The Potential of Hematopoietic Stem Cells (Hsc) Against Sars-Cov-2 (Covid-19) With Virus Isolates From Indonesia (In Vitro Study)

Purwati Sumorejo (purwati@fk.unair.ac.id)
Universitas Airlangga https://orcid.org/0000-0002-6144-2481
Disca Sandyakala Purnama
Universitas Airlangga
Andang Miatmoko
Universitas Airlangga
Nasronudin Nasronudin
Universitas Airlangga
Aristika Dinaryanti
Universitas Airlangga
Deya Karsari
Universitas Airlangga
Eryk Hendrianto
Universitas Airlangga
Igo Saiful Ihsan
Universitas Airlangga
Nora Ertanti
Universitas Airlangga
Tri Pudy Asmarawati
Universitas Airlangga
Erika Marfiani
Universitas Airlangga
Zamrotul Izzah
Universitas Airlangga
Alfian Nur Rosyid
Universitas Airlangga
Prastuti Asta Wulaningrum
Universitas Airlangga
Herley Windo Setiawan
Universitas Airlangga

Research

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Abstract

Background: The prevalence of COVID-19 cases in Indonesia until June 9th 2020, Government has confirmed the number of 32.076 positive cases from 34 provinces in Indonesia with 1.923 fatalities. Along with the development of technology, stem cell-based biological medical therapy, and stem cell-based immunotherapy were developed to find out its potential in the case of COVID-19 treatment besides using chemical drugs as a therapy.

Methods: In this study, in vitro research will be conducted to determine the potential of hematopoietic stem cells (HSC) against SARS-CoV-2 (COVID-19) viruses with virus isolates from Indonesia. The SARS-CoV-2 virus was planted in rat kidney cells and Vero cells, then cells that had been planted with the virus were given HSC cells and then evaluated at 24, 48, and 72 hours. The evaluation is done by collecting cells and supernatant from the cell plate and then evaluating the viral load using a Polymerase Chain Reaction (PCR) machine.

Results: The results showed that the addition of HSC on cells that had been infected by SARS-CoV-2 decrease in viral load within 24 to 72 hours in all variations of Multiples of Infection (MoI) values.

Conclusions: The administration of HSC cells has the potential to eliminate SARS-CoV-2 virus. Although this research is an in vitro study, this study could be the basis for the development of alternative stem cell-based therapies to handle COVID-19 cases in Indonesia.

Introduction

Recently our attention has been focused on the spread of coronavirus that occurs in the world without exception in Indonesia. The case spread is dramatically increasing in a short period of time. Starting from the outbreak case that occurred in Wuhan, China then quickly spread to other countries, and finally in March 2020 confirmed coronavirus cases occurred in Indonesia.

Coronavirus is a large family of viruses that can cause disease with mild to severe symptoms. Some examples of coronaviruses that can cause severe symptoms are Middle East Respiratory Syndrome (MERS) and Severe Acute Respiratory Syndrome (SARS). Coronavirus Disease 2019 (COVID 19) is a new type of disease that has never been identified previously caused by a virus called SARS-CoV-2. This virus is a zoonotic virus, which means transmitted between animals and humans. A common symptom of someone infected with COVID-19 is the presence of acute respiratory disorders such as fever, coughing, and shortness of breath. In severe cases, it can cause pneumonia, acute respiratory syndrome, kidney failure, and even death. Reported clinical signs and symptoms related to sufferers of this disease are fever, difficulty breathing and the presence of extensive infiltrate pneumonia in both lungs on patient's x-ray results (DJPPP Ministry of Health, 2020).

WHO determined that COVID-19 has become a global pandemic on April 2020, according to data there are positive COVID-19 cases totaling 1,524,161 cases, and cases of death from COVID-19 totaling 92,941

cases, this cased are from 213 countries who have infected by COVID-19 (WHO, 2020). The prevalence of COVID-19 cases in Indonesia until 9 June 2020, Government has confirmed 32.076 positive cases from 34 provinces in Indonesia with 1.923 death cases (Ministry of Health 2020).

