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Effective Inhibition of H5N1 Influenza Virus Infectivity using Safe and Calculated Low Power Generated from Life Restoration device under Different Conditions

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Objectives: The use of the antiviral drugs and vaccination against pathogenic viruses induce some disorders and death cases. Therefore, it is urgent to develop an alternative therapy to protect and reduce the virus side effects. Here, in this report we designated a novel device termed as Life Restoration Device (LRD) for viral infectivity reduction.
Methodology: Here in this study, we studied the efficacy of the LRD in the treatment of influenza A virus subtype (H5N1) under different conditions using plaque assay analysis.
Results: This report showed that, the infectively inhibition of the H5N1 virus was 90%, 100% and 90% for 30 min, 60 min and 90 min respectively after exposure to LRD equipped with copper rod and cell culture medium. While the copper-coated nickle rod of LRD induces the infectivity inhibition percentage into 95%, 95% and 100% after 30 min, 60 min and 90 min respectively when exposure to LRD and cell culture medium. The nucleic acids analysis using real time PCR for viral solution

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before and after treatment confirmed the data provided by the plaque assays test. This data confirms the effective inhibition of H5N1 infectivity using specific electric stimulations even under different conditions. Furthermore, the effective treatment of pathogenic viruses such as H5N1 with electric stimulation generated by LRD is effective and safe therapy but needs further future investigations.

Keywords: Life Restoration Device; codified ions; influenza virus; H5N1; viral infectivity inhibition; effective treatment.

1. INTRODUCTION

Many types of viruses had the ability to infect different body tissue compartments such as gastrointestinal tract in the digestive system or lungs and airways in the respiratory system. Most of known viruses initiate different inflammations that able to induce mild to serious abnormalities in the infected site [1,2,3]. Previously infectious pathogens such as influenza A virus subtype (H5N1) can cause mild to severe viral disorders and local or systemic toxicities. The produced inflammations by H5N1 pathogenic virus in the infected respiratory compartments cause severe respiratory dysfunction syndrome which finally, leads to increase the mortality rates of the infected cases [4,5]. Moreover, infection with H5N1 subtypes virus causes different disorders such as cardiovascular and pulmonary failures as reported by World Health Organization (WHO) [4,6,7].

Recently, it is crucial to develop a new strategy to reduce the viral infectivity using effective, low coast and lower side effects. Mild to severe side effects can be produced with different types of viruses. For example, in October 2018, the total number of confirmed Middle East Respiratory Syndrome Coronavirus (MERS-CoV) infected cases was more than 2000 cases and more than 800 with total death cases have been reported [8]. Moreover, coxsackievirus type A9 (COX A9) and herpes simplex type 1 (HSV1) viruses can cause variable symptoms including encephalitis, hepatitis and orolabial disease as reported previously [9,10]. Previously, different new strategies against pathogenic viruses were developed and improved to overcome on the changes in the pathogen's variable mutations. Vaccination considers as one of the effective strategy to protect from viral infection. Additionally, vaccination against respiratory viruses is effective and cheap method to protect from some types of viruses. Although, vaccination provide best protection method, but vaccines are not effective for some viral mutations such as COVID-19 mutations. Therefore, vaccination against viral infections needs more studies [11]. Furthermore, combination therapy using antiviral drugs for viral treatment is more effective. For example, it is well known that interferon-α and ribavirin are suitable for chronic hepatitis treatment [12]. However, combination therapy initiated many disorders especially when these systems were administrated for long time course treatment.

Recently, for more safe strategy of viral treatment, ultraviolet light treatment strategy has been raised. Interestingly, the low ultraviolet irradiation exposure inactivates non-enveloped viruses more than higher ultraviolet irradiation [13,14]. Therefore, the viral treatment with ultraviolet irradiation considers as non-invasive therapy of pathogenic viral infection. This strategy is relatively preferable due to low cost, safe, lower side effects, and effectiveness in viral inactivation compared with vaccination or antiviral drugs treatment. Furthermore, direct, or indirect treatment with electric stimulations has been raised to prevent the infectivity of human immunodeficiency virus type 1 (HIV-1) [15]. Additionally, it was reported that, the effective response to electric stimulation of chronically HIV-1 infected is higher than recently infected cells. Additionally electric stimulation could improve the respiratory functions as well as inhibit SARS-CoV-2 infectivity [16,17,18].

Therefore, in the current study, Madboly et al. 2022 successfully designed a smart device, termed as Life Restoration Device (LRD) as shown in Figs. 1 and 2. The LRD was registered in the Egyptian Patent Office, Ministry of Higher Education, Academy of Scientific Research and Technology, Cairo, Egypt under patent number: 2014101664/2014/10/20. The LRD is characterized with ability to reduce the infectivity and deactivate different types of viruses such as HIV, COVID-19, and H5N1 viruses as reported previously with our team [19,20]. The thermotical mechanism of LRD function depends on transformation of electricity with low voltage

electric impulses into codified energy (electrons). The produced codified energy is safe and coded to decompose and deactivate the biological compartments of viruses such as RNA/DNA, as well as viral envelope. Previously our team reported that, in vitro treatment of some different types of viruses such as, HIV, SARS-CoV-2 and MERS-CoV with LRD significantly reduced the viral infectivity within 30 minutes to 60 minutes [19,20]. In current study, we will evaluate the efficacy of LRD treatment in viral infectivity inhibition using H5N1 virus as model under different experimental conditions. The effect of rod materials, cell culture medium, exposure time and viral dilutions were used as different parameters to confirm the LRD efficacy to reduce H5N1 virus infectivity. The data obtained from this study will provide important information about the safe and effective alternative therapy of serious pathogenic viruses such H5N1 virus. Interestingly, the short exposure time to LRD provide effective viral treatment, decrease symptoms, and side effects.

