aerobiology

MDPI

Article

Inactivation of Avian Influenza Virus Aerosol Using Membrane-Less Electrolyzed Water Spraying

Shinhao Yang [1,*], Chi-Yu Chuang [2,*], Hsiao-Chien Huang [1] and Wei Fang [3]

[1] Environmental Sustainability Lab, Center for General Education, CTBC Business School, No. 600, Section 3, Taijiang Boulevard, Annan District, Tainan 709, Taiwan; f87541110@ntu.edu.tw
[2] Department of Occupational Safety and Health, Chang Jung Christian University, No. 1, Changda Road, Gueiren District, Tainan City 711, Taiwan
[3] Department of Bio-Industrial Mechatronics Engineering, National Taiwan University, Taipei 10617, Taiwan; weifang@ntu.edu.tw
* Correspondence: shinhaoyang@ntu.edu.tw (S.Y.); cychuang@mail.cjcu.edu.tw (C.-Y.C.)

Abstract: Avian influenza virus (AIV) can have a serious impact on both human and animal health. In this study, we used an environmentally controlled chamber and a hemagglutination assay (HA) to evaluate the ability of membrane-less electrolyzed water (MLEW) spraying to inactivate H6N1 AIV aerosol. MLEW was generated by electrolyzing sodium chloride solution, and then sprayed into the chamber at free available chlorine (FAC) concentrations of 50, 100 and 200 mg/L by means of high-pressure air pumping to inactivate airborne H6N1 AIV aerosols individually. The results showed that MLEW spraying effectively neutralized H6N1 AIV aerosol. In addition, the virucidal ability of MLEW increased as the FAC concentration increased. Five minutes after MLEW spraying at an FAC concentration of 200 mg/L, the H6N1 viral HA titer decreased from 2^4 to 2^0. Our work provides important new evidence of the value of spraying with MLEW disinfectant to protect against AIV, which may be further applied for indoor decontamination purposes to promote animal and human health.

Keywords: avian influenza; electrolyzed water; bioaerosol; H6N1; health; zoonosis; spray

Citation: Yang, S.; Chuang, C.-Y.; Huang, H.-C.; Fang, W. Inactivation of Avian Influenza Virus Aerosol Using Membrane-Less Electrolyzed Water Spraying. *Aerobiology* **2023**, *1*, 70–81. https://doi.org/10.3390/aerobiology1020006

Received: 31 August 2023
Revised: 23 October 2023
Accepted: 25 October 2023
Published: 30 October 2023

1. Introduction

Avian influenza viruses (AIV) are enveloped negative-strand RNA influenza A viruses of the *Orthomyxoviridae* family [1,2]. Influenza A viruses are divided into various subtypes based on sixteen antigenically distinct hemagglutinin (HA) antigens (from H1 to H16) and nine neuraminidase (NA) antigens (from N1 to N9). Avian influenza viruses of all subtypes are able to induce a low-pathogenicity avian influenza (LPAI) in avian populations, including chickens, ducks and turkeys [1,3,4]. Typically, LPAI presents as a mild respiratory distress with low mortality rates in poultry; however, LPAI infection may result in significant mortality rates when accompanied by other bacterial or viral infections [3,5]. H6N1, a subtype of influenza A virus, has been isolated from migrating birds and domestic poultry on many continents. H6 subtypes of AIV have been detected frequently in the live poultry markets of Asia [6]. Infection with H6N1 has been prevalent in domestic chickens in Taiwan since 1972 [2]. A H6N1 virus was also isolated during a "bird flu" incident in a poultry market in Hong Kong in 1997 [7]. A H6N1 LPAI outbreak that, within a period of two months, affected four productive brown layer flocks across three farms in the Netherlands was reported in 2019. The infections were associated with egg production drops of up to 74%, pale eggshells and persisting high mortality rates of up to 3.2% per week. Three flocks were slaughtered prematurely as they were not profitable anymore [5]. The receptor-binding analysis of H6 subtypes of AIV demonstrated a clear threat to human health via aerosol transmission [6]. Poorly ventilated spaces are conductive to the spread of airborne pathogenic viral aerosols, because people and animals may be repeatedly in

contact with airborne pathogens for extended periods of time. The aerosol transmission of AIV thus poses significant health risks to both human and avian populations [8–10].

In recent years, there has been an increasing demand for air-cleaning technology that can lower the risk of disease transmission by reducing the number of viral pathogenic aerosols in indoor environments. Consequently, a variety of physical and chemical air-cleaning technologies have been developed, and their applicability evaluated. Filtration is a commonly used method to physically separate viral aerosols from the atmosphere using various types of filters (e.g., polypropylene fiber and polycarbonate fiber) [11]. In Wenke's 2018 study, filtration technology was adopted for the supply and recirculation of air in the existing ventilation system of an animal feeding house. Barns equipped with recirculating air filtration modules recorded the lowest total concentrations of dust, and lung health was thereby enhanced, though antibodies against H1N1 and H3N2 influenza could still be detected in all animals. This air filtration system can easily be implemented and can enhance the lung health of animals in animal feeding areas [12]. However, accumulations of pathogenic aerosol on the surfaces of fibers may remain viable and contagious, given sufficient moisture and a supply of nutrients. For this reason, personal protection procedures are still necessary, and germicidal measures are also required against collected microorganisms on filters if the continued use of filtration is to be carried out safely. Photocatalysis uses light in combination with an oxidation process to destroy the cell membranes of microorganisms. Guillard and his colleagues applied TiO_2 photocatalysis to achieve a reduction in A/H5N2 AIV aerosol of about 0.7–3.1 log in a 30 min flow-through reactor experiment [13]. However, the intermediate products of the photocatalytic process, including organic and inorganic species, may prevent the desired disinfection reaction and instead result in a harmful effect. Ultraviolet light is a popular physical disinfection method in which microorganisms are inactivated by inhibiting the replication of their microbial DNA or RNA. Ultraviolet light has also been used to evaluate the susceptibility of influenza virus. McDevitt et al. used H1N1 influenza A virus aerosol to characterize its susceptibility to UV-C germicidal irradiation at a wavelength of 254 nm in a benchtop chamber. The results showed that UV-C dosage and relative humidity both play important roles in inactivating influenza A virus aerosol [14]. However, UV energy applied to a human or animal body, whether directly or by reflection, has the potential to cause dermal or ophthalmic damage. Safety precautions must be strictly observed when utilizing UV germicidal apparatus for practical application purposes [15].