Researchers are trying hard to find a drug that can be used to treat this disease. Researchers have revealed that more than 30 agents including western medicine, natural products, and Chinese medicine have the potential against COVID-19. However, until now there is no definite and recommended therapy/drug for COVID-19 because this is a new disease. Current treatment is the administration of drugs or curative therapy using antivirals, this was chosen because COVID-19 is a disease caused by a viral infection. Antiviral therapy refers to therapies used when MERS and SARS pandemics such as interferon α (IFN-α), lopinavir/ritonavir, chloroquine phosphate, ribavirin, and arbidol have been included in the latest version of the Guidelines for the Prevention, Diagnosis, and Treatment of Novel Coronavirus-induces Pneumonia issued by the Republic of China National Health Commission (NHC) for tentative treatment in handling COVID-19 (Dong et al, 2020). In addition to the use of antiviral therapy, antibiotic therapy such as amoxicillin, azithromycin, or fluoroquinolones is used (Jin et al, 2020).

The mechanism of this virus infection is virus enters the cell and reproduces itself using a host cell machine together with the damage to the host cell, this is the key to finding and developing a therapy for COVID-19 in the future. The SARS-CoV-2 virus induces the respiratory system and alveoli cells in the lungs are stem cells infected by this virus (Chhikara et al, 2020)

In patients with mild manifestations of COVID-19, there was no increase in chemokine and proinflammatory cytokines even in patients who showed symptoms. Whereas in the case of COVID-19 with severe symptoms, a lower lymphocyte count, leukocytes, and a higher neutrophil-lymphocyte ratio and a lower percentage of monocytes, eosinophils and basophils were obtained. Proinflammatory cytokines such as TNF-α, IL-6, and IL-8 and infection markers such as procalcitonin, ferritin, and C-reactive protein are also found to be higher in cases with severe clinical symptoms. The main cause of death in patients with COVID-19 is caused by ARDS (Acute Respiratory Distress Syndrome). This occurs because of a cytokine storm, which is an uncontrolled systemic inflammatory response due to the release of large amounts of proinflammatory cytokines (IFN-α, IFN-β, IL-1β, IL-2, IL-6, IL-7, IL-10, IL-12, IL-18, IL-33, TNF-α, and TGF-β) and large amounts of chemokines. This excessive immune response can cause lung damage and fibrosis thich lead to functional disability (Susilo et al, 2020).

Along with the development of technology, stem cell-based biological medical therapy, and stem cell-based immunotherapy were developed to find out its potential in the case of COVID-19 treatment besides using chemical drugs as a therapy.

Hematopoietic stem cells (HSC) are multipotent progenitor cells that can renew themselves from all different types of blood cells appear during the process of hematopoiesis. One of the important features of HSC is the ability to do self-renew, which is making copies with the same or very similar potential. The blood and the system that forms it, known as the hematopoietic system, consist of many types of cells with special functions (Domen et al., 2006).

Sources of hematopoietic stem cells are from PBMC, Bone marrow, and umbilical cord blood cells. The following are the SPH isolation and culture protocols derived from PBMCs and bone marrow stem cells which must express CD105, CD73, and CD90 and less express CD45, CD34, CD14

The mechanism of the immunomodulatory effects of HSCs is not yet fully understood, although direct and indirect effects have been demonstrated through cell-to-cell interactions or dissolved factors that create a local immunosuppressive environment. HSCs alter the cytokine secretion profile of dendritic cells, naive and effector T cells, and NK cells to induce a more anti-inflammatory and tolerant phenotype. The secretion of proinflammatory cytokines, TNF-α and IFN-γ decreases while IL-4 and IL-10 are more suppressive stimulated. Other factors involved include hepatocyte growth factor, TGF-β1, IL-10, IL-6, prostaglandin E2, nitric oxide, and possible indoleamine 2,3-dioxygenase. Although the exact mechanism has not been clarified yet, some evidence suggests that HSCs are immunosuppressive and anti-inflammatory and can be grafted between incompatible individuals

Based on the above background, an in vitro study was conducted regarding the potential hematopoietic stem cells (HSC) against SARS-CoV-2 (COVID-19) viruses with virus isolates from Indonesia.

Materials And Methods

Materials

Alfa-MEM Medium (Gibco, USA), Fetal Bovine Serum (Gibco, USA), Penicillin-streptomycin (Gibco, USA), L-Glutamine (Gibco, USA), Amphotericin B (Sigma-Aldrich, USA), NaHCO3, HEPES Medium (Gibco), Collagenase type IV (Worthington,, USA), Phosphate Buffer Saline (PBS) (Sigma-Aldrich, USA), Culture dishes (Iwaki), incubator CO2, centrifuge, microscope fluorescence, Polymerase Chain Reaction (qPCR) machine.

