

Nanoparticles–SIRT1 Prevents Endothelial Progenitor Cells Senescence by Modulating Nitric Oxide Expression and Reducing Poly ADP-ribose Polymerase: *In vitro* Study

Titin Andri Wihastuti¹, Indah Nur Chomsy², Wiwit Nurwidyaningtyas²,
Fibe Yulinda Cesa³, Hidayat Sujuti⁴, Meddy Setiawan⁵ and Kumboyono Kumboyono^{6,*}

¹Basic Nursing Department, Faculty of Health Sciences, University of Brawijaya, Malang 65145, Indonesia

²Doctoral Program in Medical Science, Faculty of Medicine, University of Brawijaya, Malang 65145, Indonesia

³Master Program in Biomedical Science, Faculty of Medicine, Brawijaya University, Malang 65145, Indonesia

⁴Department of Biomolecular-Ophthalmology, Faculty of Medicine, University of Brawijaya, Malang 65145, Indonesia

⁵Faculty of Medicine, University of Muhammadiyah Malang, Malang 65144, Indonesia

⁶Department of Nursing, Faculty of Health Sciences, University of Brawijaya, Malang 65145, Indonesia

(*Corresponding author's e-mail: abu_hilmi.fk@ub.ac.id)

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Abstract

One of cardiovascular diseases that accounts for a high global death rate is atherosclerosis. Apart from the known risk factors, the cause of this disease is uncertain. This disease is triggered by the rupture of atheroma. This condition is associated with oxidative stress, which can trigger endothelial progenitor cells (EPC) dysfunction. One of the causes of oxidative stress is hydrogen peroxide, which triggers deoxyribose nucleotide acid (DNA) damage, which will later trigger ageing in EPC. Sirtuin-1 (SIRT1) is known to be involved in EPC repair, but currently, no studies have attempted to increase SIRT1 expression *in vitro*. This study aimed to determine the effect of nanoparticles-SIRT1 (NPS1) on the expression of nitric-oxide (NO) and poly (ADP-ribose)-polymerase (PARP) in EPC cell cultures induced by hydrogen peroxide (H₂O₂). Tests were carried out *in vitro* using EPC cell cultures to determine the potential of SIRT1 (NPS1) nanoparticles as an ageing inhibitor. The research method used was pure quantitative experimental with a post-test-only control group. The expression of NO and PARP was studied using the flow cytometry method. The data obtained were analysed using parametric one-way ANOVA and post-hoc Tukey-HSD tests with a 95 % confidence interval ($p \leq 0.05$). The results showed that administration of SIRT1 nanoparticles was able to increase NO expression in EPC culture cells that induced H₂O₂ by 14.61 % and could also increase NO levels in normal EPC cells that were only given NPS1 by 38.51 % and was able to reduce PARP expression when compared with the normal group. The NPS1 given was 9.03 % for the NPS1 group and 19.78 % for the NPS1+ H₂O₂ group. This study concludes that NPS1 added to EPC cells further helps increase cell repair to prevent senescence, through increasing NO and also increasing the performance of PARP.

Keywords: Endothelial progenitor cells, Nitric oxide, Poly-(ADP-Ribose)-polymerase, Sirtuin-1, Cardiovascular disease

Introduction

Cardiovascular disease contributes to the global death rate of 17.9 million out of 39.5 million [1]. However, until now, the exact cause of this disease is unknown, but the presence of risk factors is known to trigger the emergence of this disease often. The critical factor that causes this disease to appear is when LDL (low-density lipoprotein) cholesterol is oxidized (ox-LDL), which is then phagocytosed by macrophages to form foam cells [2,3]. This condition then becomes one of the causes of atherosclerosis. Also, atherosclerosis is a chronic inflammatory vascular disease associated with oxidative stress and endothelial dysfunction [4,5]. Functionally, the quality of endothelial cells can be determined from the levels of NO produced. The lower the levels of NO expressed, the stronger the possibility that the endothelial cells will be damaged. This endothelial damage will also be responded to by poly (ADP-ribose)

polymerase (PARP) to immediately repair the endothelium [6]. This oxidative stress state will cause DNA damage to endothelial cells [7].

