Original Article

In vitro Assessment of Antiviral Activity of Cold Atmospheric Pressure Plasma Jet against the Human Immunodeficiency Virus (HIV)

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Introduction: The human immunodeficiency virus (HIV) is a threat to global health and the need for finding new methods of antivirus research in particular against HIV has increased in recent years. In this study, we investigated the ability of the cold atmospheric pressure plasma jet (CAPPJ) using helium to inhibit the replication of HIV virions. **Methods:** Single cycle replicable HIV (SCR-HIV) virions were produced by transfection of HEK293T cells with pmzNL4-3, pSPAX.2, and pMD2.G plasmids. The HeLa immortal cell line was infected with the virus SCR-HIV and then irradiated by CAPPJ at different voltages and times. The amount of the P24 antigen inside cell supernatant was measured by an ELISA method. Moreover, the cytotoxicity of this plasma jet and the viability of cells were evaluated by the XTT method. **Results:** The inhibition of HIV by CAPPJ increased with increasing the voltage and time of radiation. The highest voltage of 12 kv at 240 s caused virus inhibition; however, the cytotoxicity of HeLa cell line also elevated with increasing of voltage and time. **Conclusion:** CAPPJ substantially suppresses HIV infection *in vitro* while causing toxicity for HeLa cell line. *J Med Microbiol Infec Dis, 2016, 4 (3-4): 62-67*

Keywords: Cold atmospheric pressure plasma jet, Human Immunodeficiency Virus, Single cycle replicable virions, Plasma jet.

INTRODUCTION

The human immunodeficiency virus (HIV), the causative agent of the acquired immunodeficiency syndrome (AIDS) belongs to the retrovirus family with a genome consisting of a diploid positive single-stranded RNA. AIDS is a chronic immune deficiency illness manifested by a severe decrease in cell-mediated immunity [1-4]. The main target of HIV virions is CD4+ T lymphocytes which results in their drastic reduction during HIV infection and finally a chronic immune deficiency leading to death from opportunistic infections and tumors. It is estimated that 34 million people are living with HIV worldwide; this poses an enormous health-care challenge for many countries [5]. The process of HIV infection has three stages, namely acute HIV infection, chronic HIV infection, and AIDS [6].

The appearance of P24 antigen in the patient's blood is the first stage of the primary infection. HIV infection is treated by using a combination of anti-HIV medicines called highly active antiretroviral therapy (HAART). Although HAART is not considered a cure, it can control the virus, and the patient can live a longer and healthier life with reduced risks of transmitting HIV to others. The HAART consists of at least three HIV drugs, two nucleoside reverse transcriptase inhibitors (NRTIs) in conjunction with either a non-nucleoside reverse transcriptase inhibitor (NNRTI) and a protease inhibitor (PI) [7]. Treatment with HAART is optimally able to suppress the HIV replication, but due to the harsh side effects of drugs and development of resistant strains, investigations to discover more effective antiviral drugs against HIV are pursued in many countries.

The fourth state of matter is plasma which exists in thermal and non-thermal forms. Plasma is generated by a breakdown in a gas which contains electrons, ions and reactive chemical agents such as reactive nitrogen species (RNS), reactive oxygen species (ROS), nitric oxide (NO) as well as other short-lived and metastable species. These species attend in chemical reactions which lead to charge accumulation, ion bombardment and UV radiation processes that all can play a significant role in the treatment of the biological samples [8-14]. When a sample comes in contact with the plasma, all these factors may interact with the surface of the sample.

The artificial plasma made in laboratory conditions includes thermal and non-thermal plasmas. In thermal plasmas, the energized electrons are in the balance with the

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heavy particles, leading to quick heating and energy transfer of the gas. However, in non-thermal plasmas, such balance is not merely achieved which leaves the gas at ambient temperature [15]. Due to this characteristic, the applications of non-thermal plasmas in biomedical research have recently attracted a remarkable attention. During the last decades, plasma has drawn lots of research interest due to its promising potentials as a new biomedical treatment method. Plasma sources have been used for their potential applications in different biomedical research areas such as bacterial and fungal sterilization [16-19], suppressing the viruses [20], blood coagulation [21], wound treatment [22], surgical operations [23] and cancer treatment [24-27].