Sample Collection

Sarcov-2 isolate were collected from 3 confirmed patients in RSKI Universitas Airlangga Surabaya, with approved ethical clearance, the conscious patients sputum were collected in VTM (viral transport medium) containing Gentamycin sulfate (100µg/ml) and Amphotericin B (0,5µg/ml), after collection of samples, the virus isolated in BSL 3 Laboratory in Institute of Tropical Disease Universitas Airlangga, inside Biosafety cabinet the samples collected into new conical tube then vortexed for 5 minutes and centrifuged with 13.000 rpm for 10 minutes. Each of isolate now we labelled as P1, P2, and P3, after centrifugation the supernatant of each sample aliquoted in cryotubes and preserved in -80 ° C deep freezer.

Human Hematopoietic Stem Cell Isolation

HSC were collected from PMBC of healthy person and maintenance in Stem Cell Laboratory for 2 weeks with changing medium every 3 days and adding mitogen GM-CSF (20ng/ml) and IL-2 (100IU/ml). After 2

weeks the HSC cell confirmed with CD 34 marker by immunocytochemistry and seeded $5x10^5$ in 12 multi well-plate each well.

Cocultivation of NK cell and Sarcov-2 in Vitro

NK cells and sarcov-2 isolate were co-cultivated inside BSL 3 for 72 hours, collected supernatant were centrifuged with 5000 rpm, for 5 minutes, the supernatant and cell pellet were separated and each samples extracted with GENEAID RNA extraction Kit, then proceed to check the amount of copy DNA with qPCR.

Pro-viral Load determination

The determination of the pro-viral load was performed by SEEGENE PCR. Amplification and data acquisition were carried out using the ABI Prism 7500 Sequence detector system (Applied Biosystems).

Results

In this study, in vitro research was conducted to determine the potential of hematopoietic stem cells against COVID-19 with SARS-CoV-2 virus isolates in Indonesia. There are several procedures in this research, culturing the virus and adding hematopoietic stem cells in virus cells that have been successfully cultured.

The first stage is to carry out viral culture that begins with the sputum sampling of COVID-19 positive patients treated at Rumah Sakit Universitas Airlangga (RSUA). This patient's sputum sampling requires an ethics test that was submitted to the RSUA Ethics Commission and has been approved as evidenced by the issuance of the ethics-worthy certificate number 136 / KEP / 2020 on April 20, 2020. The sputum sampling was monitored by the RSUA Task Force Team, patients' samples were taken to carry out a viral culture of 3 patients.

After sampling the patient's sputum, the SARS-CoV-2 virus cell culture process is carried out in a laboratory with the Bio-Safety Level III (BSL3) standard of the Tropical Disease Institute. Before the virus cultured, researchers prepared cells to be used as a virus-growing medium. Cultivation of these cells was carried out in the stem cell laboratory of the Stem Cell Research and Development Center. The cells used in this study were mesenchymal stem cells derived from rat kidney cells, rat liver cells, rat lung cells, bat trachea cells, and Vero cells.

After preparing cells for the SARS-CoV-2 virus growing medium, the virus was implanted from a sample of COVID-19 positive patients. The virus is only planted in rat kidney cells and Vero cells because other cells undergo contamination so it cannot be used. The rat kidney cells and Vero cells were then planted with the SARS-CoV-2 virus and observed by taking cells and supernatants from each plate. Observations were made on cells after inoculation at 24 hours, 48 hours, and 72 hours. Supernatant and cells taken

from each plate of treatment will be used to calculate the viral load of each plate using a Polymerase Chain Reaction (PCR) machine.

After SARS-CoV-2 virus propagation in Vero cells was completed, HSC cell culture was performed. The process of HSC cell culture is by taking blood as much as 50 cc, then the culture process is carried out 1-2 weeks to then produce HSC cells. After the cells are formed cells are characterized by immunocytochemical methods using CD34.

After the HSC cells are ready, then planting the SARS-CoV-2 virus in these cells. HSC cells planted with viruses were observed microscopically at 48 hours and 72 hours.