Another method of cleaning indoor environments, which is now a mature and commonly used approach, involves the release of liquid or gaseous chemicals into the air to directly neutralize pathogenic aerosols. This may be achieved by various means, including spraying, fogging, fumigation and vaporizing. However, many chemical disinfectants are harmful to the bodies of animals, and they may also corrode objects. Such disinfectants include peracetic acid, NaOCl, hydrogen peroxide, ozone and chemical dioxide. The use of such chemicals is strongly regulated, and they should not be used in spaces occupied by animals or humans [15,16]. In contrast, natural chemical disinfectants, including botanical extracts, are potentially safe antimicrobial agents that may be regarded as low-toxicity or non-toxic products when compared with synthetic compounds such as bleach or peracetic acid. In one study, tea tree oil and eucalyptus oil were both found to exhibit > 95% antiviral activity against H11N9 influenza A virus between 5 and 15 min after application. A higher rate of inactivation effectiveness (99%) required a longer exposure time [17]. However, the inactivation performance of botanical disinfectants may be inconsistent in practice, as oil composition varies from batch to batch and with different growing conditions of plantations. In summary, although some physical and chemical air-cleaning technologies have demonstrated virucidal activity against influenza virus, a technique which can deliver an effective, low-cost, non-toxic and safe means of neutralizing AIV aerosol is still being sought today.

Electrolyzed water is generated by the electrolysis of a saline solution (e.g., NaCl or KCl) in a container fitted with anodic and cathodic electrodes, either within or without

an ion-selective permeating membrane. After the electrolyzing process, the chloride ions are converted to high oxidation/reduction potential (ORP) and free available chlorine (FAC), consisting of hypochlorous acid (HOCl), chlorine gas (Cl_2) and hypochlorite ion (OCl^-) compounds, resulting in strong antimicrobial activity [18]. The distribution of the above-mentioned chlorine-related compounds in electrolyzed water is pH-dependent and affects its antimicrobial performance. Traditional strong acidic electrolyzed water is generated by an electrolyzer within a permeating membrane, and has a good inactivating effect against most known pathogenic microorganisms due to its low pH (2–4), high ORP (>1000 mV) and higher proportion of chlorine—in gaseous form, and thus easily lost to the air—than HOCl. However, near-neutral electrolyzed water (pH 6–8) exhibits a stronger antimicrobial capacity on account of its higher proportion of HOCl [19,20]. In addition, the pH level of near-neutral electrolyzed water means that it is not as aggressively corrosive as acidic electrolyzed water, resulting in less damage to metal surfaces, and reduced irritation of the hands [21]. Near-neutral electrolyzed water can be generated simply, safely and economically by electrolyzing sodium chloride in an electrolyzer without a semi-permeable membrane (i.e., a membrane-less process). As a result, the use of membrane-less electrolyzed water (MLEW) has become popular in food and agricultural industries in recent years [22–24].

The virucidal activity of near-neutral electrolyzed water has been investigated in previous studies. Tamaki et al., 2014, found that neutral electrolyzed water with FAC > 43 ppm was capable of reducing the viral titer of H5N1 and H9N2 AIV by >5 log, one minute after mixing in test tubes. The minimum concentration of FAC required for neutral electrolyzed water to exhibit virucidal activity was estimated at around 40 ppm [25]. The authors of [21] carried out fogging treatment on ceramic tile and stainless steel using FAC 200 mg/L electrolyzed water, and recorded an RNA titer reduction of 99.9% in human norovirus, murine virus and MS2 phage. In another study, no trace of hepatitis B virus (HBV) DNA was detected on endoscopes used by patients after cleaning with electrolyzed water, thus revealing an inactivation efficacy comparable with that of 2% alkaline glutaraldehyde [26]. Similarly, the authors of [23] found that blood-borne infectious agents, including HBV and human immunodeficiency virus, were totally disinfected using FAC 4.2 mg/L electrolyzed water during in vitro experiments. In the enclosed chamber tests conducted by Urushidani and his colleagues [27], dry fog containing HOCl (FAC 125, 250 and 8700 ppm) and hydrogen peroxide (1410–56,400 ppm) was generated with an atomizing nozzle to inactivate influenza A virus, which was loaded on a 96-well plastic microplate and air-dried. The results showed that dry fogging with HOCl and hydrogen peroxide inactivated influenza A virus. In another study [24], a viral solution containing a titer of 2.8×10^6 $TCID_{50}$ influenza A virus was neutralized to below a detection limit of 1.58×10^2 $TCID_{50}$ after exposure to HOCl dry fogging for 10 min (FAC 250 ppm) and 15 min (11,280 ppm), suggesting that HOCl has high potential for application in environmental surface disinfection.

Previously, the virucidal efficacy of electrolyzed water against influenza and other avian viruses has been demonstrated in vitro by means of surface and test tube experiments. However, an application for directly inactivating AIV aerosol has yet to be fully developed. Therefore, the objective of this study was to assess the airborne virucidal efficacy of near-neutral electrolyzed water generated using a membrane−less process (membrane-less electrolyzed water, MLEW) against AIV aerosol. To this end, we evaluated the inactivation ability of mist-sprayed MLEW against H6N1 influenza aerosol in an enclosed and environmentally controlled chamber.

2. Materials and Methods

2.1. Generation of Membrane−Less Electrolyzed Water

In this study, MLEW was utilized as a mist-spray disinfectant to directly inactivate the H6N1 AIV aerosol, so that its applicability for indoor air-cleaning could be better understood. The MLEW was generated by a hand-made, membrane-less electrolyzing device based on our previous studies (see Figure 1, described in Section 2.3). Saturated NaCl

solution (6.15 M) was poured into a 1000 mL plastic container, to which metal electrodes were fitted. These were platinum and titanium base plates of 10 × 2 cm^2 size which served as cathode and anode, respectively [28,29].