2. MATERIALS AND METHODS

2.1 Materials, Viruses and Cell Lines

Madin-Derby Canine Kidney (MOCK) cell lines were purchased from the American Type Culture Collection, (Rockville, Md, USA). Beta-mercaptoethanol, crystal violet, agarose and silver nitrate solution were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Dulbecco's modified Eagle medium (DMEM) mixed with 8% fetal bovine serum and 1% antibiotic-antimycotic solutions was obtained from BioWhittaker (Walkersville, MD, USA). The RGA/Chicken/Egypt/813825A/2017 avian influenza virus (termed as H5N1) was obtained from European virus archive-global (Marseille, France).

The in vitro experiments of this report were performed to provide more information about the effectiveness of LRD and the produced low potential codified power on the H5N1 infectivity under different experimental conditions such as exposure time, cell culture medium type, LRD rod materials. The H5N1 was used as representative for enveloped RNA viruses.

2.2 Methods

2.2.1. LRD preparation for in vitro study

As shown in Fig. 1A, the LRD function is based on a cumulative application of a simple and safe range of specified energy according to a proprietary designated formula. The generated codified number of ions generated from LRD system may move through the cell culture medium penetrate the infected cells and damage the fragile protein-rich envelope and nucleic acid materials (DNA/RNA) pathogenic viruses such as H5N1 (Fig. 1B). As shown in Fig. 1 the designed glass tube will be filled with cell medium and H5N1-infected cells. Hereafter, rods made from different materials were settled with rubber plugs and inserted in each specified side of the glass tube. The codified ions generated from the LRD were applied on the medium containing infected cells with H5N1 at different time points (for example: 30 min, 60 min, 90 min or 120 min), different viral dilution and different cell culture medium. Then the treated and non-treated H5N1 virus infected cells in the medium were analyzed by calculating the viral infectivity inhibition after and before exposure into LRD respectively.

2.2.2 *In vitro* experimental design

Fig. 1C showed that, the H5N1 virus infected cells in the culture medium was poured into the designed glass tube at different cell dilutions. Both anionic and cationic plugs in the glass tube were connected with LRD's rods. The glass tube was filled with the medium containing infected cells with H5N1 virus and then the ends of glass tubes were firmly sealed to prevent leaking the medium from the glass tube (Fig. 1B and C). Then, each side of the LRD's rod was settled in the LRD specified outlets (anionic and cationic outlets) (Fig. 1A). The LRD generated low potential electrical frequencies and then this power was codified into a specified number of ions applied for 30 min, 60 min, 120 min for infected cells.

2.2.3 H5N1 virus' preparation and LRD system assembly

The H5N1 virus was diluted into different dilutions and mixed with 12 ml (DMEM containing 2% fetal bovine serum (FBS) and 1% antibiotic-antimycotic mixture (P/S) then, medium containing H5N1 virus was injected into the glass tube of LRD system. The non-treated virus in the medium were exposed to the same parameters and experimental conditions such as time and rod materials of LRD like the treated viruses and then stored at -70 ºC. In this experiment the diluted H5N1 virus was exposed to LRD in vitro for 30 min, 60 min, 90 min or 120 min and then the virus was withdrawn, aliquoted and stored in a -70 ºC freezer for further analysis.

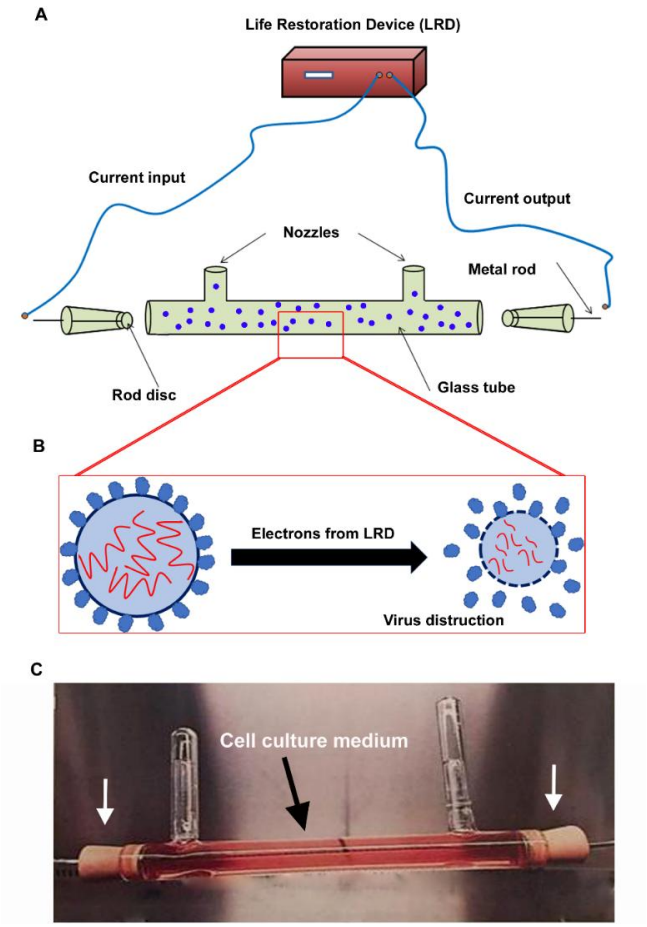

Fig. 1. (A) Diagrammatic graph showing the in vitro experiment design. The glass tube will be filled with cell culture medium and infected cells with a specified virus or virus itself. Then the two ends of nickel-coated copper rods will connect with the LRD. The electric stimulation and ions generation will be produced and controlled through the by LRD. (B) Diagrammatic drawing showing the effect of LRD treatment on the virus biological compartments. (C) A designed glass tube filled with cell culture medium and targeted virus. The glass tube will be connected with the LRD through the input and output power sources (white arrow)

2.2.4 Cell culture preparation for viral infectivity

MDCK cells in 75 cm^2 tissue culture flasks were disassociated using trypsin solution and re-suspended again to give 105 cells/ml in DMEM. Hereafter suspended cells were cultivated in 6-well tissue culture plates and incubated for 24 hrs- at 37 ºC.