Senescence or ageing in cells is an irreversible process of stopping cell division. This process is needed to maintain tissue homeostasis, heal wounds, or prevent changes in damaged cells [8]. Oxidative stress can trigger endothelial progenitor cells (EPC) dysfunction. Previous research states that one of the causes of oxidative stress is hydrogen peroxide (H_2O_2) [6,9,10]. This H_2O_2 will trigger genes that trigger deoxyribose nucleotide acid (DNA) damage and the mechanism for shortening telomeres, which accelerates cell senescence. Under these conditions, there is an imbalance between oxidative stress and NO, which further triggers the activation of senescence proteins, for example, p53, p21, and p16 [11,12]. In this condition, an enzyme detects DNA damage and activates a series of repair and maintenance mechanisms for DNA integrity. This enzyme is called Poly (ADP-ribose) polymerase (PARP). Under physiological conditions, PARP functions to maintain cell balance and function. PARP works by adding long-chain polymers to proteins, also known as ADP-ribosylation. Under physiological conditions, PARP is cytoprotective, which is beneficial for cells, but in pathological conditions, increased oxidative stress and excessive stimulation will cause the enzyme to reduce the rate of glycolysis and mitochondrial respiration, which causes cellular dysfunction [13,14].

In conditions of oxidative stress imbalance, cells respond with several proteins with antioxidant properties, one of which is Sirtuin-1 (SIRT1). SIRT1 is a member of the Sirtuin enzyme family, which acts as an NAD^+ -zavisim deacetylase. This protein plays a role in regulating the deacetylation process in proteins, which can influence gene expression and various biochemical pathways in cells, primarily to protect cells from oxidative stress [15]. Both PARP and SIRT1 proteins work to prevent cell damage. There is a connection that SIRT1 and PARP may compete for using NAD^+ molecules, which are essential cofactors for both [16]. Under conditions of stress or significant DNA damage, high PARP activity can lead to excessive use of NAD^+ , which can reduce the availability of NAD^+ for SIRT1 activity and vice versa [16]. Therefore, SIRT1 is expected to regulate PARP activity and suppress oxidative stress to slow ageing.

SIRT1 plays an essential role in regulating cell ageing. This ability is because SIRT1 is involved in DNA repair, and this activity can help maintain the integrity of the cell genome. When DNA damage occurs, SIRT1 can help repair this damage, preventing the accumulation of DNA damage that can lead to cell senescence. SIRT1 can suppress the expression of genes involved in cell senescence due to SIRT1's ability to remove acetyl groups on histones, change chromatin structure, and then influence protein transcription [17,18]. This mechanism effectively reduces the expression of genes that promote cell senescence.

SIRT1 also has a role in DNA repair. SIRT1 activity is related to DNA repair, its ability to interact with DNA repair proteins, such as p53, DNA-PK, which is involved in double DNA repair, and Ku70, to help facilitate the double DNA repair process [19,20]. Another SIRT1-mediated mechanism in DNA and cell repair inhibits the p53-p21 pathway involved in cell death after DNA damage, allowing cells to repair DNA [11,12] more effectively. These circumstances make SIRT1 expected to become a new therapeutic agent as anti-senescence at the cellular level. Other benefits of SIRT1 in protecting cells include stimulating increased NO production, anti-inflammatory activity, and reducing oxidative stress [21].