Nowadays, more attention has been directed to atmospheric pressure plasma due to its simplicity and low cost in comparison with the low-pressure plasma [28]. The atmospheric pressure plasma is a non-equilibrium plasma type, *i.e.*, without local thermodynamic equilibrium, containing hot electrons, cool ions, and neutral species within room temperature range, *i.e.*, 30-40°C [29]. From a macroscopic point of view, this type is considered as cold plasma. This features of the cold atmospheric pressure plasma have opened a wide horizon for its application in industry and medicine [30-32].

Plasma jet is an apparatus producing the atmospheric pressure plasma by using a high voltage powered electrode wrapped around an insulating tube. The plasma is created, when the gas enters the tube and is ionized by a high voltage. An ionized gas plume then is emitted out from the tube into the air which can interact with the samples under study [33]. In the current study for the first time, we investigated the inhibitory effect of the cold atmospheric pressure plasma jet (CAPPJ) on HIV replication in cell cultures with different times of plasma radiation periods and voltages in fixed frequency.

MATERIAL AND METHODS

Cell culture. Human Embryonic Kidney 293T (HEK293T) cells were cultured to produce the HIV virions. HeLa cells were also used to evaluate the antiviral effect of Cappy CAAPJ. The HEK293T cell line was chosen for this research because of its superior survival and proliferation capabilities while immortal HeLa cell line had been used in similar studies on cancer and HIV infection research [34]. Both of these cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA) and appropriate concentrations of L-glutamine (Sigma, USA) and sodium pyruvate (Sigma, USA). The cultures were maintained in a 37°C incubator with 5% CO_2.

Production of Single Cycle Replicable (SCR) HIV-1. Generation and quantification of SCR HIV-1 stocks were performed as described in the previous studies [35, 36]. Briefly, Pseudo-typed SCR HIV-1 virions were generated by co-transfecting HEK 293T cells with pmzNL4-3, psPAX2, and pM2.G plasmids using Turbo Fect Transfection Reagent (Thermo Fisher Scientific, Belgium), according to the manufacturer's instruction. The pmzNL4-3 has an HIV-1 molecular genome with a 2 kbp deletion within *pol* region, psPAX2 encoding Gag-pol polyproteins, and pM2.G encoding vesicular stomatitis virus glycoprotein (VSVG). The PmzNL4-3 can produce a viral mRNA possessing the packaging signal (ψ) while psPAX2 and pM2.G lack this packaging signal. The supernatants of the transfected cells were harvested at 48 and 72 h post transfection. The collected supernatants were then cleared by centrifugation at 5000 x *g* for 15 min. To concentrate the virus stock, the harvested virions were pelleted at 58,000 x g for 3 h at 4°C and then were resuspended in RPMI 1640 (Gibco, USA) by gentle mixing at 4ºC. For titration of the SCR HIV-1 virions, the concentrated supernatant was subjected to the measurement of HIV-1 p24 antigen, using the Gag-p24 antigen enzyme-linked immunosorbent assay kit (Advanced Bioscience Laboratories, Maryland, USA), according to the manufacturer's protocol. The viral stocks were stored at -70ºC until used. The whole process of virus production was carried out in the current study to yield enough viral stock.

CAPPJ configuration. A schematic diagram of CAPPJ is depicted in Figure 1. The plasma jet consisted of a quartz tube as an insulating tube. A copper wire is wrapped around the quartz tube as the power electrode constituting a nozzle with 10 mm width. The plasma jet was fed by a 6 kHz pulsed DC high voltage power supply with a variable voltage selector. The applied voltage on the electrodes ranged from 0 to 12 kv. The feeding gas for this essay was 99.999% pure helium (He) with 2 L/min gas flow rate.