2.2.5 H5N1 virus propagation for viral viability test

H5N1 viruses were seeded based on the previously published reports with slight modifications [21,22]. The titration of H5N1 virus and plaque infectivity test was carried out under different experimental conditions. Briefly, the seeded H5N1virus were 10 folds serially diluted in a DMEM medium without FBS and P/S. Hereafter, 100 µl from each dilution was added with 200 µl of medium containing infected MDCK cells with h5N1 virus for infectivity assay test of H5N1 virus. Control well was inoculated in the presence of 300 µl of serum-free medium only. The non-treated plate was incubated at 37 ºC under 5% CO_2 for 1 h to allow H5N1 virus adsorption and shaken every 15 min to ensure homogenous exposure of the cells to each virus and avoid drying of cells. The over-layer medium about 3 ml was added after 1 h, and the plate was shaken smoothly to allow homogenous mixing of the virus dispersion through the over-layer. Then, to allow the hardness of the agarose gel of the over-layer medium, the plate was left at 25 ºC for 10 min. After that, the wells were further incubated at a humid chamber in 37 ºC under 5%

CO_2 for 1 h. Fore cell fixation and activation of the virus, some fixation solution was added to each well for 1 h. Then, fixation solution was later washed, and the plate wells were poured and flashed with water and dried.

2.2.6 Plaque infectivity analysis

The plaque infectivity assay of H5N1 virus was carried out to investigate the viral infectivity analysis. In this assay, the cell culture medium was discarded from the cell culture plates and treated viruses were inoculated (100 µl/well) into MDCK cell line in six-well plates. After l h contact time for virus adsorption, 3 ml of DMEM supplemented with 2% agarose was added onto the targeted cell monolayer and six-well plates were left to solidify at 37 ºC till the formation of viral plaques for 3 days. Control cells were treated identically under similar conditions of treated samples. The treated plates were investigated daily under the inverted light microscope for plaques formation. Plaques were counted manually under the light microscope and percentage of plaques formation reduction in comparison to control wells was calculated based on the following equation: % Inhibition = (a-b)/a*100; [a = viral count (untreated), b = viral count (treated)]. All experiments were repeated three times under similar conditions for consistency evaluation of the experiment (n = 3).
The H5N1 virus infected cells were exposed into LRD for different time points. For illustration of the plaques, formalin (10%) was added and left for two hours for the purpose of cell fixation. Additionally, the plates including agarose gel were stained using 0.1 % crystal violet in distilled water for 5 min. After that, the dye was washed away, plate wells were washed briefly in water and left to dry. Viral plaques expressing the viral infectivity were evidenced as clear unstained spots (due to viral infection) in a violet (stained cells) background. Furthermore, the virus infectivity inhibition was calculated through the following equation: Plaque forming unit (PFU)/ml = a*b*c*10, (a = Number of plaques; b = inoculated volume of the virus; c = virus dilution; 10 = dilution factor) [23].

2.2.7 The role of LRD rod type and viral infectivity inhibition

To confirm that the viral infectivity inhibition of H5N1 virus was induced by the codified number of ions generated from LRD. Therefore, we applied generated ions from LRD to the media including infected cells with H5N1 virus through different rod materials as shown in Table 7 (Copper, nickel-coated copper and stainless-steel, and nickel-coated stainless-steel rods). Hereafter the viral load inhibition and the anti-viral activity of the precipitate formed against H5N1 were evaluated and analyzed based on each rod type.

2.2.8 Viral genome examination using PCR

Nucleic acids for both treated and non-treated H5N1 virus under different conditions were extracted and real time-PCR were carried out to determine any changes in the nucleic materials of H5N1 virus. The PCR amplicons were purified individually and analyzed using the software. Later the cycle threshold of each sample was calculated to investigate the amount of the nucleic acids of each sample separately.

3. RESULTS

3.1 LRD Design and Function

As illustrated in Fig. 1A, the theoretical idea for LRD design depends basically on a source of energy generated from dry batteries equipped with the device. The generated power will be electronically processed to the codified number of ions (Fig. 1A and Fig. 2A and B). The generated electric stimulations are coded to destroy the biological contents such as proteins, viral envelope, and nucleic acids materials of viruses such as RNA/DNA (Fig. 1B). Additionally, more development on the LRD machine to be used in the medical application for single or multiple use to save time and efforts (Fig. 2A and B). The possible medical application of LRD to destroy viral infection of human blood when infected blood passed through a designed filter and then the generated electrons from LRD will be applied (Fig. 2C).

3.2 The H5N1 Viability Assay

3.2.1 Copper rod and LRD efficacy

To confirm the efficacy of the LRD in viral viability reduction, we used different rod materials such as copper, stainless-steel and nickle. As shown in Fig. 3 and Table 1, the efficacy of LRD in treatment of H5N1 virus was similar when the materials of LRD rods changed. The viability inhibition of H5N1 virus was the same when copper rod was used to reduce the viral viability. This experiment showed that, the viability inhibition was 96%, 96% and 98% after

30 min, 60 min and 90 min exposure to LRD equipped with copper rod and cell culture medium. Additionally, when vortex during LRD exposure time was applied the viability inhibition of the H5N1 virus was 90%, 100% and 90% after 30 min, 60 min and 90 min exposure to LRD equipped with copper rod and cell culture medium. When the copper rod of LRD was coated with nickle, the viability inhibition percentage 95%, 95% and 100% after 30 min, 60 min and 90 min exposure to LRD equipped with nickle-coated copper rod and cell culture medium. This data indicating that changing the experimental condition did not affect the efficacy of the LRD in the treatment of the H5N1 virus.

