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In vitro model of HIV virus production in postmortem leucocytes identified with HIV RNA in culture

Klarisa[1, *], Budi Sampurna, Aria Yudhistira[1], Djaja Surya Atmaja[1], Budiman Bela[2]
[1]Department of Forensic Science and Medicolegal, Faculty of Medicine Universitas Indonesia-Dr. Cipto Mangunkusumo National General Hospital, Jakarta, Indonesia.
[2]Department of Microbiology, Faculty of Medicine Universitas Indonesia-Dr. Cipto Mangunkusumo National General Hospital, Jakarta, Indonesia.
E-mail: *klarisasalimdr@gmail.com*

Abstract. Purpose: The research aimed to investigate in vitro HIV replication in leucocytes imitating dead bodies' physiological oxygen deprivation condition. Methods: The research was conducted using experimental design. Blood samples were obtained from living persons infected by HIV to substitute for blood from dead bodies. Blood samples were studied for 48 hours at a temperature of 26-32°C with oxygen deprivation as a post-mortem condition. The plasma of HIV-infected blood was examined for viral load. The leucocytes were separated from the red blood cells and mixed with a plasma of HIV-positive individuals. This suspension was stored in tubes and heated cooking oil was overlayed on the surface of the plasma and leucocyte suspension to prevent oxygen exposure. Following centrifugation of suspension, the leucocytes were cultured. RT PCR was employed to detect virus production in the culture supernatant. Results: Polyacrylamide gel analysis showed that new RNA HIV was detected by RT PCR up to 48 hours of oxygen deprivation. This study also found morphologic changes in leucocytes due to cytopathic effects in the form of cell-to-cell infections. Conclusion: HIV can still replicate and produce new viruses after the isolation of leucocytes from a living person's blood with oxygen deprivation.

Keywords. Blood culture, HIV in human postmortem/dead body, HIV replication, HIV RT PCR, Oxygen deprivation.

1. Introduction

A forensic pathologist must do the post-mortem external or internal examination. One high-risk medical condition from deceased or dead bodies to handling is Human Immunodeficiency Virus (HIV) infection [1, 2]. HIV infection can occur in a cell-to-cell or cell-free manner. Cellular infections are more effective than free HIV infections. Cell-to-cell infection begins with the binding of chemokine receptors namely CCR5 and CXCR4 of healthy cells by infected cells leading to activation of ENV glycoprotein, hence fusion of the membranes of the two cells that facilitates the transfer of HIV's genetic materials into healthy cells. The fusion of healthy cells and HIV-infected cells will show the formation of syncytia, which in turn will increase in size and fuse to form multinucleated giant cells. In vitro, fusion can occur between 10-30 minutes after adding healthy cells to the cultivation of HIV-infected cells. This process of genetic transfer resembles the process of infection from free HIV to healthy cells [3-7]. The newly formed HIV virions are released via budding, in which micro-holes are formed on the host cell's surface. Consequently, the cellular ionic balance is disrupted and undergoes lysis to release the virions. This process is the final phase of HIV infection in healthy cells in which the Cytophatic Effect (CPE) occurs, causing HIV-infected white blood cells to die faster than those who are not infected. This death is not followed by stimulation of the proliferation of new white blood cells, ultimately a decrease in the number of T cells in HIV-infected patients [5-6].

During the post-mortem process, external and internal factors affect the human dead body. External factors such as temperature, humidity, and oxygen partial pressure. Internal factors like cell and tissue resistance to oxygen requirements affect cellular activity, for instance, lungs and heart tissue require a lot of oxygen around 4-14%, and bone tissue with low oxygen requirements of only around 0-4%. Generally, the hypoxic response at the cellular level will appear at an oxygen concentration of less than 1%. In each cell, factors that regulate oxygen pressure are influenced by cell types, in terms of oxygen needs, the distance between the cells and lungs, the cellular density of the vascularization around the cells, and the average oxygen consumption around the cells. In vivo, oxygen pressure is deemed physiological at a 3% level, while in vitro is around 20%. This difference would not significantly affect the viability of the cells, especially lymphocytes and monocytes stimulated by phytohemagglutinin (PHA). However, it would affect cell activity and viral proteins [3, 7-11].

