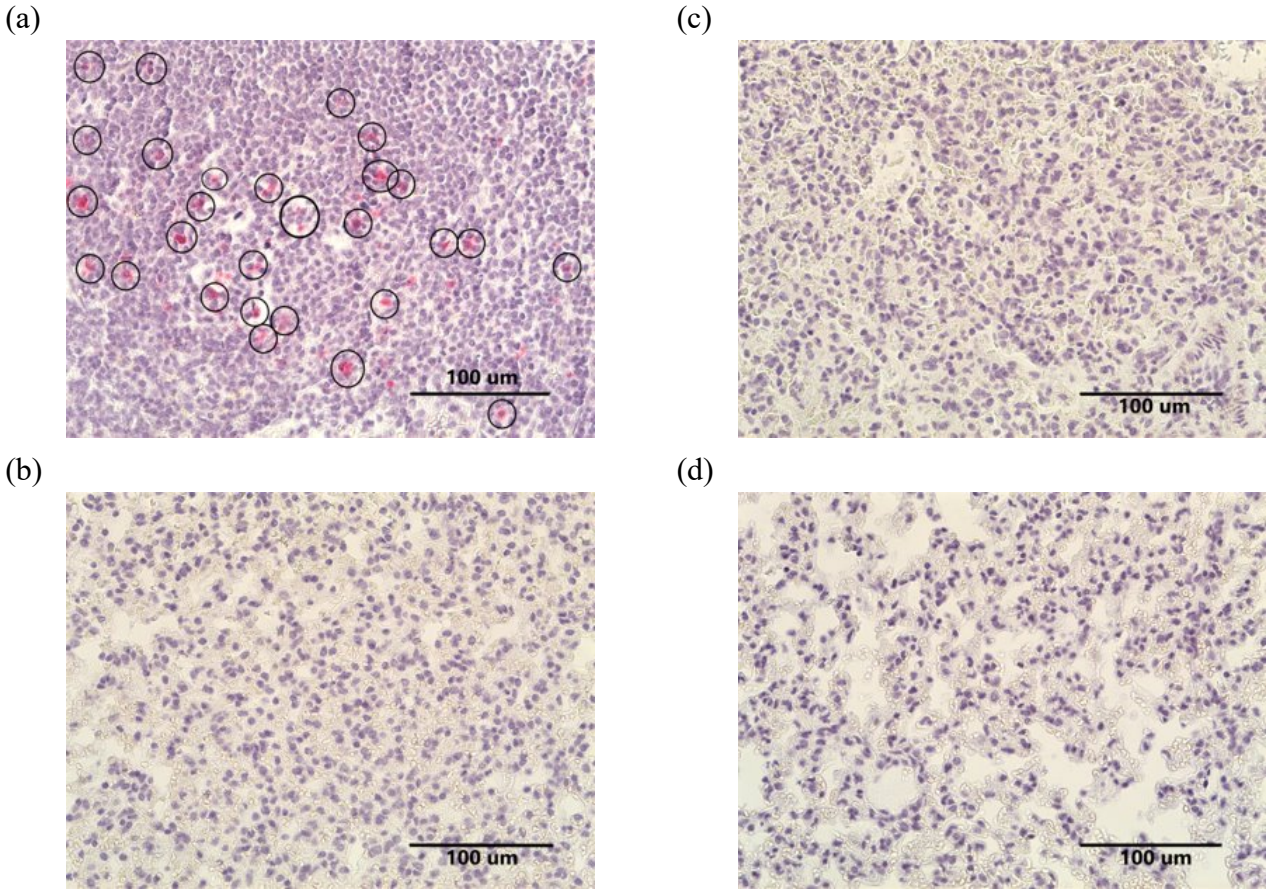

Figure 9. Active caspase-3 antibody staining for evidence of apoptosis from the lung tissue of mice. Images taken at 40x magnification. (a) "Positive" control 30% H_2O_2. (b) "Negative" control DI water. (c) Eco One solution. (d) RIPPO solution.

3.4. Cytotoxicity Tests

According to several safety data sheets (SDSs) published online, HOCl is non-toxic and possesses few safety hazards [17,18]. At high concentrations, HOCl can cause slight irritation to the eyes, skin, and lungs if inhaled. However, in general, none of these hazards are life-threatening and can usually be treated without hospitalization. In fact, HOCl is used in many pools for chlorination and is even safe to use for rinsing fruits and vegetables [2]. The purpose of performing cytotoxicity tests then was to determine if the solution from the RIPPO unit was toxic, as previous results have suggested it is producing bleach (NaOCl), which has the ability to damage living cells. For these tests, 30% hydrogen peroxide (H_2O_2) was used as the positive control (will kill the cells), and deionized (DI) water was used as the negative control (will not kill the cells). The two homemade solutions produced by the Eco One and the RIPPO were tested and compared to these two controls to complete the assay.

Lung tissue from already-sacrificed mice were dipped into equivalent amounts of these solutions for 10 seconds and then sent to a pathology lab to be spread and stained for active caspase-3 as evidence of apoptosis. Caspase-3 is a widely-studied apoptotic protein and suitable for a preliminary test like this one. Lung tissue was used

as it is believed that inhalation is the most dangerous form of chlorine exposure, especially if it exists as chlorine gas (Cl_2). The bright red spots circled in black in Figure 9a indicate the activation of caspase-3 and initiation of apoptosis after exposure to the positive control. The blue dots, on the other hand, represent intact nuclei, or nuclei that survived the treatment with 30% H_2O_2. This was expected, as was the result for the negative control that showed no red spots.

The fact that exposure to the Eco One and RIPPO solutions showed no evidence of apoptosis like the negative control is a promising initial result that the solutions used were non-toxic. However, these results do not fully dismiss the fact that the RIPPO solution could be producing bleach, nor do they claim that these solutions are completely safe. As mentioned previously, chlorine in the form of chlorine gas (Cl_2) presents the largest risks to humans and therefore the cytotoxic effects of the fogged solutions should be tested, when there is the greatest likelihood of producing gaseous chlorine.

4. Conclusion

Hypochlorous acid, due to its effectiveness against certain pathogens in the past, has been a popular area of research with the ongoing pandemic. One benefit of hypochlorous acid is the ability to produce it from common household materials (water, non-iodized salt, and vinegar). This research tested the effectiveness of a fogged application of both purchased commercial solution and two homemade solutions in their reduction of counts of *E. faecalis* and H1N1. Both the EcoLogic and Eco One solutions were able to achieve ≥99.99% reductions in *E. faecalis* and H1N1 after being fogged continuously for less than 10 minutes of fogging time, the standard disinfection time established by the EPA [15]. However, the RIPPO home unit was not effective, yielding no greater than a 1-log reduction in any of the performed tests. The pH of the RIPPO solution was measured and found to be between 8 and 9, which is likely due to the production of bleach in the unit, suggesting that pH plays a key factor in disinfecting ability. Fogging is a promising method of application because it allows larger areas to be covered in a much shorter amount of time compared to a liquid application, and can be done without sacrificing effectiveness. HOCl is cheap, easy, and safe to use, which makes it a suitable choice for fogging.

5. Acknowledgements

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