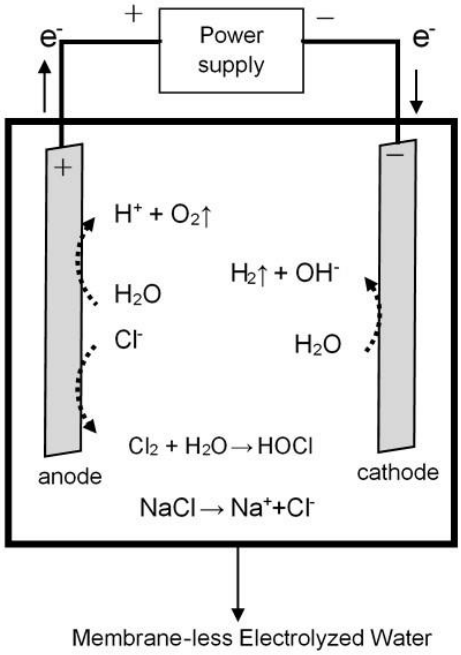

Figure 1. Schematic diagram of the membrane−less electrolyzing device.

After undergoing 30 min of electrolyzing process with a current density of 25 Amp/dm^2 (ampere per decimeters, ASD), the NaCl solution was converted to MLEW concentrate solution (FAC concentration up to 10,000 mg/L). The FAC concentration of MLEW was quantified using the N, N-dimethyl-p-phenylenediamine (DPD) colorimetric method, using a portable spectrometer (DR2800, HACH, Loveland, CO, USA). The pH of the MLEW was measured using a pH meter (CyberScan pH 510, Eutech, Inc., Singapore).

The MLEW concentrate solution was then diluted with deionized water (Milli-Q, Millipore, Billerica, MA, USA) to FAC 50, 100 and 200 mg/L, thus producing a ready-to-spray MLEW disinfectant.

A 100 mL amount of this ready-to-spray MLEW disinfectant (with pH values ranging from 7.3 to 7.5) was then pumped via an orifice nozzle of 8 µm diameter under 70 kg/cm^2 air pressure, and sprayed into the enclosed chamber (shown in Figure 2, described in Section 2.3) to inactivate suspended H6N1 viral aerosol by direct airborne contact.

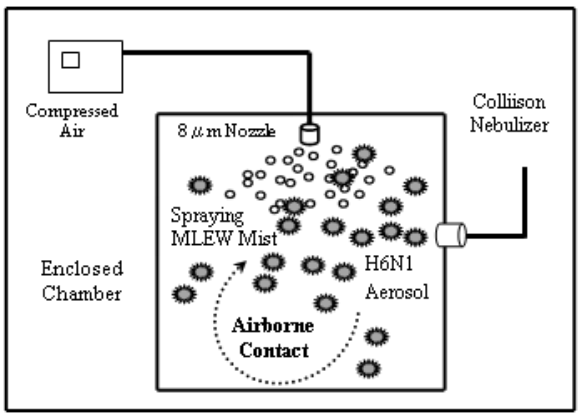

Figure 2. Schematic diagram of MLEW mist−spraying in the enclosed chamber.

2.2. H6N1 Viral Preparation and Viability Assay

H6N1 avian influenza was selected as a test viral strain; this was provided by Zoonoses Research Center, National Taiwan University. The H6N1 avian influenza strain was inoculated and propagated in special pathogen-free embryonated chicken eggs, which were 9–11 days old. Inoculated SPF eggs were incubated at 37 °C and candled twice a day for 7 days [30]. The infectious allantoic fluid (AF) was harvested and stored at −70 °C prior to usage. Both viral viability and infectious titer were used as indexes to evaluate the inactivation ability of MLEW in this study. Infectious AF was collected and assessed using a hemagglutination assay (HA) examination based on the WHO's guidelines for the diagnosis and surveillance of animal influenza, published in 2002 [31]. A 25 μL amount of phosphate-buffered saline buffer (PBS, pH 7.2) was added into each well of a V-bottom 96-well microtiter plate. Then, 25 μL of infectious AF was placed in the first well of each row. Two-fold serial dilution across the plate was then carried out by transferring 25 μL from the first wells in lettered rows to wells in the next rows. Then, 25 μL of fresh PBS was added to each well. Finally, a 25 μL amount of chicken red blood cell (RBC) suspension was added to each well, and the plate was allowed to stand for 45 min at 25 °C until settling was complete.

A settled pattern was determined as positive (i.e., the virus remained infective and complete hemagglutination had taken place) if a hazy film was observed in the bottom of the well. In contrast, a negative result (i.e., the virus was noninfective, and RBC was not agglutinated) was determined when a sharp button of RBC was observed in the well. The settling endpoint of serial dilution was also identified. The highest dilution of virus, which caused complete hemagglutination, was considered the HA titer endpoint—the HA titer being the reciprocal of the dilution of virus in the last well with complete hemagglutination. For example, if a one-in-sixty-four (1/64) dilution contained 1 HA unit, the HA titer of the infectious AF was therefore the reciprocal of 1/64 = 64 = 2^8.

2.3. Environmentally Controlled Experimental Setup

The environmentally controlled experimental setup consisted of a stainless steel enclosed chamber (80 × 80 × 80 cm^3, shown in Figure 2), a make-up air unit, an aerosol nebulizer, a charge neutralizer, an MLEW mist-spraying nozzle and a bioaerosol sampler, all of which were fabricated based on previous studies (shown in Figure 3) [11,29,32]. Before the experiment, the chamber was purged and well stabilized by HEPA-filtrated make-up air. The initial relative humidity inside the chamber was set to 30% by changing the ratio of the flow rates of both a dry gas stream and a humidified gas stream generated by a water vapor saturator. The relative humidity and temperature inside the chamber were measured using Q-trak (Model 8550, TSI Inc., Shoreview, MN, USA).

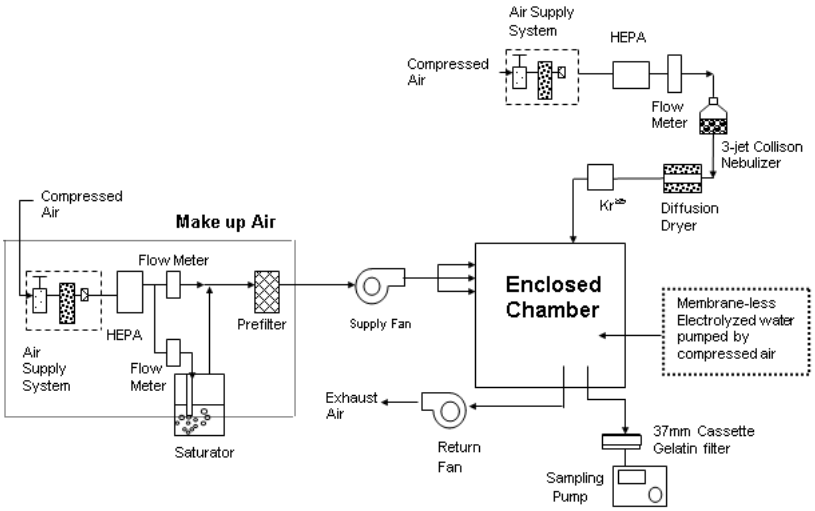

Figure 3. Schematic diagram of experimental setup.