Previous studies used blood samples taken from human dead bodies or postmortem at lower temperatures and only detected the expression of the virus at the beginning of infection, without detecting the viability of HIV from its replicability. Also, monocyte from in vitro blood samples from living people stored at 24-26° C and monocytes in vitro blood samples taken from a dead body that has been stored for 21 hours at room temperature has the same morphological picture within 6 hours, while lymphocytes within 21 hours [8].

This study was conducted to determine the viability of HIV characterized by its ability to replicate within white blood cells following cessation of oxygen exposure until 48 hours at 26-30° C temperature.

2. Methods

This research used an experimental study design under a controlled environment with an average room temperature in Indonesia of around 26-30° C. Blood samples used were taken from living patients infected with HIV with a viral load

of more than 10^3 copies of (Ribonucleic Acid) RNA/mL blood. As much as 12 mL blood was taken, 3 mL for viral load examination and 9 mL for RPMI 1640 medium culture and (Reverse Transcription Polymerase Chain Reaction) RT PCR examination using the Applied Biosystems® instrument. Viral load was calculated using the Cobas Taqman® device in the Rumah Sakit CiptoMangunkusumo, Jakarta, HIV Integrated Services Unit. Culture and RT PCR was conducted at the Virology and Cancer Pathobiology Research Centre Laboratorium of the Faculty of Medicine Universitas Indonesia.

Samples were obtained from 2 HIV-infected patients, then divided into 2 tubes and coded with C1, C2, D1, and D2. The alphabetic indicates individual patient and the number indicates the aliquotes. Each sample would then be isolated to obtain PBMC. A portion of HIV-infected PBMCs isolated from blood samples of HIV-infected patients was then separated into six different tubes based on six intervals, i.e., 0, 2, 4, 6, 24, and 48 hours. To resemble a post-mortem atmosphere, the infected PBMC was suspended with the plasma of the infected patient in sterilized 1.5 ml screw cap microtubes, and the surface of the suspension was overlayed with approximately 250 µl cooking oil that had been sterilized by autoclave at 121° C. All of the procedure was performed aseptically in a Class II biosafety cabinet.

To maintain the temperature of the suspension at 26-32°C, the tube was placed in a heat block with a temperature of 30°C. Temperature control was carried out by placing a tube containing water and a thermometer in one of the heat block wells. All samples were placed in the heat block until the specified time interval since the beginning of oxygen deprivation by overlaying of cooking oil, except the hour-0 tube which was directly cultured. Before culture, the leucocyte and plasma suspension were centrifuged at a speed of 1000 RPM for 10 minutes. Subsequently, the overlayed cooking oil and the plasma were removed. The pellet was then washed with Phosphate Buffer Saline (PBS) 1: 1 three times and then centrifuged at a speed of 1000 RPM for 10 minutes. The resulting PBS wash supernatants were collected and stored at -80° C. The leucocyte pellets were resuspended using 1 ml of complete medium (Gentamicin, FBS 20%, IL-2 20%, Penstrep 1%, Amphotericin 1%, RPMI 1640).

Culture of oxygen-deprived leucocytes was carried out using the microculture technique [13], briefly, cell suspension containing $1x10^6$ infected Peripheral Blood Mononuclear Cells (PBMC) was added with 1 x 10^6 PHA stimulated PBMC and complete medium to a volume of 2 mL at each time interval. Incubation of the culture was carried out at 37°C±2°C, 5% CO_2, and 95% humidity. On each following day, 1 mL of supernatant was taken and stored at -80°C for the first 7 days and on the 21st day. The supernatant that had been taken was then replaced by the same amount of culture medium. Every 3-4 days $5x10^5$ PHA-stimulated donor PBMC was added to each culture. Observation to identify the presence of bacteria and/or fungi in the culture well plate was carried out by microscopic examination while taking the supernatant. Should there be any sign of contamination, the culture was disposed of immediately according to the laboratory decontamination standard and sterilized to prevent contamination of other samples. On the seventh day of each interval, 1 mL of the supernatant was taken, divided into two of 500 µL each, and stored at -80°C. On the tenth day of the culture, RT PCR was performed.