So far, efforts to increase SIRT1 in the body have been carried out by administering resveratrol [22]. However, until now, no research has shown that the SIRT1 protein can be packaged and applied independently as a therapeutic agent for cardiovascular disease. Meanwhile, nanoparticles that function as drug carriers have great potential as carrier agents for active ingredients or drugs [23]. In biomedical applications, the small size of nanoparticles (ranging between 100 nm) and their physicochemical structure make this drug delivery system advantageous [24]. Therefore, nanoparticles are considered a good alternative for adding exogenous SIRT1 protein. Our primary concern is to learn more about the effect of SIRT1 nanoparticles on NO and PARP expression in their role as senescence blockers in EPCs by maintaining SIRT1 levels to keep EPC cells alive and functioning optimally.

Materials and methods

Endothelial progenitor cell culture

A 5 mL venous blood sample of a healthy woman who was menstruating was taken at the Central Laboratory of Biomedical Sciences, Faculty of Medicine, Brawijaya University. We used menstruating women as the subjects of this research because, during menstruation, there is an increase in EPC as part of the regeneration process of endometrial wall epithelial cells, which are shed during menstruation [25]. The venous blood sample was then added with 5 mL Lymphoprep (1:1), centrifuged at 1600 rpm for 30 min and pipetted using a micropipette slowly to collect part of the buffy coat layer. After that, the buffy coat was transferred to a 15 mL propylene tube, and 5 mL of PBS was added and centrifuged at 1200 rpm for 10

min. EPC culture was carried out for 7 days on well-plate 24. From the buffy coat obtained, cells were counted using a hemocytometer. First, the PBMC isolated pellet was resuspended in EBM-2 complete medium to 2.4 mL. Then, cell viability was calculated using the staining method of 20 μ L of 0.05 % trypan blue solution, then incubated for 2 - 3 min. The solution is then injected into the hemocytometer, and the cells in the box are counted (live cells = clear, dead cells = blue). The cells were then planted in well-plate 24, and the medium was changed routinely every 2 days and immediately incubated in a CO₂ incubator. EPC culture observations were documented using an inverted microscope. The culture medium used in this study was Lonza™ EBM-2 Endothelial Cell Growth Basal Medium-2 (Basel, Switzerland), added with 20 % Fetal Bovine Serum (FBS), 1 % Penicillin-Streptomycin and 1 % L-glutamine. The culture medium was transferred to a sterile tube using Minisart® Syringe Filters 0.22 μ m.

Nanoparticles SIRT1 (NPS1) manufacture

The manufacture of nanoparticles is based on the nanoprecipitation method, a solvent displacement method with the principle of diffusion of organic solvent into the aqueous phase in the presence or absence of surfactants. This nanoprecipitation method refers Luque-Alcaraz *et al.* [26] with several modifications. The preparation carried out is by preparing organic solvents, acetone and methanol, in a ratio of 3:2. After that, the organic phase (FO) was made, which consisted of chitosan and phosphatidylcholine which was mixed with 5 mL of organic solvent until dissolved. Dissolution can be assisted by using a sonicator or stirring on a hotplate stirrer for 1 - 2 h. After everything was dissolved, 7.5 μ L of SIRT1 recombinant protein was added and mixed with FO. After mixing, an aqueous phase (FA) is prepared, which consists of 1 % Tween 80 (1 g of Tween 80 dissolved in 100 mL water for injection). Comparison FA: FO = 20:1. If the FO is dissolved in 5 mL, then prepare the FA 20 times (100 mL, after that, add the FO to the FA slowly while stirring). The stirring was continued with an ultraturax at 8000 rpm for 5 min for 18 h and maintained not to overheat to prevent the protein from degrading. After 18 h of stirring, the nanoparticles are ready to be harvested. The characteristics of NPS1 produced in this study are pH 5.418 ± 0.55 , with a deep yellow color; smells like egg yolk (phosphatidylcholine-like), which is obtained through organoleptic tests.