After the virus has been planted on HSC cells, cells and supernatants are taken from existing cell plates and then the viral load is calculated from all plates using a Polymerase Chain Reaction (PCR) machine.

Table 4.1: Viral Load Data on Cells and Supernatants After Addition of HSC Cells

No.	Virus Dosage	MoI	Period	Viral Load
1.	100µl	0,00416	0 hours	20,8 Copy/µl
			24 hours	0 Copy/µl
			48 hours	0 Copy/µl
			72 hours	0 Copy/µl
1.	150µl	0,00624	0 hours	20,8 Copy/µl
			24 hours	0 Copy/µl
			48 hours	0 Copy/µl
			72 hours	0 Copy/µl
2.	200µl	0,00832	0 hours	20,8 Copy/µl
			24 hours	0 Copy/µl
			48 hours	0 Copy/µl
			72 hours	0 Copy/µl

Discussion

Figure 4.2 (b) shows that rat kidney cells after inoculation of the SARS-CoV-2 virus for 24 hours have been deformed, which is indicated by a change in the mesenchymal cells of the rat kidney cells. This also occurs in Vero cells after 24 hours of inoculation shown in Figure 4.3 (b). Then in Figure 4.3 (c) which is Vero cells after inoculation for 48 hours shows that the Vero cells are loose and clustered to form clumps of cells. Then after 72 hours of inoculation, microscopically in Figure 4.3 (d) there is a change in the

shape of the cell structure in which the Vero cell experiences a cytopathic effect (CPE). Cytopathic effect (CPE) is a change in the structural form of host cells that are formed due to an infection from a virus. CPE occurs when an infection caused by a virus causes lysis of the host cell or occurs when the cell dies without lysis due to the inability of the cell to multiply itself.

Hematopoietic stem cells (HSC) are cells that can renew and rearrange and differentiate into progenitors from all adult blood cell lineages. HSC is probably the best type of stem cell and is currently the only stem cell with an extensive application for the treatment of various diseases. HSC alters the cytokine secretion profile of dendritic cells, T-naive cells, and effectors, and NK cells to induce a more anti-inflammatory and tolerant phenotype, proinflammatory cytokine secretion, TNF-α, and IFN-γ decrease, whereas IL-4 and IL -10 which is more suppressively stimulated (Lozito, 2008). Other factors involved include hepatocyte growth factor, TGF-β1, IL-10, IL-6, prostaglandin E2, nitric oxide, and the possibility of indoleamine 2,3-dioxygenase. Although the exact mechanism has not yet been clarified, some evidence suggests that HSC is immunoregulatory and anti-inflammatory (Lozito et al, 2008).

Examination of cytokines found that an increase in IL-10 levels, then a decrease in TNF-α levels and IL-2 levels in all-time variations and MoI. Based on the data in table 4.1 shows a decrease in the value of the viral load, the amount of viral load at the time of initial administration of HSC cells was 20.8 copies / µl in all variants of MoI, then after administration of HSC cells, the viral load became 0 copies / µl in all-time variants such as 24 hours, 48 hours and 72 hours. It indicates that the addition of HSC causes HSC to eliminate viruses in cells and supernatants after 24 hours inoculation. Binding of virus to the plasma membrane of cells is the crucial step in viral infection. Different surface receptor expression contributes to the susceptibility of cells to infection. Particularly, integrin adhesion receptors have been shown to mediate binding and internalization of a variety of viruses. It was shown that different integrin receptors are already expressed on early HSC. Therefore, it is conceivable that these receptors mediate the interaction between HSC and a variety of viruses (Annete and Werner, 2003).

Another mechanism of HSC in eliminating viruses is by increasing anti-inflammatory cytokines and decreasing inflammatory cytokines that are so strong, so the virus becoming inactive. HSC cells differentiate into the lineage of immune cells which make phagocytosis of the virus occurs and the damaged virus is removed from the cell. Based on these results it is recommended that HSC be given to COVID-19 patients in conjunction with other therapeutic regimens.

Conclusion

Based on the results of the evaluation in this study it was found that the administration of HSC cells has the potential to eliminate SARS-CoV-2 virus. Although this research is an in vitro study, this study could be the basis for the development of alternative stem cell-based therapies to handle COVID-19 cases in Indonesia.