To investigate the inhibitory effect of plasma on HIV replication, the samples were treated by plasma jet at three different voltage discharges; namely 9, 10 and 12 kv in five different plasma exposure times 10, 30, 60, 120, and 240 s for every mentioned voltage. The space between the nozzle tip and the samples was 10 mm that was kept unchanged to have the same treatment situation for all the samples.

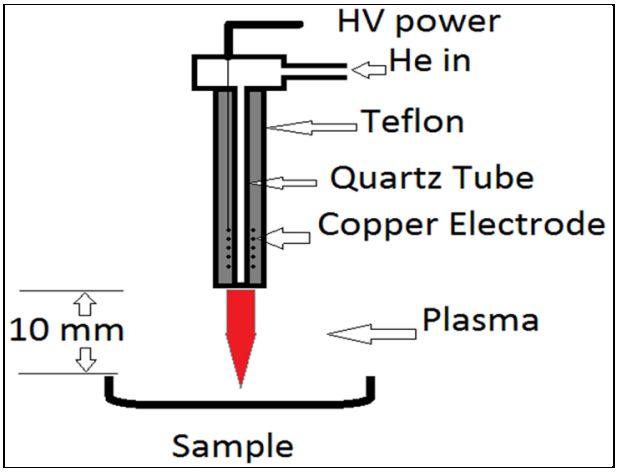


Fig. 1. A schematic diagram of CAPPJ. It shows sites of High Voltage Power, Helium gas input, Teflon, Quartz Tube, Copper Electrode and irradiation plasma on the sample.

Effect of CAPPJ exposure on HIV replication. The inhibitory effect of CAPPJ against HIV-1 was studied by a single cycle replication assay. Briefly, HeLa cells were

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seeded in a 96 well plate (8×10^3 cells/100µLper well). The day after, HeLa cells were infected with SCR HIV virions (120 ng P24 for each well). The CAPPJ was used to irradiate the 5 groups of samples at different exposure times of 10, 30, 60, 120, and 240 sec and voltages of 9, 10 and 12 kv with 6 kHz fixed frequency in five different modes as reflected in Table 1.

Twenty-four hours after the infection, the cells were washed twice with pre-warmed DMEM (to remove the unbound virions) and then 250 µl DMEM were added to all wells. The supernatants were collected 72 h postinfection and were tested for the p24 antigen by a quantitative ELISA according to the manufacturers' instructions (Biomerieux, France). The inhibition rate (%) of the p24 expression was then evaluated.

Cell proliferation assay (XTT method). The cytotoxicity of CAPP irradiation on HeLa Cells was assessed by the Cell Proliferation Kit II (XTT) (Roche, Germany) according to the manufacturer's instruction.

The HIV replication assay plates were straightly evaluated for cytotoxicity assay after determination of P24 load. XTT solution was added to the wells, and then the plates were incubated for 4 h at 37°C. The optical density (OD) of the plate was measured using microplate reader (BioTek, Finland) at 450 nm.

Statistical analysis. The results of quantitative experiments were reported as mean ± SD using GraphPad Prism 6 software (San Diego, CA). All tests were repeated at least three times. To statistical evaluation of data, one-way analysis of variance (ANOVA) with Dunnett's post-hoc test was applied. In all cases, $P<0.05$ was taken as the level of significance.

Table 1. The groups that were exposed to the plasma radiation

Group Number	Group Description
1	The SCR virions which were irradiated before infection (to evaluate virucidal effect)
2	HeLa cells that were irradiated to assess the cytotoxicity of irradiation
3	HeLa cells which were irradiated immediately after infection
4	HeLa cells which were irradiated two hours after infection
5	HeLa cells which were irradiated six hours after infection

RESULTS

The cytotoxicity assay was carried out to find the voltage and time of exposure that is safe for the HeLa cell line. The anti-HIV activity of CAPPJ was measured by determining the inhibitory effect on the replication of HIV-1. In the first group, The SCR HIV were irradiated by CAPPJ before infection (adding to cells) (Fig. 2). The results of the first group showed that by increasing the voltage and time of irradiation on the virus directly, the virus became weaker and destroyed. For example, inhibition of the virus replication in 9 kv/240s was 64.5%, in 10 kv/240s 69.7%, and in 12 kv/240s 73.5%. Furthermore, due to the lack of direct plasma radiation on the wells, by increasing the voltage and time of irradiation on the virus directly, a better viability was observed.