The H6N1 viral test aerosol was generated by adding 0.1 mL of infectious AF and 50 mL of PBS buffer (final HA titer 2^9) into a three-jet Collison nebulizer (BGI Inc., Waltham, MA, USA), which was operated at a flow rate of 6 L/minute. The viral test aerosol was dried by the diffusion dryer. The dried test aerosol was then passed through a Kr-85 radioactive source, so that it was neutralized to the Boltzmann charge equilibrium. After passing through the charge neutralizer, the aerosol was delivered into the enclosed chamber so that it was suspended in the air. The sampling method for the H6N1 viral test aerosol was adopted from the manual of the American Conference of Governmental Industrial Hygienists, as well as from previous studies [33]. Air was drawn from the chamber by a Gilian sampler at 5 L/min through a manifold attached to an SKC 37 mm polyethylene cassette (No. 225-2050LF, KC Inc., Columbus, OH, USA) loaded with a gelatin filter (No. 225-9552, SKC Inc., Eighty Four, PA, USA). Airborne viral samples were collected at 30-minute intervals for natural deposition testing, and were collected at 5-minute intervals after 2 min spraying. Sampled gelatin filter was then dispersed into sterile PBS buffer at 30 °C for 15 min and processed using the HA examination described above. For the purposes of the study, natural deposition (without any physical or chemical disinfection intervention), pure water (sterile, and deionized for FAC-free treatment) and MLEW groups with FAC concentrations of 50, 100 and 200 mg/L were applied so that the inactivation efficacy of MLEW could be assessed 2 min after H6N1 viral aerosol nebulization. All experiments were repeated three times, and statistical values were expressed as the mean and standard deviation. A chi-square test was used to determine the difference in HA titers between various FAC groups. Values of $p < 0.05$ were considered as statistically significant differences in all data analyses.

3. Results

3.1. Natural Deposition of H6N1 Viral Aerosol inside the Enclosed Chamber

The H6N1 aerosol deposition effect caused by gravity in the enclosed chamber is shown in Figure 4. The initial airborne viral HA titer (0 min), prior to applying 60 min of aerosol nebulization, was 2^4. The HA titer declined to $2^{3.1}$ and 2^2 in the 30 and 60 min after viral nebulization, respectively. We set a lower relative humidity (30%) for the initial condition inside the chamber to prevent interference from particle condensation. It can be seen from Figure 4 that the viral aerosol exhibited a slowly declining deposition characteristic inside the chamber.

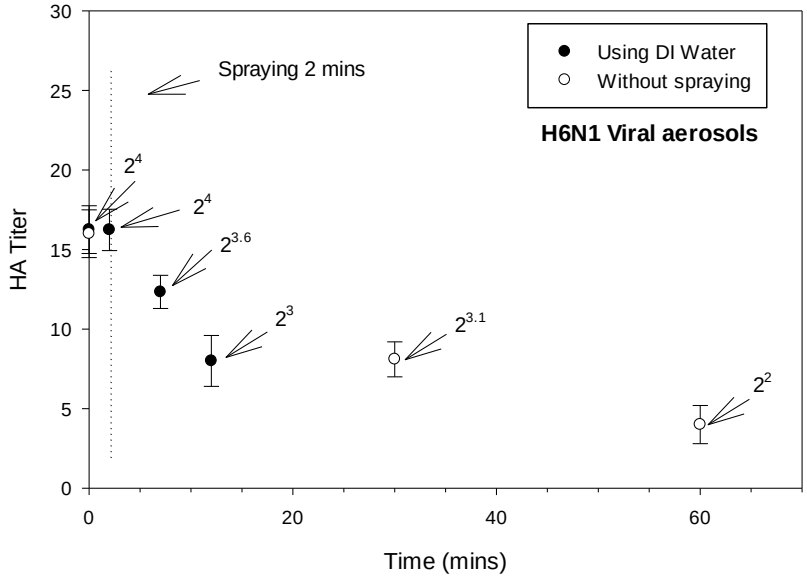

Figure 4. Natural deposition and pure water mist-spraying of H6N1 viral aerosol in the chamber.

3.2. The Inactivation Ability of Pure Water Spraying against H6N1 Viral Aerosol

Figure 4 also shows the viability of H6N1 viral aerosol under pure water spraying. The initial HA titer of the viral aerosol after nebulization was 2^4. Two minutes after pure water mist-spraying, the HA titer was still 2^4, indicating a non-significant inactivation effect. Finally, the HA titer of the viral aerosol decreased to $2^{3.6}$ and 2^3 after 5 and 10 min, respectively. These results suggest that pure water spraying may achieve the effect of cleaning contaminated air, without the need for any chemical disinfecting agent. Moreover, the relative humidity inside the chamber increased to 99.9% during pure water mist-spraying, further suggesting that the reduction of the HA titer may have been the result of the physical interception, coagulation and deposition of particles, rather than any chemical inactivation process.

3.3. The Inactivation Effect of MLEW Spraying against H6N1 Viral Aerosol

The viability of H6N1 viral aerosol under MLEW mist-spraying at FAC concentrations of 50, 100 and 200 mg/L is shown in Figures 5–7, respectively. It can be seen in Figure 5 that when MLEW was mist-sprayed at FAC 50 mg/L, the initial HA titer was 2^4. Two minutes after spraying, the HA titer decreased to 2^3. Ten minutes after spraying, the HA titer decreased to 2^1. FAC 50 mg/L MLEW spraying caused a statistically significant reduction in the HA titer of H6N1 viral aerosol inside the enclosed chamber ($p < 0.05$).