3.2.2 Stainless-steel rod and LRD efficacy

From another hand the viability inhibition percentage of H5N1 virus was the lower at short time exposure with LRD when copper rod was replaced with stainless-steel. While the viability inhibition percentage was highly increased after 90 min exposure like copper rod data. In details, the viability inhibition was 50%, 50% and 98% after 30 min, 60 min and 90 min (respectively) exposure to LRD equipped with stainless-steel rod and cell culture medium. Additionally, when vortex during LRD exposure time was applied, the viability inhibition of the H5N1 virus was 50%, 80% and 97.5% after 30 min, 60 min and 90 min (respectively) exposure to LRD equipped with stainless-steel rod and cell culture medium.

3.2.3 Nickle rod and LRD efficacy

Furthermore, when the stainless-steel rod of LRD was coated with nickle, the viability inhibition percentage 78%, 98.8% and 98% after 30 min, 60 min and 90 min (respectively) exposure to LRD equipped with nickle-coated stainless-steel rod and cell culture medium. In general, changing the materials of LRD rod did not change the efficacy of the LRD in H5N1 treatment (Fig. 3 and Table 1). The viability inhibition of LRD equipped with nickle rod was 78%, 98.9% and 98% after 30, 60, 90 min exposure to the LRD respectively. These data indicating that, changing the LRD rod materials did not change the LRD efficacy against the H5N1 viral activity.

Table 1. The H5N1 viral viability inhibition after treatment with LRD under different conditions. The viral viability inhibition increases when the exposure time increase. The rod materials, coating meatal or vortex during treatment did not affect the viral inhibition

Experiment conditions	Virus type	Rod material	Initial virus count (virus/ml)	Exposure time	Initial virus count (virus/ml) after treatment	% of virus inhibition
Cell culture medium	H5N1	copper	$10x10^4$	30	$4x10^3$	96
				60	$4x10^3$	96
				90	$2x10^3$	98
Cell culture medium	H5N1	Copper+ vortex	$1x10^7$	30	$1x10^6$	90
				60	0	100
				90	$1x10^6$	90
Cell culture medium	H5N1	Nickle-coated copper	$2.2x10^4$	30	$1x10^3$	95
				60	$1x10^3$	95
				90	0	100
Cell culture medium	H5N1	Stainless-steel	$2.5x10^7$	30	$1.25x10^3$	50
				60	$1.1x10^7$	50
				90	$5x10^5$	98
Cell culture medium	H5N1	Stainless-steel + vortex	$1x10^8$	30	$50x10^6$	50
				60	$20x10^6$	80
				90	$3x10^6$	97.5
Cell culture medium	H5N1	Nickle-coated stainless-steel	$2.6xl0^5$	30	$5.5x10^4$	78
				60	3100	98.8
				90		98

Table 2. The average of infectivity inhibition of H5N1 virus before and after treatment with LRD for 30 min and 60 min in culture medium and under different viral dilution as shown in figure 2D

Experiment condition		Initial virus conc. (PFU/ml)	Exposure time	Virus conc. (PFU/ml) after treatment	Percent of virus inhibition (%)
Copper rod	Cell culture medium	$1x10^7$	30 min	$1x10^5$	90
			60 min	0	100

Table 3. The average of infectivity inhibition of H5N1 virus before and after treatment with LRD for 60 min in egg allantoic fluid and under different viral dilution as shown in figure 6

Experiment condition	Initial virus conc. (PFU/ml)	Exposure time	Virus conc. (PFU/ml) after treatment	Percent of virus inhibition (%)
Egg allantoic fluid	$2.5x10^7$	60 min	0	100

Table 4. The cycle threshold value of Real-time PCR of the treated virus solution with LRD equipped with copper rod and under different viral dilutions for 120 min. The concertation of nucleic acid material of H5N1 virus decreased after treatment with LRD

Initial viral concentration	Before treatment		After treatment	
$1x10^7$	Viral dilution log	CT value	Viral dilution log	CT value
	10^1	22.76	Stock	21.19
	10^2	23.49	1	24.22
	10^3	26.24	2	27.58
	10^4	29.12	3	31.79

Table 5. The average of infectivity inhibition of H5N1 virus before and after treatment with LRD equipped with stainless-steel rod for 30 min and 60 min in culture medium and under different viral dilution as shown in figure 5

Experiment conditions		Initial virus conc. (PFU/ml)	Exposure time	Virus conc. (PFU/ml) after treatment	Percent of virus inhibition (%)
Stainless-steel rod	Cell culture medium	$100x10^6$	30 min	$100x10^6$	0
			60 min	$2.51x10^6$	33

Table 6. The cycle threshold value of Real-time PCR of the treated virus solution with LRD equipped with stainless-steel rod and under different viral dilutions and for 120 min. The concertation of nucleic acid material of H5N1 virus decreased after treatment with LRD

Initial viral concentration	Before treatment		After treatment	
	Viral dilution log	CT value	Viral dilution log	CT value
$1x10^7$	10^1	20.39	Stock	20.82
	10^2	22.97	1	26.27
	10^3	26.68	2	27.79
	10^4	32.72	3	32.54
	10^5	31.42		36.01

Table 7. The average of infectivity inhibition of H5N1 virus before and after treatment with LRD equipped with nickel rod for 30 min and 60 min in culture medium and under different viral dilution as shown in figure 6

Experiment conditions		Initial virus conc. (PFU/ml)	Exposure time	Virus conc. (PFU/ml) after treatment	Percent of virus inhibition (%)
Nickel rod	Cell culture medium	$4.3x10^6$	30 min	$4.3x10^6$	0
			60 min	$2.51x10^6$	41