The second group was used as a control, in which the plasma was irradiated on HeLa cells in the wells. The results showed by increasing the voltage and time of irradiation on the HeLa cells, the viability of the cells reduced. For example, the cytotoxicity for HeLa cells in 9 kv/10s was 0.8% and at 12 kv/240s 41% (Table 2).

The results of the third group showed that by increasing the voltage and time of irradiation on the wells containing infected cells, the virus was destroyed and the viability of the cells decreased. Here, the inhibition of the virus replication at 9 kv/240s, 10 kv/240s, and 12 kv/240s were 61.9%, 68.2%, and 73.3% respectively (Fig. 3). Moreover, the cytotoxicity of HeLa cells in 9 kv/240s, 10 kv/240s, and 12 kv/240s was 30.4%, 34.4%, and 40.5% respectively (Fig. 4).

Results of the fourth group showed that by increasing the voltage and time of irradiation on the infected HeLa cells two hours post infection, the virus titer decreased significantly. For instance, the inhibition of the virus replication at 9 kv/240s, 10 kv/240s, and 12 kv/240s was 68.4%, 71.3%, and 74.7%, respectively (Fig. 5). The cytotoxicity of HeLa cells at 9 kv/240s was 33.8%, at 10 kv/240s 36.7% and 12 kv/240s 40.9% (Fig. 6).

The results of the fifth group showed that by increasing the voltage and time of irradiation on the infected HeLa cells 6 h post infection, the virus replication decreased drastically. In this group, the inhibition of the virus replication at 9 kv/240s, 10 kv/240s, and 12 kv/240s was 70.1%, 73.8%, and 78.2%, respectively (Fig. 7). The cytotoxicity of HeLa cells at 9 kv/240s was 48.9%, at 10 kv/240s 55.2% and at 12 kv/240s 57.3% (Fig. 8).

Table 2. The cytotoxicity effects of CAPPJ on group 2 in which only HeLa cells were irradiated

9 kv	10 kv	12 kv
10s= 0.8%	10s= 4.4%	10s= 14.7%
30s= 4.4%	30s= 14.2%	30s= 21.6%
60s= 21.4%	60s= 23.5%	60s= 28.9%
120s= 26%	120s= 28.9%	120s= 30.2%
240s= 32%	240s= 35.5%	240s= 41%

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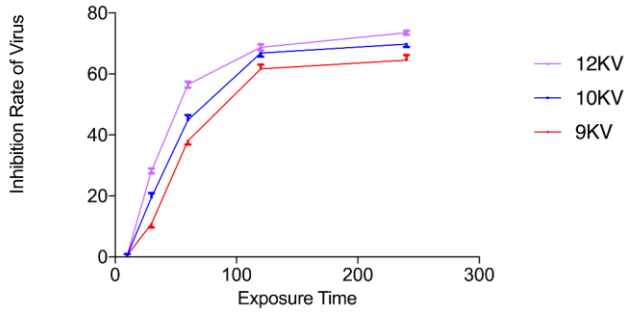


Fig. 2 Inhibition rate of HIV replication by CAPPJ in group 1 for evaluation virucidal effect of irradiation before infection.

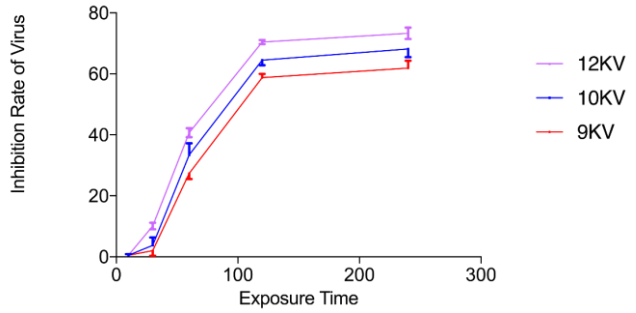


Fig. 3. Inhibition rate of HIV replication by CAPPJ in group 3; HeLa cells were irradiated immediately after infection by SCR virions.