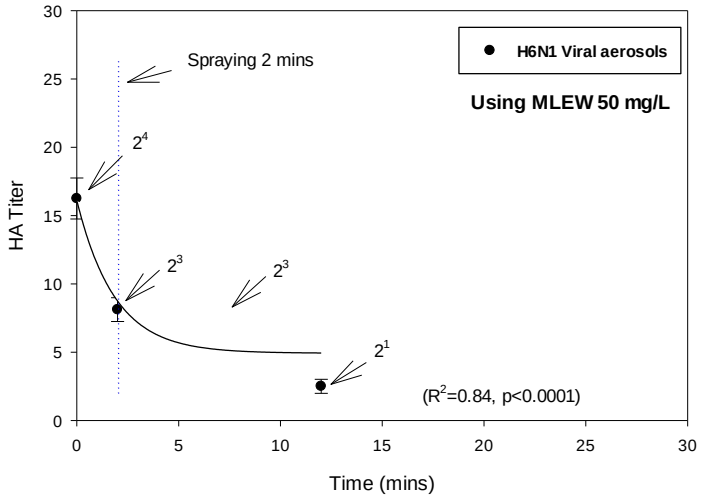

Figure 5. FAC 50 mg/L MLEW mist-spraying of H6N1 viral aerosol in the chamber.

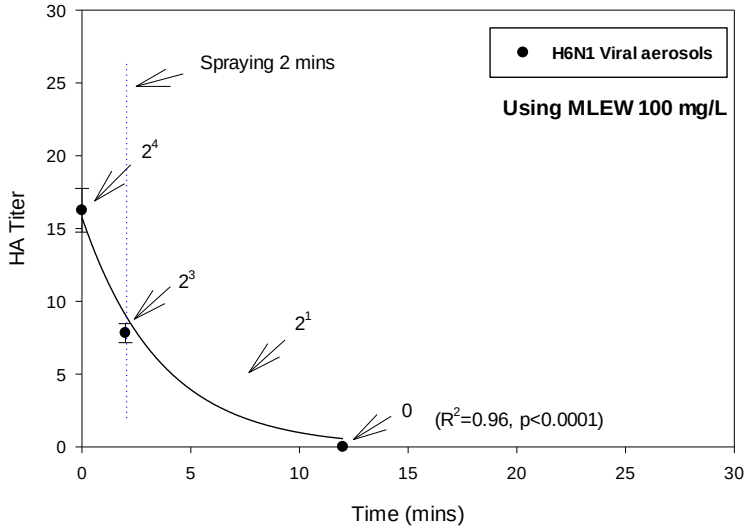

Figure 6. FAC 100 mg/L MLEW mist-spraying of H6N1 viral aerosol in the chamber.

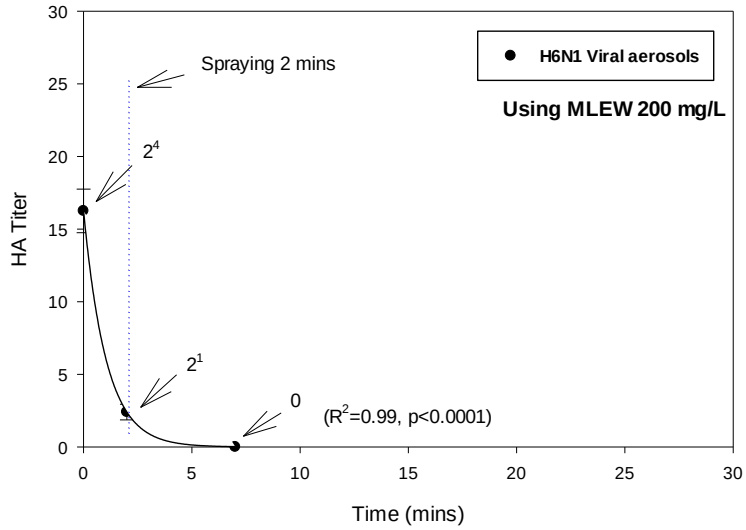

Figure 7. FAC 200 mg/L MLEW mist-spraying of H6N1 viral aerosol in the chamber.

Figure 6 shows the susceptibility of H6N1 viral aerosol to MLEW mist-spraying at FAC 100 mg/L. Again, the initial HA titer was 2^4. This decreased to 2^2 in the five minutes after spraying, and reached 2^0 after ten minutes, indicating that the viral aerosol was no longer infective. Similarly, with the FAC 50 mg/L group, FAC 100 mg/L MLEW spraying caused a statistically significant reduction in the HA titer of H6N1 viral aerosol inside the enclosed chamber ($p < 0.05$).

Figure 7 shows the susceptibility of H6N1 viral aerosol to MLEW mist-spraying at FAC 200 mg/L. Again, the initial HA titer was 2^4. However, at this level of FAC concentration, the viral aerosol became non-infective just 5 min after spraying. Similarly, with the FAC 50 and 100 mg/L group, FAC 200 mg/L MLEW spraying caused a statistically significant reduction in the HA titer of H6N1 viral aerosol inside the enclosed chamber ($p < 0.05$). Overall, when comparing the HA titer decaying trend across the pure water and MLEW mist-spraying tests, a significant susceptibility of H6N1 viral aerosol to MLEW can be identified. MLEW exhibited a strong ability to inactivate H6N1 viral aerosol through airborne contact, with higher FAC concentrations producing greater effects.

4. Discussion

The purpose of this study was to evaluate the inactivating ability of mist-sprayed MLEW against H6N1 aerosol in an indoor environment-simulating chamber. Firstly, and as illustrated in Figure 4, the natural depositions of H6N1 AIV aerosol were measured after nebulization in an enclosed chamber which simulated a room without any fresh air intake (see Figure 3). The initial HA titer of H6N1 AIV aerosol was 2^4. Thirty minutes after aerosol nebulization, the HA titer decreased to $2^{3.1}$. Sixty minutes after aerosol nebulization, the HA titer decreased to 2^2. Because there was no intervention by any chemical disinfectant, the viability of the AIV aerosol was not additionally affected. In this experiment, the airborne concentration of AIV aerosol was the only factor related to the HA titer. Thus, the decrease in the HA titer of the AIV aerosol could be attributed to natural deposition (including gravity, well-loss and desiccation inside the enclosed chamber). The further decline in HA titer from 2^4 to 2^2 indicated that about 50% of the AIV aerosol did not remain suspended in the air. These trends are also observed in our previous bacterial aerosol deposition studies [29,34].

However, even when the HA titer decreased from 2^4 to 2^2, the indoor air was still contaminated and infective, and suspended viral aerosol was still present even after 60 min. In our environmentally controlled chamber experiment, the relative humidity was set to 30% to prevent particle condensation. However, previous studies involving environmental factors have suggested that the transmission efficiency and viability of viral bioaerosols

decreases with increasing relative humidity [35,36]. This implies that the H6N1 viral aerosol is able to remain suspended in the air for longer time periods in areas with higher relative humidity, additionally increasing the risk of aerosol transmission.

