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Gene Expression of SOX2, OCT4, and Nanog by Small Molecule Compound VC6TFZ on Peripheral Blood Mononuclear Cell

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ABSTRACT

Peripheral blood mononuclear cells are a potential source of cells to be induced into pluripotent stem cells because the collection procedure is accessible, minimally invasive, and can be stored in a frozen form. Small molecule compound VC6TFZ consisting of Valproic Acid (VPA), CHIR990210 (CHIR), 616452, Tranylcypromine, Farsokline, 3-deazaneplanocin (DZnep) and TTNPB has been shown to induce pluripotency in mouse fibroblasts; however, this has not been proven in peripheral blood cells. Therefore, this chemical reprogramming strategy can be used in producing the desired functional cell types for clinical applications. This study aimed to determine whether the small molecule compound VC6TFZ can induce pluripotency of peripheral blood mononuclear cells to become induced pluripotent stem cells. Mononuclear cells were isolated from peripheral venous blood by density gradient centrifugation method. The cells were grouped into 4 groups. Group 1 was the control group, which was not exposed to the small molecule. Groups 2-4 were experimental groups exposed to different doses of the small molecule VC6TFZ. Identification of induced pluripotent stem cells was carried out by identifying colony morphology and pluripotent gene expression of Octamer-binding transcription factor-4 (OCT4), Sex-determining region Y-box 2 (SOX2), and Nanog using Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Colonies with a round shape, large, cobble stone like, and clear boundaries resembling pluripotent stem cell colonies were observed on the 9th day of the induction process. In addition, OCT4 and Nanog gene expression were significantly increased in the treatment group compared to the control.

Keywords: Induced pluripotent stem cells, peripheral blood mononuclear cells, small molecule, VC6TFZ, gene expression of OCT4, SOX2, Nanog

INTRODUCTION

The development of induced Pluripotent Stem Cells (iPSCs) in 2006 by Shinya Yamanaka was a remarkable breakthrough made possible by many research findings by past and current scientists in related fields. Much progress has been made ever since to improve the efficiency of reprogramming and reduce the risk associated with the technology. Novel strategies already employed to improve reprogramming include inhibiting barriers to reprogramming, using non-integrative delivery methods, overexpression of enhancing genes, and specific small molecules that enhance reprogramming.[1]

Human-induced Pluripotent Stem Cells (hiPSCs) have been an alternative source of pluripotent stem cells, which tackled the ethical issue because it originates from somatic cells. Human-induced pluripotent stem cells are potential as a source of cells for cell transplantation therapy, disease modeling, and drug screening.[2] The source of somatic cell type to be reprogrammed may include skin fibroblasts, Peripheral Blood Mononuclear Cells (PBMCs), and epithelial cells. Most cells containing a nucleus can be reprogrammed to hiPSCs, including PBMCs such as lymphocytes or monocytes. This method is partly because blood draws are safe, accessible, and fast outpatient procedures that allow for the bulk isolation of PBMCs that could be used for reprogramming. Contrastingly, skin biopsies need to procure dermal fibroblasts, an approach that can be more painful, less sterile, and leaves scars.[3]

Insertional mutagenesis due to the integration of viral vectors into critical sites of the host genome leading to malignant transformation has been observed in preclinical and clinical gene therapy trials. Because of these limitations and safety concerns, alternative methods of iPS cell generation have been sought. They have focused on eliminating the integration of retroviral and lentiviral vectors from the reprogramming procedure.[4] Various approaches were made to improve the efficiency and safety of iPSCs for clinical applications, one of the

strategies was the use of small molecules.[5]

Although human iPSCs using chemicals have not been developed yet, human stem cells studied with small molecules reveal further details about epigenetic remodeling.[6] The combination of several small molecules could induce pluripotency despite no use of exogenous transcription factors.[7] Small molecules are cell-permeable, non-immunogenic, more cost-efficient, easy to manufacture, store, and standardized. Another advantage is that it does not involve genetic manipulation; therefore, it is safer, provides an immediate biological effect, and is reversible. The result can be controlled by modifying dosage and combinations.[8]