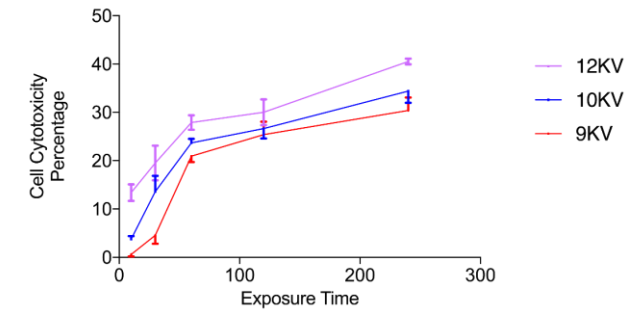


Fig. 4. Cytotoxicity effects of CAPPJ on HeLa cells in group 3; HeLa cells were irradiated immediately after infection by SCR virions.

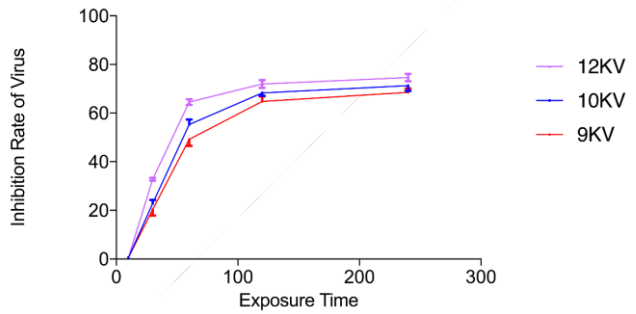


Fig. 5. Inhibition rate of HIV replication by CAPPJ in Group 4; HeLa cells were irradiated 2 h after infection.

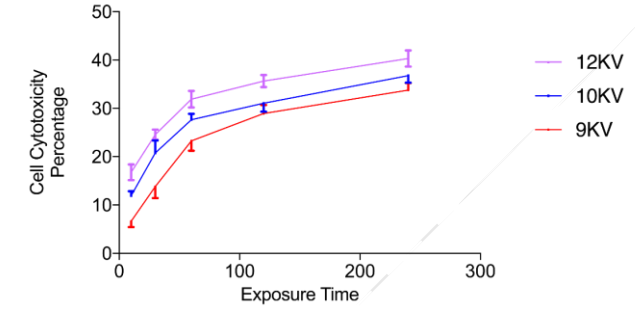


Fig 6. Cytotoxicity effects of CAPPJ on HeLa cells in group 4; HeLa cells were irradiated 2 h after infection.

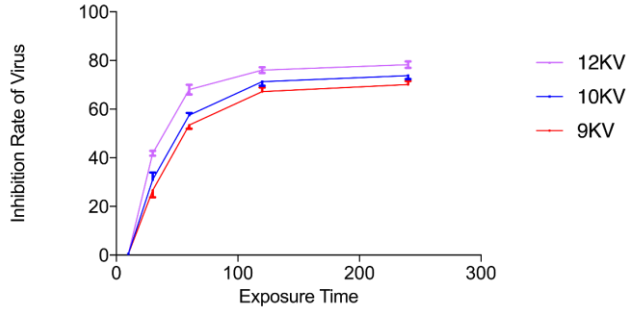


Fig.7. Inhibition rate of HIV replication by CAPPJ in Group 5; HeLa cells were irradiated 6 h after infection.