Secondly, pure water was used to determine its effect on the viability of H6N1 AIV aerosol. Figure 5 shows that direct contact with pure water mist did not have any inactivating effect on AIV aerosol, because the HA titer remained at 2^4. Moreover, after 10 min of pure water spraying, the HA titer of AIV aerosol decreased to 2^3. This result suggested that, during this period of time, the environmental deposition of AIV aerosol was promoted by pure water mist-spraying, rather than any by any chemical inactivation. Particle aggregation and condensation may have occurred inside the enclosed chamber, and this may explain the promotion of deposition, because relative humidity increased to a level of 99% after pure water mist-spraying. Oswin et al., 2022, suggested that the loss of infectivity in virus aerosol at a high relative humidity is caused by the volatilization of CO_2 from bicarbonate buffers within droplets [37]. In general terms, water spraying may be described as a low-cost, non-residual technique which is commonly used to control dust levels in animal facilities. These results suggest that it might also be helpful in reducing the risk of aerosol transmission of AIV in indoor environments.

In the third part of this study, MLEW mist was delivered into the enclosed chamber to evaluate its inactivating ability against H6N1 aerosol. As can be seen in Figure 6, the HA titer of AIV aerosol decreased from 2^4 to 2^3 after 2 min of spraying with MLEW at FAC 50 mg/L. When Figure 6 is compared with Figure 5, it can be seen that the FAC 50 mg/L MLEW mist had a statically significant inactivating effect compared with pure water ($p < 0.05$). In previous studies, the minimum concentration of FAC in neutral electrolyzed water required for a virucidal effect was found to be approximately 40 ppm; this is consistent with the results of the present study [25,38]. In the 10 min after FAC 50mg/L MLEW spraying, the HA titer further declined to 2^1. This meant that, even 10 min after spraying, the FAC 50 mg/L MLEW remained suspended in the air and was not lost due to Cl_2 volatilization, indicating that a prolonged inactivating capacity of MLEW could also be expected.

In Urushidani's 2022 study, FAC 125 ppm HOCl fogging effectively achieved inactivating effects against influenza A virus over 5 min. All influenza A virus was neutralized after 15 min of fogging [27]. In this study, when the FAC concentration in MLEW was further raised to 100 mg/L, its inactivating efficacy also significantly increased. In the 5 min after MLEW mist-spraying, the HA titer decreased to 2^2, as can be seen in Figure 7. After 10 min, the AIV aerosol was totally neutralized and non-infective. This difference may have been caused by less chlorine loss in the MLEW airborne particles transmitted via high-pressure spraying rather than fogging [39]. A similar trend can also be observed in Figure 7. When MLEW spraying was carried out with an FAC concentration of 200 mg/L, an even stronger inactivating efficacy was revealed. The HA titer of AIV aerosol decreased to 2^2 in the 2 min after contact with MLEW mist; after 5 min, a zero figure was recorded. This result shows that spraying with FAC 200 mg/L MLEW delivers faster and stronger airborne inactivating efficacy against AIV aerosol compared to FAC concentrations of 50 and 100 mg/L, determined with the chi-square test (statistically significant, $p < 0.05$).

The germicidal characteristics of the electrolyzed water were obtained from free chlorine components (including ClO^-, HOCl, Cl_2). Although the distribution proportion of free chlorine components is dependent on pH value, some studies have suggested that the FAC concentration in the solution is the most important factor when determining the antimicrobial ability of neutral electrolyzed water [25,40,41]. In the disinfection study conducted in a henhouse field by Zhao et al., 2014, the antimicrobial component of MLEW may have been lost during spraying. The antimicrobial ability may decrease over the distance traveled by the mist due to FAC loss, which is exacerbated by higher air temperatures. In short, an adequate FAC concentration and contact time are the most important operating parameters to achieve effective airborne disinfection [39]. The operating parameters of the present study were as follows: an orifice nozzle of 8 μm diameter was used under 70 kg/cm^2 to

spray MLEW at a minimum FAC concentration of 50 mg/L MLEW. These parameters were based on our previous work on bacterial inactivation [28]. By such means, we obtained observable evidence of the ability to inactivate H6N1 AIV aerosol.

The US Environmental Protection Agency (US EPA) has recently sanctioned the utilization of HOCl as an antiviral disinfectant. A concentration of 200 ppm of HOCl was directly applied to cleanse the wounds of surgical patients, with no adverse effects reported [42]. Consequently, the concentration range (FAC 50~200 mg/L) employed in this study can be considered safe and is suitable for implementation in real-world environmental conditions. In light of the increasing environmental burden caused by the intensive use of chemical disinfectants against infectious microorganisms, MLEW might become an alternative air-cleaning disinfectant, on account of its favorable, ecologically friendly characteristics [43,44].

5. Conclusions

In light of the threat posed by the airborne infectious agent H6N1 avian influenza, MLEW may be recommended to be utilized for cleaning indoor environments because of its economic production and environmentally friendly characteristics. The present study provides evidence of the efficacy of MLEW mist-spraying to neutralize H6N1 aerosol based on HA titer evaluation. At FAC concentrations of up to 200 mg/L, MLEW rapidly and effectively inactivated the viral aerosol. It is recommended that the methods described here be more widely applied for hygiene interventions in agricultural and healthcare facilities. To this end, we shall investigate other microbial species and different operating parameters (e.g., nozzle type, pressure, contact time and ventilation) in our future work.

Author Contributions: Conceptualization, S.Y. and C.-Y.C.; methodology, S.Y. and C.-Y.C.; writing—original draft preparation, S.Y., H.-C.H., W.F. and C.-Y.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Bureau of Animal and Plant Health Inspection and Quarantine, Council of Agriculture, Taiwan, Republic of China, under Contract No. 98AS-9.6.1-BQ-B3.

Institutional Review Board Statement: This study did not require ethical approval.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the results in the current study are available from the corresponding author on reasonable request.