3.3 Plaque Infectivity Assay of H5N1 Virus

3.3.1 H5N1 infectivity using copper rod in culture medium and vortex

To confirm the efficacy of the LRD in viral infectivity inhibition in vitro, several dilutions of the H5N1 virus were used in plaque assays. The plaque assays showed that there was a significant reduction in the viral load of H5N1 and cellular infectivity after exposure to LRD for 30 min and 60 min (Fig. 4A). Quantitatively, the exposure of infected cells with H5N1 to LRD reduced the virus infectivity titer o to 90% and 100% after 30 min and 60 min respectively compared to the non-treated virus (Fig. 4B). The obtained data of this experiment indicated that electric stimulations produced by LRD effectively reduced the H5N1 infectivity even at different viral concentrations. Additionally, as illustrated in Table 2, the percentage of viral infectivity inhibition after 30 min and 60 min was 90% and 100% respectively confirming the data of plaque assay test.

3.3.2 H5N1 infectivity using copper rod in egg allantoic fluid for longer time exposure

In this experiment we changed the cell culture medium to confirm the efficacy of the LRD in H5N1 infectivity reduction in vitro as shown in Fig. 5 and Table 3. Several dilutions of the H5N1 virus in egg allantoic fluid instead of the culture medium were used in plaque assays. The plaque assays of this experiment showed that there was a higher reduction in the viral infectivity of H5N1 after exposure to LRD for 120 min (Fig. 5A). Quantitatively, 120 min exposure of infected cells with H5N1 to LRD reduced the virus infectivity titer into 100% compared to the non-treated virus (Fig. 5B). This data indicated that electrons generated by LRD effectively reduced the H5N1 infectivity even in different medium and longer time. Additionally, as illustrated in Table 3, the higher percentage of viral infectivity inhibition after 120 min was 100% confirming the data of plaque assay test.

3.3.3 Nucleic acid analysis after LRD equipped with copper rod treatment

To confirm the decomposition of the nucleic acid materials of exposed H5N1 virus into specified number of ions generated from LRD, we quantified the amount of nucleic acid proteins of treated viruses using real-time PCR. As shown in Fig. 6 and Table 4, the value of cycle threshold of the treated samples with LRD equipped with copper rod was higher than that of the control (Fig. 6). The cycle threshold increase using LRD treatment was confirmed when we different concentration of H5N1 virus were exposed to the LRD equipped with copper rod for 120 min (Table 4). The increase in the cycle threshold value of the treated samples indicated that the LRD decreased the nucleic acid materials of the treated H5N1 virus compared with the control.

3.3.4 H5N1 infectivity titer using stainless-steel rod in culture medium

As shown in Fig. 7 and Table 5, to emphasis on the efficacy of the LRD against viral treatment in vitro, stainless-steel rod was used instead of copper rod to perform plaque assays test. In this experiment, the plaque assays showed that there was no reduction in the viral load of H5N1 and cellular infectivity after exposure to LRD equipped with stainless-steel rod for 30 min. While after 60 min exposure to LRD equipped with stainless-steel showed slight infectivity inhibition (Fig. 7A). Quantitatively, as shown in Table 5 the exposure of infected cells with H5N1 to LRD equipped with stainless-steel reduced the virus infectivity titer o to 33% after treatment for 60 min compared to the non-treated virus (Fig. 7B). The obtained data of this experiment indicated that electric stimulations produced by LRD equipped with stainless-steel rod reduced the H5N1 infectivity for 33% after 60 min but not after 30 min (Table 5). these data indicating that stainless-steel rod might significantly reduce the infectivity inhibition of H5N1 virus after long time exposure such as 90 min into 98% as shown in Table 1. However, the short exposure time of the LRD treatment showed lower, reduction of viral inhibition but longer time such as 90 min showed higher reduction of viral inhibition similar to that of cooper rod as shown in Table 1.

3.3.5 Nucleic acid analysis after LRD equipped with stainless-steel rod treatment

As shown in Fig. 8 and Table 6, the change in the nucleic acid materials of exposed H5N1 virus into LRD equipped with stainless-steel rod was confirmed from the cycle threshold using real-time PCR. The value of cycle threshold of the treated samples with LRD equipped with stainless-steel rod was higher than that of the non-treated viruses (Fig. 8). It well clear that, the cycle threshold increased when different

concentrations of H5N1 virus were exposed to the LRD equipped with copper rod for 120 min (Table 6). The increase in the cycle threshold value of the treated virus indicated that the LRD decomposed and decreased the nucleic acid materials of the treated H5N1 virus compared with the non-treated viruses.

Fig. 2. (A), Photos of primary models of single and multiple use LRD (B), Photos of new models of single and multiple use LRD. (C), A future possible home-treatment design of human infected viruses such as hepatitis viruses

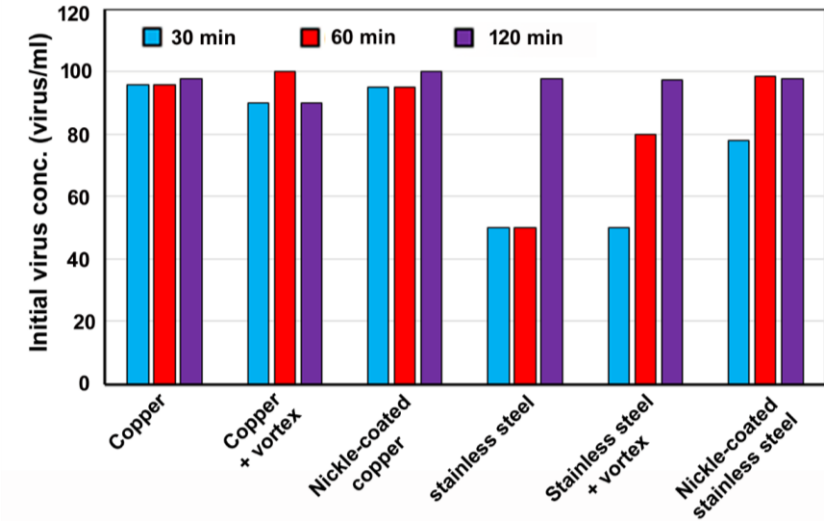

Fig. 3. H5N1 virus viability inhibition test after different conditions of LRD treatment. The copper rod inhibits the viral viability more compared with stainless-steel rod. The viability inhibition increases with increasing the exposure time especially for stainless-steel rod materials