VC6TFZ is a combination of small molecules consisting of Valproic Acid (VPA), CHIR990210 (CHIR), 616452 (Repsox), Tranylcypromine, Farsoklin, 3-deazaneplanocin (DZnep), and TTNPB. The combination of these small molecules could induce pluripotency in Mouse Embryonal Fibroblasts (MEFs) with higher efficiency (0.02%) than the Yamanaka protocol that used exogenous transcription factors (0.001%-0.01%). CHIR, Farsoklin, and 616452 (Repsox) components induce the expression of Sall4 and SOX2 pluripotent genes in the early phase of the cellular reprogramming process. Concomitant overexpression between Sall4 and SOX2 can activate OCT4 promoter-driven luciferase reporter, making it sufficient for OCT4 induction. Expression of OCT4 increases significantly with the addition of DZnep. OCT4 and SOX2 then activate other pluripotent genes such as Nanog. SOX2, OCT4, and Nanog activate each other to form a pluripotency circuit that maintains the pluripotent nature of a cell.[9] This study was the first study aimed to prove whether the small molecule compound VC6TFZ could induce pluripotency in human peripheral blood mononuclear cells.

METHODS

This in-vitro study was conducted from June to July 2019 using a post-test control group only design. This study protocol consisted of several steps, including PBMCs isolation, PBMCs culture, pluripotency induction using small molecules, and iPSCs identification. Blood samples were drawn from a healthy female volunteer aged 31 years old in the Tropical Diseases Center, Universitas Airlangga.

Peripheral blood mononuclear cells isolation: Blood was collected into heparin-coated tubes. Collected blood was diluted with 5 mL Phosphate Buffered Saline (PBS) and centrifuged through a Ficoll gradient for 30 minutes at 1600 xg. PBMCs were collected and transferred to a new tube, and 10 mL PBS was added. Centrifugation was done for 5 minutes at 2000 xg. The supernatant was aspirated and resuspended at 10 mL PBS.

Peripheral blood mononuclear culture: PBMCs were cultured at Roswell Park Memorial Institute (RPMI) medium enriched with L-Glutamin 1mL/100mL, a supplement containing insulin, holo-transferrin (iron-saturated), and selenium in phosphate-buffered saline (ITS) 1 mL/100mL, Fibroblast Growth Factor (FGF) 5 ng/uL, ascorbic acid 5 mg/100mL, Granulocyte-Macrophage Colony-Stimulating Factor (GMCSF) 50 uL/100 mL, and dexamethasone 100 uL/100mL. Mononuclear cells were cultured for six days, and the medium was changed every three days. After 6 days of culture at RPMI medium, PBMNCs were cultured using the hanging drop method for two days. Hanging drop culture was carried out by dripping the cell suspension using the pipet tip on the inside of a 5 cm Petri dish lid. Cells were incubated at 37°C CO^2 for 48 hours without the addition of a medium.

Pluripotency induction using small-molecule compound VC6TFZ: 5000 mononuclear cells in suspension were transferred to an M-6 well plate precoated with vitronectin and feeder cells overnight. Feeder cells were made from mitosis-inactivated rabbit adipose mesenchymal cells using mitomycin-C 2 μg/mL for 30 minutes. ReproTesR medium containing small molecule VC6TF was added. On day 7, DZnep was added. Cells were exposed to small molecules for 14 days. The medium was changed every four days. For example, on day 14, VC6TFZ was changed to 2i medium (PD0325901 and CHIR) for seven days.

The cells were grouped into four groups. Group 1 (P1) was the control group that was not exposed to a small molecule. Group 2 (P2) was the experimental group exposed to small molecule dosage valproic acid (VPA) 0.5 mM, CHIR990210 (CHIR) 5 uM, 616452 (Repsox) 5 uM, Tranylcypromine, 2.5 uM, Farsoklin (FSK) 20 uM, 3-deazaneplanocin (DZnep) 20 nM, and TTNPB 5 uM. Group 3 (P3) was the experimental group exposed to small molecule dosage valproic acid (VPA) 0.75 mM, CHIR990210 (CHIR) 10 uM, 616452 (Repsox) 7.5 uM, Tranylcypromine 5 uM, Farsoklin (FSK) 40 uM, 3-deazaneplanocin (DZnep) 50 nM, and TTNPB 5 uM. Group 4 (P4) was the experimental group exposed to small molecule valproic acid (VPA) 1 mM, CHIR990210 (CHIR) 20 uM, 616452 (Repsox) 10 uM, Tranylcypromine, 10 uM, Farsoklin (FSK) 60 uM, 3-deazaneplanocin (DZnep) 100 nM, and TTNPB 5 uM.