Abbreviations

HSC : Hematopoetic Stem Cell

MoI : Multiple of Infection

PCR : Polymerase Chain Reaction

SARS : Severe Acute Respiratory Syndrome

MERS : Middle East Respiratory Syndrome

IFN : Interferon

TNF : Tumor Necrosis Factor

IL : Interleukin

ARDS : Acute Respiratory Distress Syndrome

TGF : Transforming Growth Factor

CD : Cluster of Differentiation

PBMCs : Peripheal Blood Mononuclear Cells

BSL-3 : Bio Safety Level 3

RNA : Ribonucleic acid

DNA : Deoxyribonucleic acid

CPE : Cytopathic Effect

Alpha MEM : Alpha Minimun Essential Medium

GM-CSF : Granulocyte-Macrophage Colony Stimulating Factor

HEPES : (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) Medium

$NaHCO_3$: Natrium bicarbonate

RSKI : Rumah Sakit Khusus Infeksi Universitas Airlangga, Surabaya

Declarations

Ethics approval

The patient's sputum sampling requires an ethics test that was submitted to the Rumah Sakit Universitas Airlangga (RSUA) Ethics Commission and has been approved as evidenced by the issuance of the ethics-worthy certificate number 136/KEP/2020 on April 20, 2020, following the regulatory guidelines of the country.

Consent to publication

Not applicable.

Availability of data and material

Availability of data and materials.

Competing interests

The authors report no conflicts of interest in this work.

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Figures

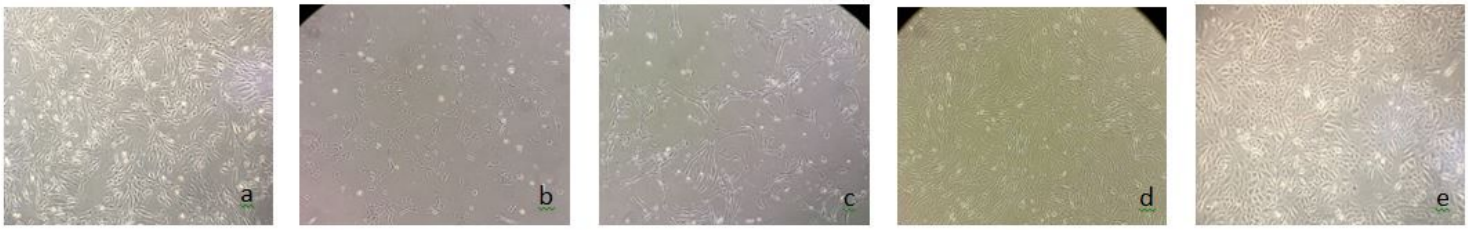

Figure 1

Microscopic Image of Mesenchymal Stem Cell, (a) Rat Kidney Cells, (b) Rat Liver Cells, (c) Rat Lung Cells, (d) Bat Tracheal Cells, (e) Vero Cells

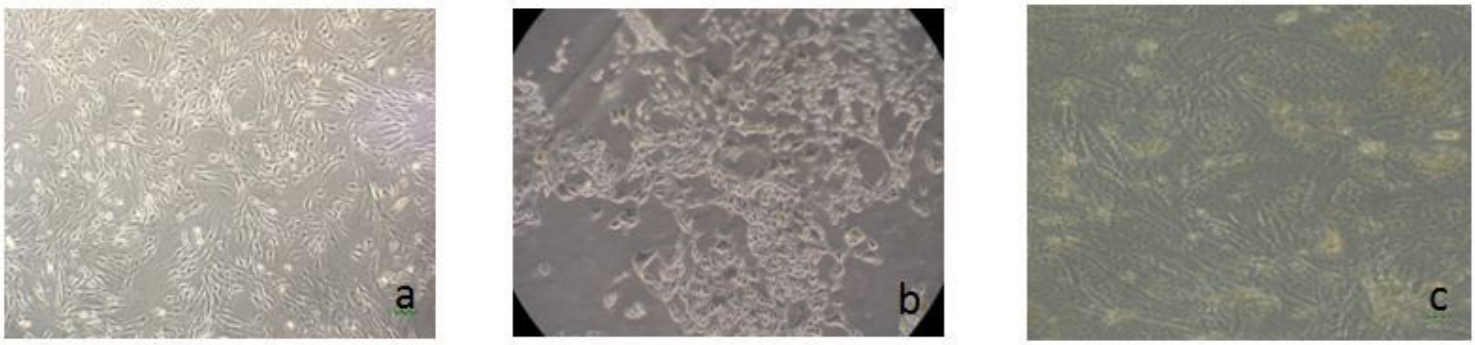

Figure 2

Microscopic Images of Mouse Kidney Cells That Have Been Planted with Viruses (a) Before Inoculation, (b) Inoculation 24 hours, (c) Cells and supernatants collected for PCR examination