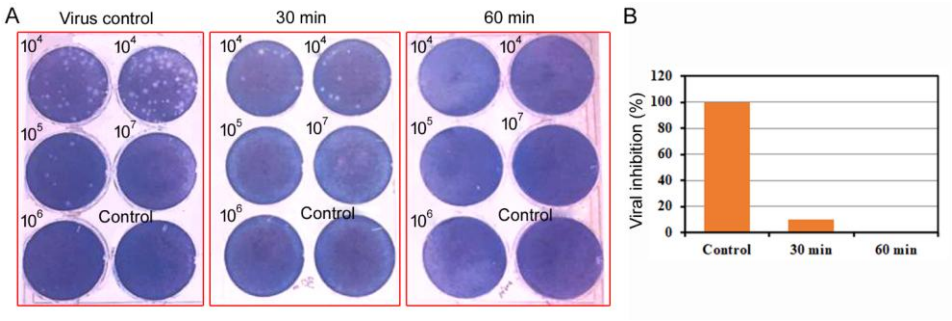

Fig. 4. (A) Plaque assay titration of H5N1 with different viral dilutions before and after treatment with LRD equipped with copper rod for 30 min and 60 min in culture medium with vortex. After 60 min the viral infectivity inhibition percentage was higher than that of 30 min. (B) A graph showing the inhibition percentage of H5N1 virus in culture medium after 30 min and 60 min treatment with LRD

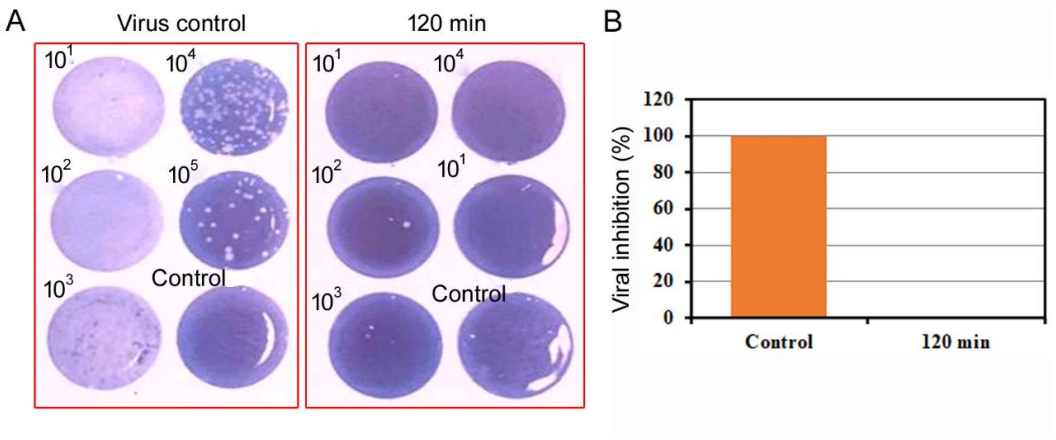

Fig. 5. (A) Plaque assay titration of H5N1 with different viral dilutions before and after treatment with LRD equipped with copper rod for 120 min in egg allantoic fluid. After 120 min the viral infectivity inhibition percentage was 100%. (B) A graph showing the inhibition percentage of H5N1 virus in egg allantoic fluid after 60 min treatment with LRD

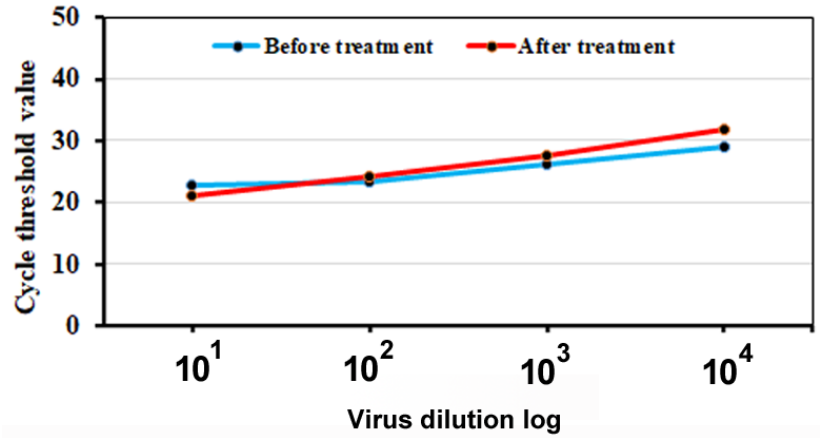

Fig. 6. Real-time PCR of the treated virus solution showing the value of cycle threshold value of different H5N1 viral dilutions after treatment with LRD equipped with copper rod for 120 min. The higher PCR cycle threshold value indicated lower nucleic acid material concentration indicating the effect of LRD treatment