Induced pluripotent stem cells identification: Identification of iPSCs was carried out by morphology identification and determination of

gene expression of SOX2, OCT4, and Nanog using RT-PCR. Induced pluripotent stem cell colonies had significant characteristics, tight and precise borders, and a cobblestone-like appearance. In addition, in iPSCs colonies, cells had a small size with a large percentage of cytoplasmic nuclei.

RT-PCR: Total RNA from an entire well of the colony was isolated using the RNA simple Total RNA Kit (TIANGEN). RNA was converted to cDNA using iScript cDNA Synthesis (Bio-Rad). PCR was carried out using Evagreen supermix (Bio-Rad) and performed on a CFX96 Realtime PCR System (BioRad) Sequence Detection System. The primers used for real-time PCR are listed in Table 1, with GAPDH as a housekeeping gene.

Statistical analysis: Collected data were coded, tabulated, and analyzed using SPSS version 24. Gene expressions of OCT4, SOX2, and Nanog were presented as mean+SD. Data normality test was done using Kolmogrov-Smirnov test. Differences in OCT4, SOX2, and Nanog gene expressions between the four groups will be analyzed by oneway ANOVA test if the data were normally distributed and Kruskal-Wallis if the data were not normally distributed. The difference with p <0.05 was significant (Figure 1).

This study was approved by the Ethics Committee for Health Studies Dr. Soetomo General Academic Hospital, Surabaya with number 1214/KEPK/V/2019.

RESULTS AND DISCUSSIONS

Induced pluripotent stem cells (like colonies) were observed on day nine after exposing PBMCs to the small-molecule compound VC6TFZ. Colonies had large and round morphology with a cobblestone-like appearance (Figure 2).

Table 1. The primers used for real-time PCR

Gen	Forward Primer	Reverse Primer
SOX2	5'-GCCGAGTGGAAACTTTTGTCG-3'	5'-GCAGCGTGTACTTATCCTTCTT-3'
OCT4	5'-TCGCAAGCCCTCATTTCACC-3'	5'-GCCAGGTCCGAGGATCAAC-3'
NANOG	5'-TTTGTGGGCCTGAAGAAAACT-3'	5'-AGGGCTGTCCTGAATAAGCAG-3'
GAPDH	5'-ATCACCATCTTCCAGGAGCGA-3'	5'-TTCTCCATGGTGGTGAAGACG-3'