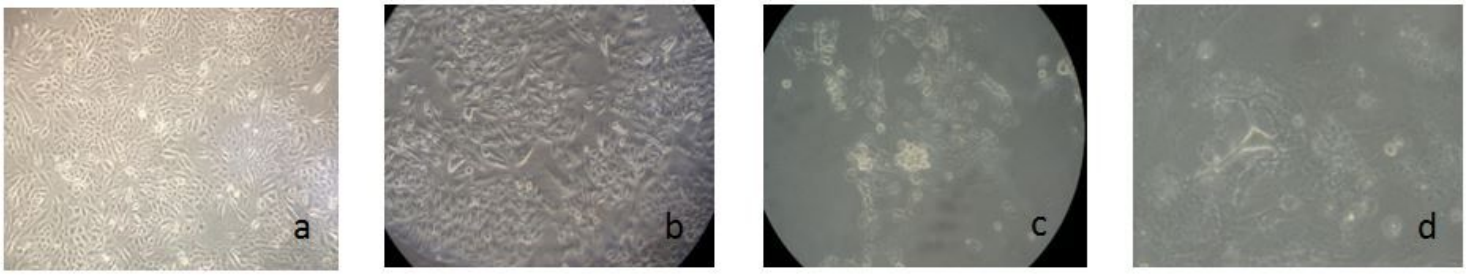

Figure 3

Microscopic Images of Vero Cells that Have Been Planted with Viruses (a) Before Inoculation, (b) 24-hour inoculation, (c) 48-hour inoculation, (d) 72-hour inoculation

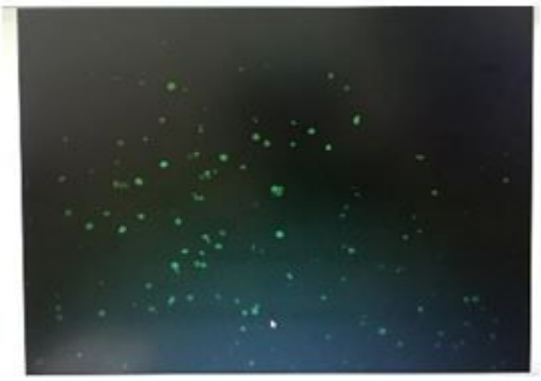

Figure 4

Results of HSC Cell Characterization using CD34

Figure 5

Microscopic Images of HSC Cells After Planting Viruses After 48 hours (a) Control HSC, (b) HSC with 50µl virus, (c) HSC with 100µl virus, (d) HSC with 150µl virus, (e) HSC with 200µl virus

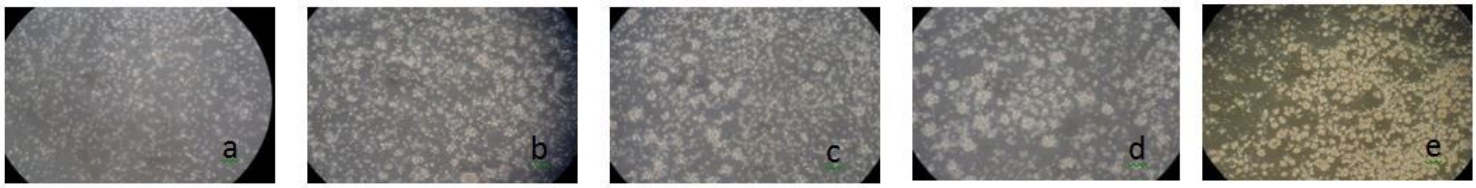

Figure 6

Microscopic Images of HSC Cells After Planting Viruses After 72 hours (a) Control HSC, (b) HSC with 50µl viruses, (c) HSC with 100µl viruses, (d) HSC with 150µl viruses, (e) HSC with 200µl viruses