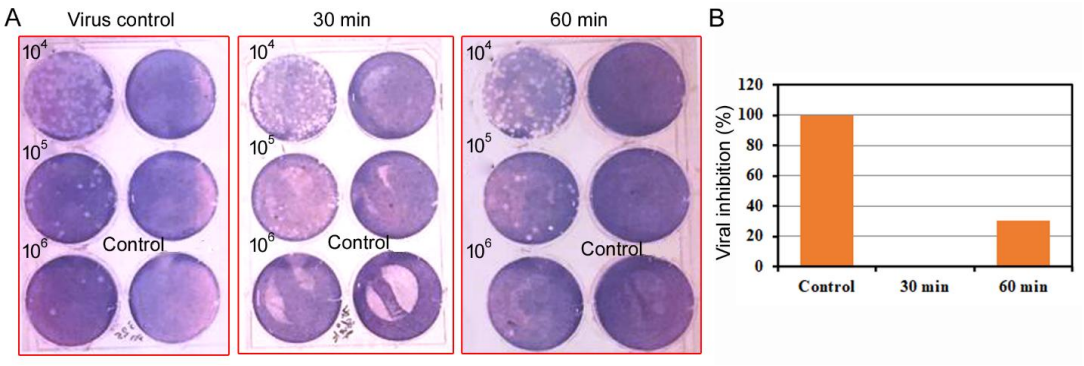

Fig. 7. (A) Plaque assay titration of H5N1 with different viral dilutions before and after treatment with LRD equipped with stainless-steel rod for 30 min and 60 min in culture medium with vortex. After 60 min the viral infectivity inhibition percentage was higher than that of 30 min. (B) A graph showing the inhibition percentage of H5N1 virus in culture medium after 30 min and 60 min treatment with LRD

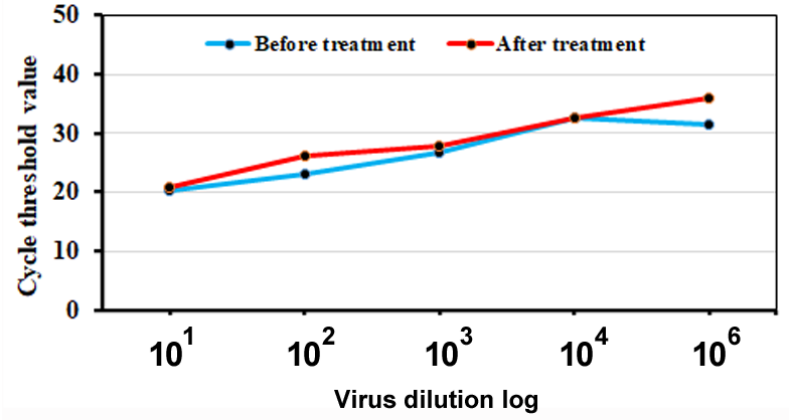

Fig. 8. Real-time PCR of the treated virus solution showing the value of cycle threshold value of different H5N1 viral dilutions after treatment with LRD equipped with stainless-steel rod. The higher PCR cycle threshold value indicated lower nucleic acid material concentration indicating the effect of LRD treatment

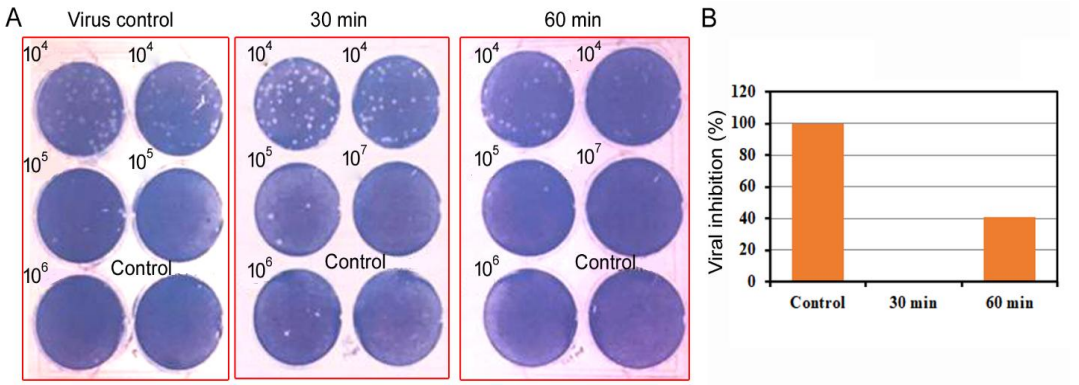

Fig. 9. (A) Plaque assay titration of H5N1 with different viral dilutions before and after treatment with LRD equipped with nickel rod for 30 min and 60 min in culture medium. After 60 min the viral infectivity inhibition percentage was higher than that of 30 min. (B) A graph showing the inhibition percentage of H5N1 virus in culture medium after 30 min and 60 min treatment with LRD equipped with nickel rod

3.3.6 H5N1 infectivity using nickle rod in culture medium

To confirm the efficacy of the LRD to inhibit the infectivity of H5N1 virus in vitro, nickle rod was used to perform plaque assays. Similar to stainless-steel rods, the plaque assays showed that there was no reduction in the viral load of H5N1 and cellular infectivity after exposure to LRD equipped with nickle rod for 30 min. While after 60 min exposure to LRD equipped with nickle rod, the plaque assays showed a slight infectivity inhibition (Fig. 9A). Quantitatively, as shown in Table 7 and Fig. 9B the exposure of infected cells with H5N1 to LRD equipped with nickle rod reduced the virus infectivity titer o to 41% after treatment for 60 min compared to the non-treated virus. The obtained data of this experiment indicated that electric stimulations produced by LRD equipped with nickle rod reduced the H5N1 infectivity for 41% after 60 min but not after 30 min (Table 7). These data indicating that nickle rod might significantly reduce the infectivity inhibition of H5N1 virus after long time exposure such as 90 min into 98% as shown in Table 1. However, the short exposure time of the LRD treatment showed lower reduction of viral inhibition but longer time such as 90 min showed significant reduction of viral inhibition similar to that of copper rod as shown in Table 1. The infectivity reduction profiles using LRD equipped with stainless-steel and nickle rods were the same compared with LRD equipped with copper rod. Additionally, treatment with LRD equipped with copper rod showed effective viral infectivity reduction after short times while the treatment with LRD equipped with stainless-steel or nickle rods showed effective inhibition after longer time.