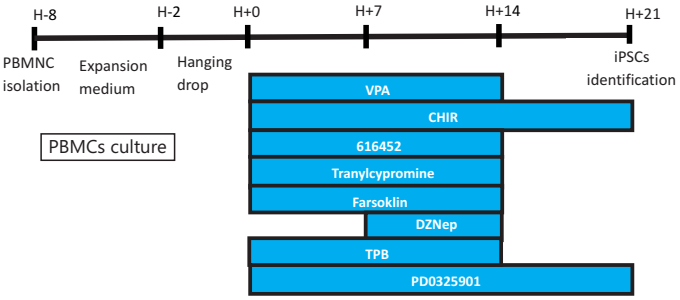

Figure 1. Study protocol

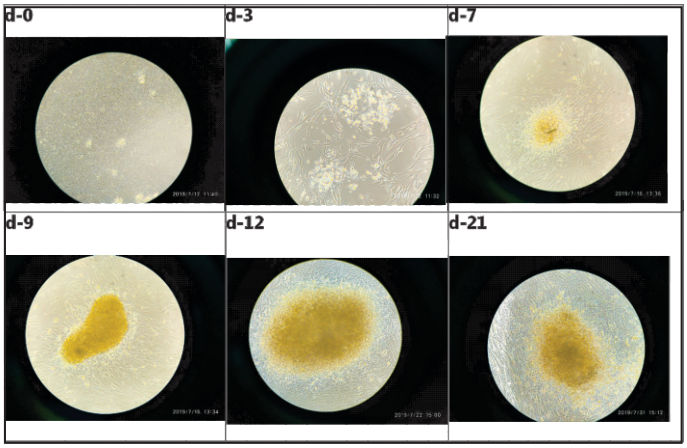

Figure 2. Changes in colonies morphology from day 0 to day 21 (the colonies were not visible on days 0 and 3, colony appeared on day 7 with morphology cobblestone-like appearance. On days 9, 12, and 21 the colonies seemed bigger)

OCT4 gene expression was found in all treatment groups, with the most robust expression in the P3 group. Significant differences were found between the P3 and P4 groups with the control group (p=0.024, p=0.030), while there was no significant difference between the P2 group and the control (p=0.054) (Table 2).

Table 2. Comparison of OCT4 expression among research groups

Control Group	VC6TFZ Experimental Group Mean±SD OCT4	p-value
34.41±0.1	P2: 39.13±2.45	0.054
	P3: 40.57±2.2	0.024
	P4: 40.2±1.14	0.030

SOX2 gene increased in all treatment groups compared to the control group, with the strongest expression found in the P3 group. However, there was no significant difference between the control group with P2, P3, and P4 (p=0.234, p=0.415, p=0.185) (Table 3).

Table 3. Comparison of SOX2 expression among research groups

Control Group	VC6TFZ Experimental Group Mean±SD SOX2	p-value
36.61±2.06	P2: 40.9±1.76	0.234
	P3: 39.39±5.45	0.415
	P4: 41.5±0.55	0.185

Nanog gene expression was elevated in all treatment groups, with the strongest expression in the P3 group. Significant differences were found between the P2, P3, and P4 groups with the control group (p=0.025, p=0.009, and 0.011) (Table 4).

Table 4. Comparison of Nanog expression among research groups

Control Group	VC6TFZ Experimental Group Mean±SD Nanog	p-value
32.72±0.71	P2: 34.6±0.76	0.025
	P3: 35.26±0.23	0.009
	P4: 35.1+0.14	0.11

It was found in this study that the small-molecule compound VC6TFZ could induce pluripotency in human PBMCs. It was proven by forming colonies

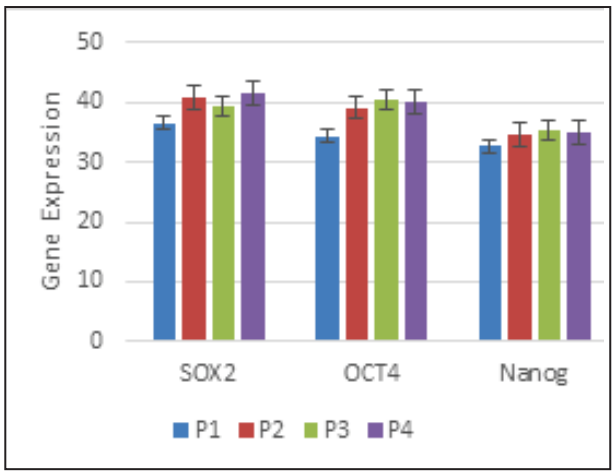

Figure 3. Absolute expression of pluripotency-related genes in the four groups

that resembled iPSCs morphology and the increased expression of OCT4, SOX2, and Nanog genes (Figure 3). It was also found that the colony began to appear on the 7th day of induction and increasingly enlarged on day 9. The colonies had round morphology, cobblestone-like appearance, and clear edges. This colony morphology resembles iPSCs.[1]