EPC treatment with hydrogen peroxide (H₂O₂) and NPS1

EPC cells were induced with 200 μ L of H₂O₂ for 24 h in the H₂O₂-induced group. After 24 h of incubation in an O₂ incubator, the cells were harvested using 200 μ L of Trypsin-EDTA per well and waited 5 min. After that, 200 μ L of culture medium was added per well and centrifuged for 5 min at 2,500 rpm (29 °C). These cells were tested for parameters using flow cytometry. The groups in this study were divided into 4 groups, including the control group, the group given H₂O₂ (H₂O₂), the normal group given SIRT1 (NPS1), and the group induced by H₂O₂ and given NPS1 (NPS1+ H₂O₂).

Measurement of NO and PARP by flowcytometry

The cells that have been harvested are added to intracellular staining at 500 μ L per well. After that, it was incubated for 5 min and vortexed, then centrifuged for 5 min at 2500 rpm, and the supernatant was discarded. Then, the primary antibody was added to the pellet and incubated for 20 min. After that, secondary antibodies were added and incubated for 20 min. For reading, 400 μ L PBS was added to each sample. Tools and materials used in this process include a Becton Dickinson™ FACS-Calibur flowcytometer (San Jose, CA, USA) and data analyzed with Paint-a-Gate software, Mikro 22R Refrigerated Centrifuge, PE mouse anti-Cleaved PARP (Asp214) from BD Pharmingen™ (Franklin Lakes, New Jersey, USA) and Nitric Oxide Assay Kit (Flowcytometry).

Statistical analysis

In this study, data analysis used the parametric one-way ANOVA test. Data must first meet the normality test and homogeneity test. If the data is normally distributed and homogeneous, then testing continues using one-way ANOVA. The data presentation then uses mean and standard deviation as a pair of measures of centering and dispersion. If the ANOVA test shows a *p*-value ≤ 0.05 , a further test is carried out, the Tukey-HSD post-hoc test.

Ethical Clearance

This research has received approval from the Universitas Brawijaya Research Ethics Commission No. 1206-KEP-UB.

Results and discussion

Following are the results of microscopic observations with 40× and 100× magnification. At this magnification, it is shown that the particle shape is round (red arrow) at each magnification observed.

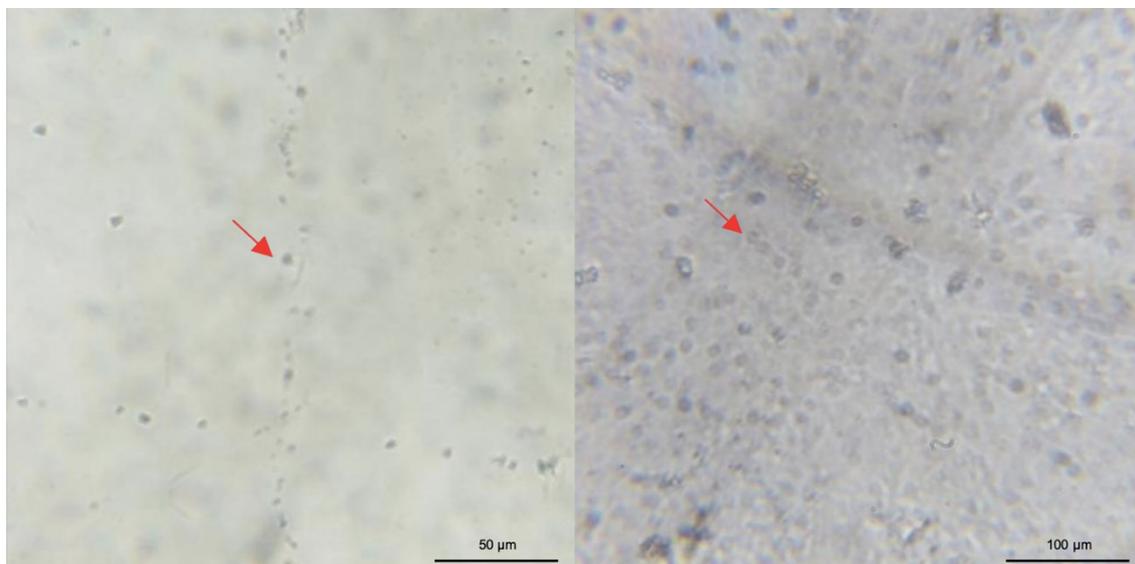


Figure 1 Results of microscopic observations of SIRT1-nanoparticles. (A) 40× magnification; (B) 100× magnification.