Acknowledgments: We acknowledge Zoonoses Research Center, School of Veterinary Medicine, National Taiwan University for providing the H6N1 avian influenza viral strain.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Li, Y.T.; Linster, M.; Mendenhall, I.H.; Su, Y.C.F.; Smith, G.J.D. Avian influenza viruses in humans: Lessons from past outbreaks. *Br. Med. Bull.* **2019**, *132*, 81–95. [CrossRef]
2. Wei, S.H.; Yang, J.R.; Wu, H.S.; Chang, M.C.; Lin, J.S.; Lin, C.Y.; Liu, Y.L.; Lo, Y.C.; Yang, C.H.; Chuang, J.H.; et al. Human infection with avian influenza A H6N1 virus: An epidemiological analysis. *Lancet Respir. Med.* **2013**, *1*, 771–778. [CrossRef]
3. Pantin-Jackwood, M.J.; Swayne, D.E. Pathogenesis and pathobiology of avian influenza virus infection in birds. *Rev. Sci. Tech.* **2009**, *28*, 113–136. [CrossRef]
4. El Zowalaty, M.E.; Bustin, S.A.; Husseiny, M.I.; Ashour, H.M. Avian influenza: Virology, diagnosis and surveillance. *Future Microbiol.* **2013**, *8*, 1209–1227. [CrossRef]
5. Landman, W.J.M.; Germeraad, E.A.; Kense, M.J. An avian influenza virus H6N1 outbreak in commercial layers: Case report and reproduction of the disease. *Avian Pathol. J. W.V.P.A* **2019**, *48*, 98–110. [CrossRef]
6. Wang, G.; Deng, G.; Shi, J.; Luo, W.; Zhang, G.; Zhang, Q.; Liu, L.; Jiang, Y.; Li, C.; Sriwilaijaroen, N.; et al. H6 influenza viruses pose a potential threat to human health. *J. Virol.* **2014**, *88*, 3953–3964. [CrossRef]
7. Cheung, C.L.; Vijaykrishna, D.; Smith, G.J.; Fan, X.H.; Zhang, J.X.; Bahl, J.; Duan, L.; Huang, K.; Tai, H.; Wang, J.; et al. Establishment of influenza A virus (H6N1) in minor poultry species in southern China. *J. Virol.* **2007**, *81*, 10402–10412. [CrossRef]
8. Herfst, S.; Schrauwen, E.J.; Linster, M.; Chutinimitkul, S.; de Wit, E.; Munster, V.J.; Sorrell, E.M.; Bestebroer, T.M.; Burke, D.F.; Smith, D.J.; et al. Airborne transmission of influenza A/H5N1 virus between ferrets. *Science* **2012**, *336*, 1534–1541. [CrossRef]

9. Jegede, A.; Fu, Q.; Lin, M.; Kumar, A.; Guan, J. Aerosol exposure enhanced infection of low pathogenic avian influenza viruses in chickens. *Transbound. Emerg. Dis.* **2019**, *66*, 435–444. [CrossRef]
10. Herfst, S.; Imai, M.; Kawaoka, Y.; Fouchier, R.A. Avian influenza virus transmission to mammals. *Curr. Top. Microbiol. Immunol.* **2014**, *385*, 137–155. [CrossRef]
11. Kim, S.Y.; Kim, M.; Lee, S.; Lee, J.; Ko, G. Survival of microorganisms on antimicrobial filters and the removal efficiency of bioaerosols in an environmental chamber. *J. Microbiol. Biotechnol.* **2012**, *22*, 1288–1295. [CrossRef]
12. Wenke, C.; Pospiech, J.; Reutter, T.; Altmann, B.; Truyen, U.; Speck, S. Impact of different supply air and recirculating air filtration systems on stable climate, animal health, and performance of fattening pigs in a commercial pig farm. *PLoS ONE* **2018**, *13*, e0194641. [CrossRef] [PubMed]
13. Guillard, C.; Bui, T.H.; Felix, C.; Moules, V.; Lina, B.; Lejeune, P. Microbiological disinfection of water and air by photocatalysis. *Comptes Rendus Chim.* **2008**, *11*, 107–113. [CrossRef]
14. McDevitt, J.J.; Rudnick, S.N.; Radonovich, L.J. Aerosol susceptibility of influenza virus to UV-C light. *Appl. Environ. Microbiol.* **2012**, *78*, 1666–1669. [CrossRef]
15. Megahed, N.A.; Ghoneim, E.M. Indoor Air Quality: Rethinking rules of building design strategies in post-pandemic architecture. *Environ. Res.* **2021**, *193*, 110471. [CrossRef]
16. Song, L.; Zhou, J.; Wang, C.; Meng, G.; Li, Y.; Jarin, M.; Wu, Z.; Xie, X. Airborne pathogenic microorganisms and air cleaning technology development: A review. *J. Hazard. Mater.* **2022**, *424*, 127429. [CrossRef]
17. Usachev, E.V.; Pyankov, O.V.; Usacheva, O.V.; Agranovski, I.E. Antiviral activity of tea tree and eucalyptus oil aerosol and vapour. *J. Aerosol. Sci.* **2013**, *59*, 22–30.
18. Huang, Y.; Hung, Y.; Hsu, S.; Huang, Y.; Hwang, D. Application of electrolyzed water in the food industry. *Food Control* **2008**, *19*, 329–345.
19. Kormányos, B.; Nagypál, I.; Peintler, G.; Horváth, A. Effect of Chloride Ion on the Kinetics and Mechanism of the Reaction between Chlorite Ion and Hypochlorous Acid. *Inorg. Chem.* **2008**, *47*, 7914–7920.
20. Len, S.; Hung, Y.; Chung, D.; Anderson, J.; Erickson, M.; Morita, K. Effects of storage conditions and pH on chlorine loss in electrolyzed oxidizing (EO) water. *J. Agric. Food Chem.* **2002**, *50*, 209–212. [CrossRef]
21. Ayebah, B.; Hung, Y. Electrolyzed water and its corrosiveness on various surface materials commonly found in food processing facilities. *J. Food Process. Eng.* **2005**, *28*, 247–264. [CrossRef]
22. Sun, J.; Jiang, X.; Chen, Y.; Lin, M.; Tang, J.; Lin, Q.; Fang, L.; Li, M.; Hung, Y.C.; Lin, H. Recent trends and applications of electrolyzed oxidizing water in fresh foodstuff preservation and safety control. *Food Chem.* **2022**, *369*, 130873. [CrossRef]
23. Jemni, M.; Otón, M.; Ramirez, J.G.; Artés-Hernández, F.; Chaira, N.; Ferchichi, A.; Artés, F. Conventional and emergent sanitizers decreased Ectomyelois ceratoniae infestation and maintained quality of date palm after shelf-life. *Postharvest Biol. Technol.* **2014**, *87*, 33–41. [CrossRef]
24. Vásquez-López, A.; Villarreal-Barajas, T.; Rodríguez-Ortiz, G. Effectiveness of Neutral Electrolyzed Water on Incidence of Fungal Rot on Tomato Fruits (*Solanum lycopersicum* L.). *J. Food Prot.* **2016**, *79*, 1802–1806. [CrossRef]
25. Tamaki, S.; Bui, V.N.; Ngo, L.H.; Ogawa, H.; Imai, K. Virucidal effect of acidic electrolyzed water and neutral electrolyzed water on avian influenza viruses. *Arch. Virol.* **2014**, *159*, 405–412. [CrossRef]
26. Lee, J.H.; Rhee, P.L.; Kim, J.H.; Kim, J.J.; Paik, S.W.; Rhee, J.C.; Song, J.H.; Yeom, J.S.; Lee, N.Y. Efficacy of electrolyzed acid water in reprocessing patient-used flexible upper endoscopes: Comparison with 2% alkaline glutaraldehyde. *J. Gastroenterol. Hepatol.* **2004**, *19*, 897–903. [CrossRef]
27. Urushidani, M.; Kawayoshi, A.; Kotaki, T.; Saeki, K.; Mori, Y.; Kameoka, M. Inactivation of SARS-CoV-2 and influenza A virus by dry fogging hypochlorous acid solution and hydrogen peroxide solution. *PLoS ONE* **2022**, *17*, e0261802. [CrossRef]
28. Chuang, C.; Yang, S.; Chang, M.; Huang, H.; Luo, C.; Fang, W.; Hung, P.; Chung, P. Applying the Membrane-Less Electrolyzed Water Spraying for Inactivating Bioaerosols. *Aerosol Air Qual. Res.* **2013**, *13*, 350–359. [CrossRef]
29. Chuang, C.; Yang, S.; Chang, M.; Huang, H.; Luo, C.; Hung, P.; Fang, W. Inactivation efficiency to *Bacillus subtilis* and *Escherichia coli* bacterial aerosols of spraying neutral electrolyzed water. *J. Air Waste Manag. Assoc.* **2013**, *63*, 1447–1456. [CrossRef]
30. WHO. *Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza*; World Health Organization: Geneva, Switzerland, 2011.
31. WHO. *Manual on Animal Influenza Diagnosis and Surveillance*; World Health Organization: Geneva, Switzerland, 2002.
32. Hogan, C.; Kettleson, E.; Lee, M.H.; Ramaswami, B.; Angenent, L.; Biswas, P. Sampling methodologies and dosage assessment techniques for submicrometre and ultrafine virus aerosol particles. *J. Appl. Microbiol.* **2005**, *99*, 1422–1434.
33. Verreault, D.; Moineau, S.; Duchaine, C. Methods for sampling of airborne viruses. *Microbiol. Mol. Biol. Rev.* **2008**, *72*, 413–444. [CrossRef]
34. Chuang, C.-Y.; Hsu, Y.-F.; Hung, P.-C.; Dai, Y.-T.; Yang, S. Evaluation of the Inactivating Ability of Carbon-Nanotube Coated Plasma Treatment on Bioaerosols for Indoor Air Cleaning. *Coatings* **2022**, *12*, 1497. [CrossRef]
35. Pica, N.; Bouvier, N.M. Environmental factors affecting the transmission of respiratory viruses. *Curr. Opin. Virol.* **2012**, *2*, 90–95. [CrossRef]
36. Gralton, J.; Tovey, E.; McLaws, M.-L.; Rawlinson, W.D. The role of particle size in aerosolised pathogen transmission: A review. *J. Infect.* **2011**, *62*, 1–13. [CrossRef]