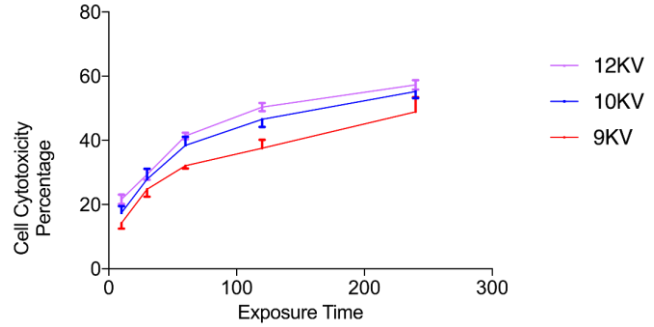


Fig. 8. Cytotoxicity effects of CAPPJ on HeLa cells in group 5; HeLa cells were irradiated 6 h after infection.

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DISCUSSION

The use of non-thermal plasmas in biomedical applications holds a significant promise; this method has generated much interest in the recent years. The considerable heterogeneity and rapid turnover of HIV in infected people and a very high frequency of mutations during replication have made this virus capable of rapid development of resistance to the existing drugs. Therefore, the introduction of new medicine and inhibition methods is essential to fight against HIV infection. In this regard, in the current study, the antiretroviral effect of CAPPJ was evaluated. The efficiency of CAPPJ depends on the factors such as the applied voltage, gas flow velocity, the type of gas and the distance between the electrodes. In the present survey, we used helium gas with variable voltages while the gas flux was fixed. The results of previous studies have shown that plasma can help to inactivate or reduce the growth of microorganisms such as bacteria, fungal and viruses [16-20]. The effect of plasma on cancer cells are shown to be more pronounced compared to the normal cells [24-27]. A variety of charged particles such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and UV Light can promote oxidative stress and play a role in cell apoptosis and cell death. For instance, Kim *et al.* investigated the effects of non-thermal plasmas on cancer and normal cells and showed the antiproliferative and apoptotic effects of plasma on the cancer cells [25]. Moreover, Lapa and colleagues exhibited the effect of cold plasma jet of helium/oxygen on cancerous cells and demonstrated that this approach could be instrumental in the treatment of the tumors [37]. Also, Mirpour and others showed that plasma has a higher cytotoxicity effect on the breast cancer cells comparing the normal cells (approximately 32% higher) and also revealed that the plasma even during 300 s of irradiation could cause morphological changes in the malignant cell lines [33].

In the present study, irradiation of HIV virions by the cold plasma before infection (virucidal effect), inactivated viral infection up to73.5% (at 12 kv and 240 s). This viral inactivation was in agreement with another study in which the activity of Dielectric Barrier Discharge Atmospheric Cold Plasma (DBDACP) cytopathic effect on herpes simplex virus (HSV) was investigated. The data showed that by increasing the time of radiation, replication of HSV decreased on epithelial cells of the cornea dramatically [20].

As expected in group 1, where the virus was first irradiated and subsequently added to the wells containing HeLa cells, higher percentages of the virions were destroyed with increasing the voltage and the irradiation time (Fig. 2). In groups 3, 4 and 5, the destructive effect of the irradiation affected both the virions and the HeLa cells. So in this survey, we pursued the irradiation voltage and exposure time with the least cytotoxic effect and the highest viral inhibitory simultaneously. So in group 2, the CAPPJ effect was investigated on HeLa cells to determine the cytotoxic consequences of the plasma. Likewise, with increasing voltage and irradiation time, the viability of the cells decreased (Table 2). This experiment revealed that little difference was detectable comparing with the groups where the virions were present. In groups 3-5, with increasing voltage and irradiation time, the viability of the cells decreased, and the percentages of the destructed virions increased at the same time (Fig. 3-8).

The results of group 5 showed the higher cytotoxic effect on the HeLa cells comparing with other groups. Besides irradiation effect on the viability of the cells, the cytolytic effect of replicative HIV virions might play a role in this phenomenon.

The results of this study showed that by increasing the voltage and time of CAPPJ, HIV could be destroyed *in vitro*. However, the adverse effects of CAPPJ on the HeLa immortal cells were higher than the normal cells.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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