Observations of culture cells were carried out when changing the medium, namely every 2 days. On the first day, PBMC cells have not yet differentiated and are shown in a round shape. On the third day, there began to be changes in the cells, which began to differentiate, taking the shape of balls more significantly than the previous day. On the 7th day, there are spindle-like cells, characteristic of early EPC cells.

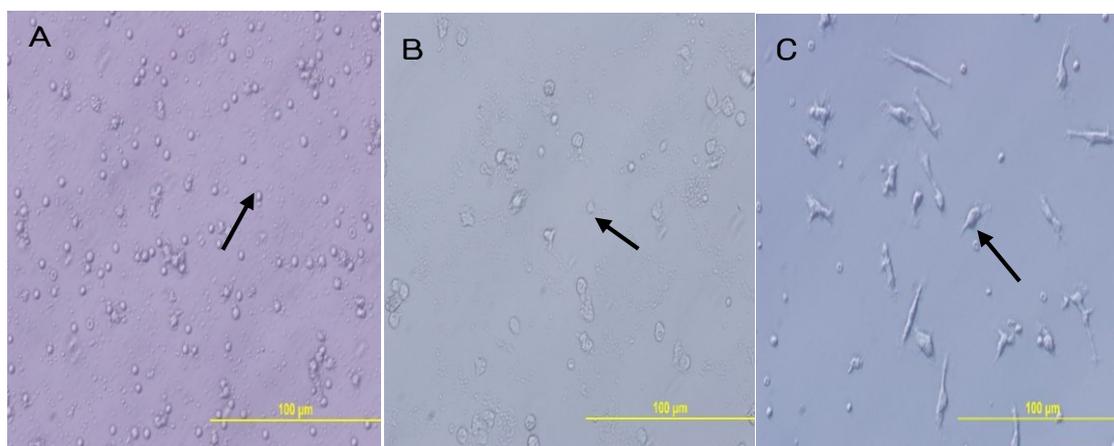


Figure 2 Culture observation results: (A) Day 1 cells are round (pointed by arrow); (B) On day 3, the cells have started to differentiate into EPC cells (pointed by arrow); (C) On day 7, the EPC cells have differentiated into spindle-shaped early EPCs (spindle-like; pointed by arrow).