HIV growth was assessed by RT PCR examination using culture supernatant; it was determined as positive if HIV RNA components were found. Plasmid Deoxyribonucleic Acid (DNA) pNL43 and plasma samples tested positive for HIV RNA were used as positive controls. The negative controls used were distilled water and PHA-stimulated donor cells. Supernatants from cultures at each time interval of oxygen deprivation, and PBS were used to wash the pellets, and positive control samples were centrifuged at 21,000 xg for 75 minutes at 4°C. The samples were extracted using the QIAamp Viral RNA Mini Kit (qiagen®), followed by the addition of RNase-free DNase and RNase inhibitors. The sample that was added to both enzymes was divided into 2 and subsequently underwent Polymerase Chain Reaction (PCR) and RT PCR. PCR was conducted to detect any presence of DNA bands. The primary sequencing used was the GAG primer.

The data were processed using SPSS version 24. Data analysis was conducted by paired categorical comparative analysis of 2 groups. Hence Cochran-Q test was used. Significant results were indicated by the value of $p < 0.05$, and post hoc analysis using the McNemar was conducted to find the relationship between significant results.

3. Results

Visualization using acrylamide gel showed that all samples showed bands at each time interval, except for the last sample in which the band did not appear at the 48th hour interval.

Visualization of RT PCR using acrylamide gel showed that in all four samples, there were bands up to the 48th time interval except for sample D2.

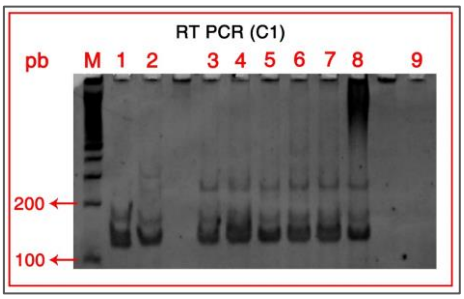

Figure 1. RT PCR Sample C1

Lane 1-2 Control (+); 1: pNL43 DNA, 2: HIV RNA positive human plasma. Lane 3-8 sample C1 according to oxygen deprivation

time interval; 3: 0-hour, 4: 2-hour; 5: 4-hour; 6: 6-hour; 7: 24-hour; 8: 48-hour. Lane 9 Control (-) H_2O.

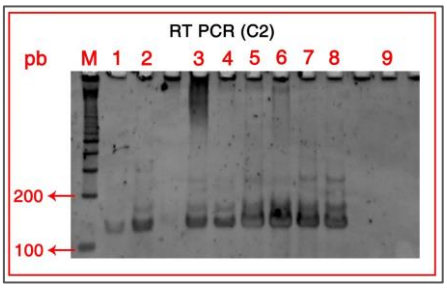

Figure 2. RT PCR Sample C2

Lane 1-2 Control (+); 1: pNL43 DNA, 2: HIV RNA positive human plasma. Lane 3-8 sample C2 according to oxygen deprivation time interval; 3: 0-hour, 4: 2-hour; 5: 4-hour; 6: 6-hour; 7: 24-hour; 8: 48-hour. Lane 9 Control (-) H_2O.

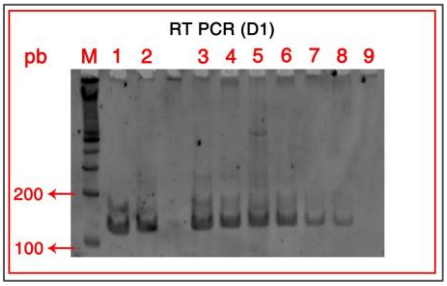

Figure 3. RT PCR Sample D1

Lane 1-2 Control (+); 1: pNL43 DNA, 2: HIV RNA positive human plasma. Lane 3-8 sample D1 according to oxygen deprivation time interval; 3: 0-hour, 4: 2-hour; 5: 4-hour; 6: 6-hour; 7: 24-hour; 8: 48-hour. Lane 9 Control (-) H_2O.