4. DISCUSSION

The treatment of severe disorders caused by infection with different types of viruses such as HIV-1, SARS-CoV-2, MERS-CoV, H5N1, COX-A9 and HSV1 need more investigation and improvement. Previously the WHO announced that the COVID-19 virus and its mutations caused many infection cases and death cases and the current strategy of COVID-19 treatment with vaccination or antiviral drugs is not ideal [24]. Therefore, it is crucially to develop a new strategy to treatment the viral infection effectively, low side effects and with low coast. Therefore, we recently designated a novel device called LRD for treatment of different types of viruses such as HIV-1, SARS-CoV-2, MERS-CoV, H5N1, COX-A9 and HSV1. The viral infectivity inhibition with LRD is characterized with non-invasive, low side effect and higher efficacy [19,20].

The LRD design in this report depends mainly on production of codified number of ions that can destroy the biological compartments of biological materials such as DNA/RNA or envelope of the exposed viruses rather than the infected cells as reported previously [19,20]. In the current study, infectivity inhibition rate of H5N1 viruses treated with LRD equipped with cooper rod or copper rod with vortex was significantly (about 98) high as compared with the non-treated cells. In this experiment the short time exposure either after 30 min or 60 min was the same. From another point the infected cells with H5N1 virus treated with LRD equipped with stainless-steel rod showed lower infectivity inhibition at short exposure time (30 min or 60 min) but it showed significant higher infectivity inhibition rate after 90 min. Furthermore, LRD equipped with nickle rod showed the same infectivity inhibition rate of stainless-steel ones. These results indicated that, treatment with LRD under different condition showed effective treatment of the viral infectivity due to the generated electrons but not from the metal rod or chemical int the culture media. Additionally, the full detailed mechanism of viral infectivity reduction using electric stimulation is still unobvious and needs more clarifications. There are big differences between the biological structure and function between the viral envelope (H5N1 in this study) and eukaryotic cell membrane (infected cells in this study). In the current report we hypothesized that, the specificity of the viral envelope into codified ions generated by LRD is higher than the specificity of electrons to infected eukaryotic cells plasma membrane. Furthermore, another reason of the specific viral infectivity inhibition is due to the differences in molecular structure between both viral envelope and cellular membrane of the infected cells. These differences might be due to changes in the fluidity ratios of each membrane.

The data of the current report and our hypothesis to explain the mechanism of LRTD function against viral treatment were in consistent with previously published reports. Interestingly, the stimulation of some cells using specified electric frequencies reduced the side effects generated from lung inflammations after infection with SARS-CoV-2 virus [25]. Additionally, it was reported previously in variable studies exposure of infected cells with HIV-1 for low electric frequencies for short time daily showed lower

disorders and decrease in the viral infectivity [16,26]. Interestingly, previous reports support the function mechanism of our LRD and its function which based on the use of the codified number of ions to change the compartment of the treated viruses rather than the infected cells. The difference in the infectivity inhibition rate between copper, stainless-steel and nickle rods is due the electrons conductivity of copper than that of the other two metals [27,28]. The data of current report is in consistent with previously reported data which showed the influence of the electric stimulations on the infectivity inhibition of the pathogenic viruses [15,25,26]. The suggested hypothesis was confirmed previously from published studies which showed that the electric frequencies applied on the infected cells with HIV-1 can make some destructions on the cell membrane or the envelope fluidity of the nutrients and as a result the HIV-1 infectivity was reduced [29,30]. Furthermore, the thickness of the viral envelope (H5N1 in this study) measured 6.34 ± 0.49 nm in diameter and composed of lipid bilayer membranes [31]. While the plasma membrane of eukaryotic cells (infected cells in this study) thickness measured 5 nm to 10 nm and composed from variable compartment such as glycolipids, phospholipid bilayer, glycoproteins, lipoproteins [32]. Furthermore, the lower potential sensitivity of HIV-1 envelope fluidity is higher than that of infected cells plasma membrane fluidity due the thickness and molecular structure of each compartment [30,33,34]. Therefore, we could confirm our hypothesis reporting that the codified number of ions generated by LRD can specifically dismiss the viral envelope lipid bilayer and entered deeper into the nucleic acid materials rather than those of the infected cells. Another possible hypothesis of the LRD viral infectivity inhibition in this study is the production of the reactive oxygen species (ROS) by electric stimulations. Previously, the production of ROS destroyed the nucleic acid materials after viral exposure to electric stimulations [13, 35].

5. CONCLUSION

The virus's infectivity treatment along with lower disorders and higher efficacy is a great challenge and need more studies. The results from this study revealed a significant viral infectivity inhibition after short time exposure into of LRD. The effective infectivity inhibition of H5N1 under different conditions indicated that LRD treatment is crucial for further pathogenic viruses' treatment. Changing the materials of LRD rod or cell culture medium or viral dilution showed the same effective treatment profiles. We hypothesized that, the induction of H5N1 viral using LRD might be due to the specifically of a codified number of ions generated by LRD to the viral envelope fluidity. Generally. The results of this study are helpful to design a therapy for pathogenic viruses' treatment. Additionally, this type of novel treatment is safe with lower side effects compared with the antiviral drugs therapy or vaccination. Moreover, it is possible that, in the future LRD system can be designed for viral treatment at home or hospitals with affective treatment.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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