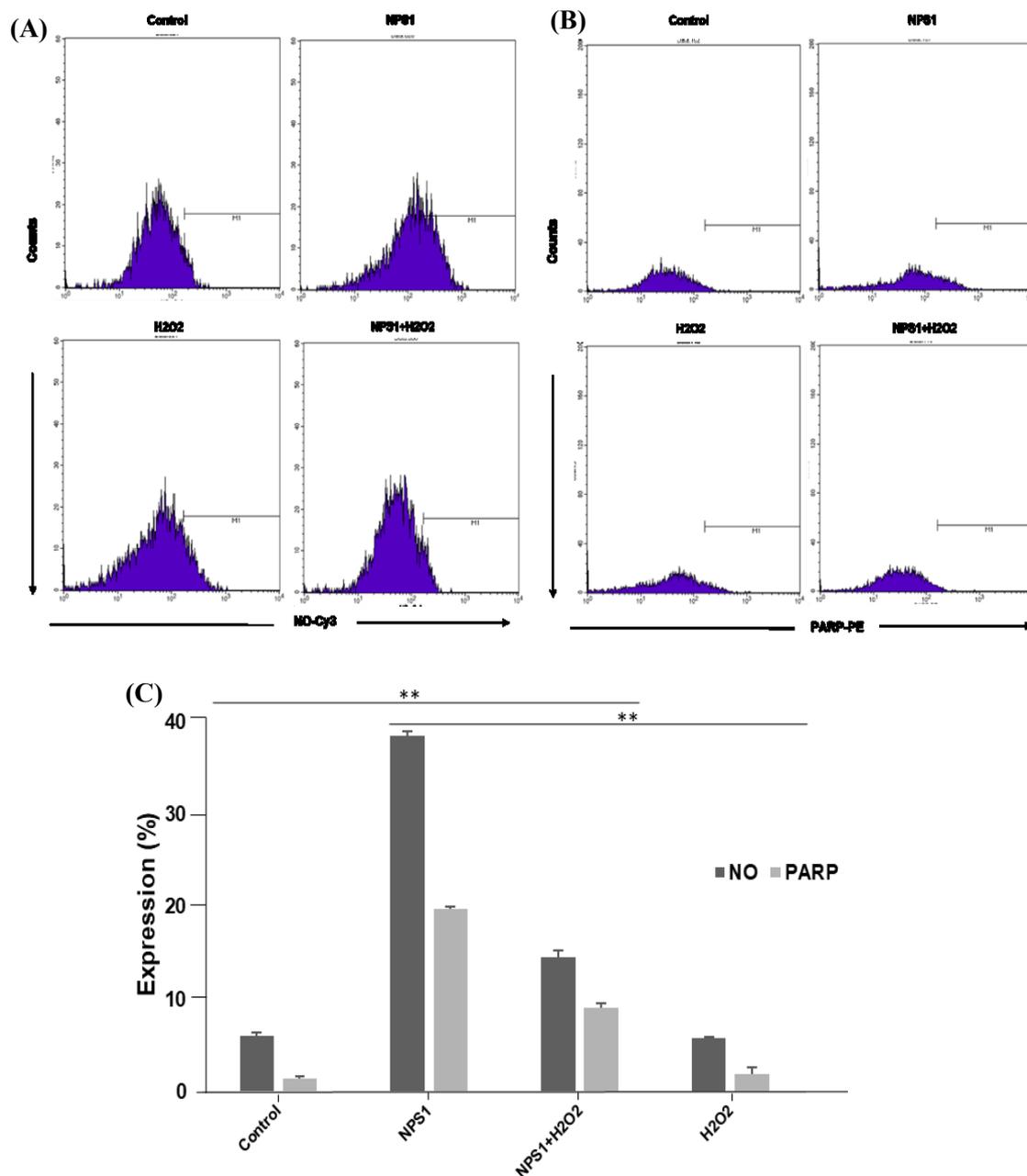


Figure 3 (A) Results of NO flow cytometry data; (B) PARP flow cytometry data results; (C) Bar graph shows % expression of NO and PARP in each treatment group (N = 5); Control group (NO = 6.02 ± 0.48 %; PARP = 1.39 ± 0.24 %); NPS1 (NO = 38.51 ± 0.40 %; PARP = 19.78 ± 0.81 %); NPS1+ H₂O₂ (NO = 14.61 ± 0.73 %; PARP = 9.03 ± 0.53 %); and H₂O₂ (NO = 5.75 ± 0.17 %, PARP = 1.87 ± 0.28 %). The ANOVA test showed that the treatment group had a significant difference from one of the control groups in the NO and PARP groups ($p < 0.001^{**}$). For the post hoc-Tukey HSD test, groups NPS1, NPS1+ H₂O₂, and H₂O₂ on NO and PARP had differences ($p < 0.001^{**}$); There were differences between the NPS1, NPS1+ H₂O₂, and control groups in NO and PARP ($p < 0.001^{**}$); there were no significant differences between control and H₂O₂ for NO ($p = 0.83$) and PARP ($p = 0.51$).