The colonies in this study express pluripotency with the increased gene expression of OCT4, SOX2, and Nanog. The expressions of each gene were analyzed using RT-PCR, and higher expressions were found in all treatment groups compared to the control group. There were significant differences in OCT4 expression between the control group and the P3 and P4 groups (p=0.024, p=0.030). There were significant differences in Nanog expression between the control and all treatment groups (p=0.025, p=0.009, and 0.011). However, there were no significant differences in SOX2 expression between the control group and all treatment groups (p=0.234, p=0.415, p=0.185). The expression levels of pluripotency-related genes, Sall4 and Sox2, were most significantly induced in the early phase in response to VC6TF.[9] No significant differences in Sox2 expression between the control group and all treatment groups in this study might be due to the determination of gene expression only at the end of the study, not monitored daily, and Sox2 might be increased in the early phase of this research. In these cells, the expression levels of most pluripotency marker genes were elevated, but there was still no significant increase in expression, suggesting an incomplete reprogramming state.[9]

Reprogramming using small molecules is easy to control in a concentration and time-dependent manner.[10] In a study by Hou, colonies began to

appear on day 20, while this study showed that the colonies appeared earlier on day 7. This result was likely due to the optimized culture method and the hanging drop culture method. The induction was carried out in only 21 days, faster than the protocol by Hou *et al*., which required 36 days because the colonies in this study appeared earlier. Optimization of PBMC culture plays an essential role in the reprogramming process. Peripheral blood mononuclear cells in this study were cultured for 6 days in an expansion medium. During the culture period, the longer duration led to more dead cells. However, the number of living cells remained constant, so the optimal course of culture time was needed. This 6-day duration was based on research conducted by Gu *et al*., which found that the 6 day culture time showed the number of colonies that most expressed TRA 1-60 and AP compared to days 4, 8, or 10.[11] The hanging drop technique is a well-established method used in microbiology, which allows the drops of medium to be maintained with minimal evaporation and without spreading. It was initially developed and used for neural tissue culture in the early 20th century.[12] Hanging drop culture method allows the formation of colonies through cell aggregation induced by gravity.[13] This method requires no specialized equipment or reagents and is highly cost-effective. In addition, the simplicity of the method minimizes potential pitfalls.[14] Another critical advantage of the hanging drop culture is that all cell types are maintained in the same microenvironment, in which cell-cell interactions are intact. Its simplicity in simulating the effects of sophisticated 3-D cellular culture is particularly appealing.[12]

In this research, the small molecule compound VC6TFZ was a combination of VPA, CHIR, 616452, Tranylcypromine, FSK, and ZDNet. Hou and his colleagues had succeeded in generating iPSCs derived from MEF using VC6TFZ without any exogenous transcription factors. SOX2, OCT4, and Nanog activate each other to form a pluripotency circuit that maintains the pluripotent nature of a cell.[15]

One of the challenges in reprogramming PBMCs was the non-adherent nature of these cells.[16] To overcome this problem, this study coated the good walls using vitronectin and feeder cells to enable the PBMCs to attach to the well. This feeder cell produces stemness-supporting factors that prevent spontaneous differentiation. This feeder cell has adhesion molecules and an extracellular matrix that increases attachment of iPSCs, which supports growth and survival.[17,18]

RT-PCR is a powerful technique that can be used as a quantitative method of gene expression analysis to investigate iPS reprogramming.[19] This technique requires a normalization strategy to ensure the reliability of the data.[20,21] One common approach is to rely on comparing the target gene with an endogenous control (reference gene) in the same sample. At present, so-called genes are universally used as a reference.[22] It requires validation of reference genes for the accurate assessment of target genes' expression, and one of the most housekeeping stable genes, GADPH, was used in this research.[19] The limitations of this study were that determining expressions of related genes using RT-PCR was required to be monitored daily, not only at the end of the study.

CONCLUSIONS AND SUGGESTIONS

In conclusion, this was the first study that showed small molecule compound VC6TFZ could induce pluripotency in human PBMCs. Further research for determining expressions of related genes using RT-PCR was required at the end of the study and at intervals of several days to determine the exact time and dose for administering small molecules. In addition, further studies were needed to analyze the molecular profile, differentiation, and self-renewal ability of these cells. The following steps toward clinical application will involve confirming the possibility of inducing tumorigenicity and determining the proper concentration, combination of small molecules, and treatment times in-vivo.

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