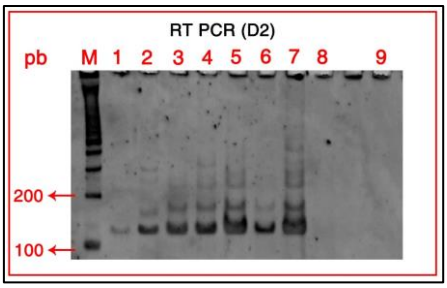

Figure 4. RT PCR Sample D2

Lane 1-2 Control (+); 1: pNL43 DNA, 2: HIV RNA positive human plasma. Lane 3-8 sample D2 according to oxygen deprivation time interval; 3: 0-hour, 4: 2-hour; 5: 4-hour; 6: 6-hour; 7: 24-hour; 8: 48-hour. Lane 9 Control (-) H_2O.

The statistical analysis resulted in $p = 0.416$ which means there is no significant difference in the number of samples experiencing HIV replication between 5 measurements compared to the hour-0.

On the 21st day, several cells displayed changes; namely, there were larger cells with several nuclei on them or becoming multinucleated giant cells.

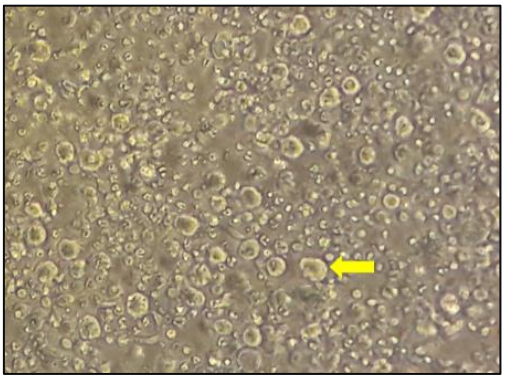

Figure 5. Sample C2 day-21, the time interval of hour-24 *Multinucleated Giant Cell* (yellow arrow) among smaller cells.

In contrast to the image of cells in the negative control containing normal PBMCs, the cells multiply without the

presence of a multinucleated giant cell.

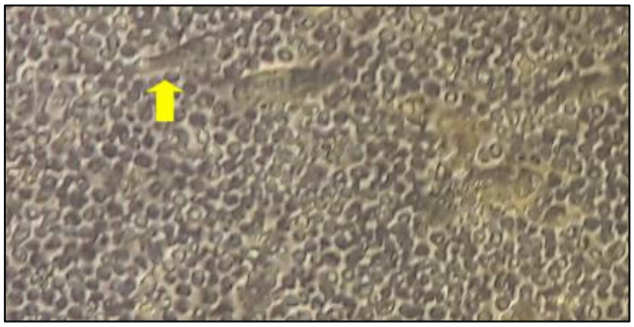

Figure 6. Negative control. Normal PBMC appeared dense with spindle cells (yellow arrow).

The appearance of cells in culture looks different in the culture of sample D2, in which a group of cells clustered together and appear darker (more brownish) than the surrounding cells, also known as ghost cells.

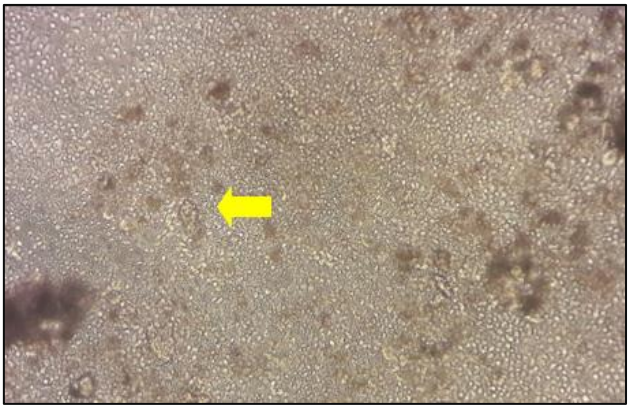

Figure 7. Sample D2 day-7, time interval of hour-48. Ghost cell pointed by the yellow arrow.