The research results in **Figure 3(C)** show that administration of SIRT1 nanoparticles increased NO expression in the normal EPC group given NPS1 and the EPC group induced by H₂O₂ and given NPS1. These results illustrate that the increase in NO is maximum for physiological conditions, namely 38.51 %, but can still increase NO levels in oxidative stress conditions by 14.61 %. This increase is thought to be related to the ability of NPS1 to increase SIRT1 levels in cells so that they can work optimally under

physiological conditions. The administration of NPS1 in this study showed that when compared with the control group and the EPC group induced with H₂O₂, the administration of NPS1 still significantly affected EPC cells.

The research results in **Figure 3(C)** show that administering SIRT1 nanoparticles reduced PARP expression compared to the 2 treatment groups, namely the NPS1 group and the NPS1+ H₂O₂ group. The results from PARP were quite significantly different compared to the NO group, whose NPS1 performance was directly proportional to treatment. This stat is fascinating because it turns out that PARP can also increase under normal conditions, which means that NPS1 is thought to be able to induce PARP. When compared between the treatment group and the control group, the percentage of PARP continues to increase, namely 1.39 % in the control group (normal EPC), 1.86 % in the positive control group, namely with hydrogen peroxide induction, 9.03 % when administering NPS1 to EPC cells that have the condition. Oxidative stress exposed to hydrogen peroxide also increased in healthy cell conditions given NPS1 by 19.78 %

NPS1 increases NO expression *in vitro*

Previous *in silico* studies have shown that based on surface markers using an immunophenotype approach, the SIRT1 protein does not always play a role in cell signalling [27]. For this reason, in the *in vitro* study in this research, SIRT1 packaging uses the drug carrier method with the nanoprecipitation method as a carrier for the SIRT1 protein to penetrate the EPC cell membrane. Nitric oxide (NO) is a signalling molecule vascular endothelial cells produce that maintains vascular tone. NO also participates in other physiological processes, such as cell survival, proliferation, and migration [28,29]. If NO levels are maintained in optimal conditions in cells, then it can be said that survival can also be better. In **Figure 3(C)**, the histogram shows that giving NPS1 nanoparticles to the induced group increased NO levels. This increase is thought to be due to increased EPCs when PBMC isolation is carried out in menstruating women. The increase in EPC quality will also be followed by increasing NO levels. This result is supported by previous research conducted by Souza *et al.* [30] which states that a positive correlation exists between the number of circulating EPCs and NO in healthy children.

Although the increase did not appear to be as prominent in the EPC group induced with hydrogen peroxide as in the normal group, when compared with the EPC group induced with H₂O₂, there were significant differences, respectively, between the groups, namely 5.75 and 14.61 %. Oxidative stress conditions will increase reactive oxygen species (ROS), which will cause telomere shortening and DNA damage. This results in the activation of Vascular Endothelial Growth Factor (VEFG) via the PI3K/Akt pathway, which will cause the formation of unpaired eNOS and a decrease in NO in the cell. Later, if this situation continues, it will disrupt cell proliferation and cause senescence of EPCs. With the increase in NO in the hydrogen peroxide-induced group, it can be said that NPS1 can increase SIRT1 levels in cells to inhibit the senescence of EPC and function optimally as a senescence blocker through increasing NO.

Previous research has proven that SIRT1 will decrease in conditions of oxidative stress. Meng, Qin and Liu research showed that SIRT1 can stimulate eNOS and inhibit NOX and mTOR to trigger an anti-oxidant protective response [31]. Endothelial nitric oxide synthase (eNOS) is the main enzyme-producing NO, which participates in mitochondrial biogenesis and has various anti-atherosclerotic functions [31,32]. The increase in NO in this study is thought to be due to an increase in eNOS stimulated by increased levels of SIRT1 added exogenously through the administration of NPS1. With this stimulation, even in conditions of oxidative stress, EPC cells can still maintain their stability by adding NPS1, which stimulates eNOS to increase NO expression in EPC cells and prevent premature senescence or premature cell ageing. This outcome aims to minimize cell ageing will improve the quality of EPCs to later differentiate into endothelial cells and increase their role in re-endothelialization of injured vessels. However, conditions of excessive NO will lead to detrimental things. NO functions as a regulator in blood vessels and one of them functions as vasodilation [33,34]. Blood vessels with NO levels that are too high will, of course, also be detrimental, potentially causing hypotension [34]. Further study of the supporting variables is needed to prove that the expression of NO, which in this study was 38.51 %, is at the optimal increase stage.