37. Oswin, H.P.; Haddrell, A.E.; Otero-Fernandez, M.; Mann, J.F.S.; Cogan, T.A.; Hilditch, T.G.; Tian, J.; Hardy, D.A.; Hill, D.J.; Finn, A.; et al. The dynamics of SARS-CoV-2 infectivity with changes in aerosol microenvironment. *Proc. Natl. Acad. Sci. USA* **2022**, *119*, e2200109119. [CrossRef]
38. Abadias, M.; Usall, J.; Oliveira, M.; Alegre, I.; Vinas, I. Efficacy of neutral electrolyzed water (NEW) for reducing microbial contamination on minimally-processed vegetables. *Int. J. Food Microbiol.* **2008**, *123*, 151–158. [CrossRef]
39. Zhao, Y.; Xin, H.; Zhao, D.; Zheng, W.; Tian, W.; Ma, H.; Liu, K.; Hu, H.; Wang, T.; Soupir, M. Free chlorine loss during spraying of membraneless acidic electrolyzed water and its antimicrobial effect on airborne bacteria from poultry house. *Ann. Agric. Environ. Med.* **2014**, *21*, 249–255. [CrossRef]
40. Thorn, R.M.S.; Lee, S.W.H.; Robinson, G.M.; Greenman, J.; Reynolds, D.M. Electrochemically activated solutions: Evidence for antimicrobial efficacy and applications in healthcare environments. *Eur. J. Clin. Microbiol.* **2012**, *31*, 641–653. [CrossRef]
41. Oomori, T.; Oka, T.; Inuta, T.; Arata, Y. The efficiency of disinfection of acidic electrolyzed water in the presence of organic materials. *Anal. Sci.* **2000**, *16*, 365–370. [CrossRef]
42. Block, M.S.; Rowan, B.G. Hypochlorous Acid: A Review. *J. Oral Maxillofac. Surg.* **2020**, *78*, 1461–1466. [CrossRef]
43. Morita, C.; Nishida, T.; Ito, K. Biological toxicity of acid electrolyzed functional water: Effect of oral administration on mouse digestive tract and changes in body weight. *Arch. Oral Biol.* **2011**, *56*, 359–366. [CrossRef] [PubMed]
44. Saitoh, Y.; Harata, Y.; Mizuhashi, F.; Nakajima, M.; Miwa, N. Biological safety of neutral-pH hydrogen-enriched electrolyzed water upon mutagenicity, genotoxicity and subchronic oral toxicity. *Toxicol. Ind. Health* **2010**, *26*, 203–216. [CrossRef] [PubMed]