4. Discussion

The sample used was white blood cells from living patients with HIV infection, based on a study conducted by Dokgöz et al., in which morphological changes of white blood cells from cadavers and a living person stored at 24°C - 26°C were almost the same [8]. The hour-0 point was then determined when blood was drawn, and PBMC was separated from all components of the whole blood. The blood drawing process will change the blood environment from oxygen-rich into anaerobic, the accumulation of carbon dioxide, changes in acid-base balance, and the accumulation of toxic metabolites from the bodies that have already occurred [9, 10].

The interval between blood drawing, transportation to the laboratory, up to the separation of PBMC was considered neglect since blood was stored under 4°C. This indicated the metabolic process was in a minimal condition, and it did not resemble the condition after death. The effect of this interval was not analyzed in this study.

The results of this study proved that up to hour-48 samples in C1, C2, and D1 (figure 1.1, 1.2,1.3), there was still detectable RNA of HIV as a product of replication. The possibility that it was due to the remaining fragments and genetic material of the HIV and proviral DNA of HIV-infected patients was excluded by pellet washing using PBS and the addition of RNase-free DNase and RNase inhibitor [11][12]. Otherwise, the possibility of RNA remaining from PBMC samples was excluded by negative results from PBS-washed RT PCR testing (data not shown). This result is also consistent with Dokgöz et al, that monocytes can be detected until 48-72 hours and lymphocytes until more than 96 hours in vivo and in vitro blood drawn in human and human dead bodies [8], which HIV more stable lived in monocyte and lymphocyte. In this research, we conducted only 48 hours.

Additionally, the discovery of multinucleated giant cells (Figure 1.5) in this study confirmed cell-to-cell infection occurred, thus indicating positive results occurred from replication [4, 13-14]. On the contrary, the negative control PBMC culture did not show any multinucleated giant cells, instead spindle cells (Figure 1.6) were seen. This cell is a monocyte-derived macrophage, part of tissue macrophages, and can be obtained from in vitro blood [13, 15-16].

Negative results in the hour-48 interval of sample D2 could be justified by many reasons, such as the formation of ghost cells (figure 1.7) from donor PBMCs, contaminants such as *Mycoplasma sp*. which is relatively antibiotics resistant and has a CPE effect causing cells to die [17-19], the effect of donor PBMC factor that has a CCR5-delta 32 genetic polymorphism that is relatively resistant to HIV infection due to a loss of 32 pb in the CCR5 gene coding region, hence interfere with HIV binding to CCR5 receptor, but this polymorphism is most common in Caucasians while all donors have Mongoloid race [19]. Other various explanations behind sample D2 show negative results, however, statistically, they do not affect other results significantly.

The purpose of giving cooking oil is to reduce oxygen levels obtained from the air. Even though the cooking oil

overlay may not be very effective for the prevention of oxygenation. Cooking oil contains oxygen but can be reduced by heating at 25°C - 120°C [20, 21]. Reducing oxygen infiltration to cooking oil and samples in this model, however, oxygen deprivation was secured by using a sealed screw cap after cooking oil overlay.

5. Conclusion

HIV in human white blood cells in HIV-infected blood plasma was still able to replicate and produce new viruses for at least 48 hours after cessation of oxygen exposure. The replicability of HIV is accompanied by morphological changes in infected cells in PBMC cell cultures. Despite the finding by Dokgöz et al that in 48 hours was leucocytes from cadavers and living individuals showed signs of deterioration, we found that the HIV still able to produce new RNA.

Based on this study, there is a need to increase knowledge and implementation of Occupational Safety and Security for workers working with infected bodies or bodies suspected of being infected with HIV. HIV screening should always be performed before external examinations and/or internal examinations. Further research on the end time limit of HIV replication ability should be done. Lastly, handling bodies with HIV infection should be done in a health facility with a good infectious waste treatment system, and it is better if the body is buried straight after being washed or put in a tightly closed chest.

Acknowledgments

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Conflict of Interest

The authors whose names are listed immediately below certify that they have no affiliations with or involvement in any organization or entity with any financial interest

Author contribution

Klarisa and Budiman Bela designed the experiments, performed the simulations, and carried them out. Klarisa prepared the manuscript with contributions from all coauthors.

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