NPS1 decreased PARP expression when compared to the treatment groups

Poly-(ADP-ribose)-polymerase (PARP) is a genome-stabilizing enzyme that catalyzes the covalent transfer of mono- or poly-adenosine diphosphate (ADP) from NAD⁺ to glutamate or aspartate residues in target proteins [6]. This process will later produce protein-conjunct chains from poly ADP-ribose (PAR) polymers. PARP is responsible for DNA repair, and when DNA damage occurs, it will cause excessive consumption of NAD⁺. From the research results, **Figure 3(C)** shows that PARP increased when NPS1 was administered to both normal EPC cells and cells not induced with hydrogen peroxide. The increase in

PARP levels is a signal from the cells that the cells are experiencing damage. However, in the mechanism of action, SIRT1's performance is hampered because NAD⁺ cell levels increase. Therefore, this also shows that the administration of NPS1 does not hamper the DNA repair process carried out by PARP. This result also shows that under normal conditions, cells will still respond to NPS1 as a foreign object that causes DNA damage, thereby stimulating SIRT1 to immediately stimulate repair through increasing NO [6].

This increase in PARP administration also proves that the performance of SIRT1 and PARP does play a role in cell repair. PARP works directly to repair DNA, while SIRT1 is an active protein that stimulates cell repair by increasing several pathways [35,36]. One of them is increasing NO via the P13K/Akt pathway, as discussed in the previous chapter. The statement that the performance of SIRT and PARP is supported by other research conducted by Zha *et al.* [6] which attempted to inhibit the performance of PARP by using the PARP1 inhibitor PJ34, which aims to improve the function of NAD⁺ levels and increase SIRT1 activity.

With this increase, reduced PARP levels are expected to improve the performance of SIRT1. In this research, NPS1 has been added so that it is thought to no longer require NAD⁺ conditions. Therefore, this will not inhibit the performance of PARP to repair non-excessive DNA. With the results presented, PARP decreased compared to the inter-treatment group and increased compared to the control group. This outcome is an exciting discovery because, with the presence of NPS1, it will not interfere with the performance of PARP in repairing DNA but will also increase NO so that, in this case, a discovery is made that the performance of PARP and SIRT1 can still be improved as long as they do not influence each other in the presence of NAD⁺ competition.

Excessive PARP activity will certainly cause the opposite effect to that desired. So, PARP appears to be a two-sided coin. Thus, PARP has the potential to respond to DNA damage, but excessive activation can cause cell death [37]. Modifying PARP activity through inhibition or cleavage can cause apoptosis by preventing DNA repair carried out by PARP. However, recent reports suggest that PARP may lead to PARP1-dependent cell death, which is reported to be different from apoptosis, necrosis or autophagy [38, 39]. Increased apoptosis in conditions of increased PARP refers to the study, which states that it is possible that PARP, which is increased and has excessive expression, will cause toxicity in cells. This condition needs to be reviewed further to determine the level of cell apoptosis.

Conclusions

This research succeeded in modulating NO levels with a significant increase in NO. This study concludes that NPS1 added to EPC cells further helps increase cell repair to prevent senescence, through increasing NO and also increasing the performance of PARP as an enzyme that functions in the DNA repair response at levels that are not excessive and does not exceed SIRT1 levels in cells. Future research must further study the levels of peroxynitrite (ONOO⁻) and PAR as confirmation of the cause of the increase in NO and PARP levels in EPC cell cultures.

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