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Pregnant Human Myometrial 1-41 Cell Viability Test on Vitamin D Administration

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Abstract

Background: Preterm labor is one of the universal causes of perinatal mortality worldwide. One of the causes of preterm labor is uterine muscle integrity problems. Some mechanistic studies show insight into vitamin D activity's possible role in the injured muscle. This study aimed to determine whether vitamin D can increase muscle cell viability.

Methods: This experimental research used human smooth muscle uterine myometrium cell line pregnant human myometrial (PHM) 1-41. The cells were cultured for 24 hours in hypoxia condition, then incubated with several doses of vitamin D. The PHM1-41 cell viability was measured using spectrophotometry. Data analysis was conducted using IBM SPSS 24.0. A p-value <0.05 was considered statistically significant.

Results: The result showed that the minimum level of muscle cell viability after vitamin D incubation was with 300 nM administration, and the maximum level was after 10nM (88.57%+4.48 and 96.21%+2.13 respectively).

Conclusions: Vitamin D at a specific dose can improve cell availability. The optimal dose to improve cell viability is 10 nM.

Keywords: Oxidative stress, PHM1-41 cell viability, preterm labor, vitamin D

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Introduction

Preterm labor, occurring between 20–36 weeks of gestational age, is one of the most common causes of neonatal death and is still a longstanding global problem. According to the World Health Organization (WHO), approximately 15 million neonates were born prematurely, with the ratio of preterm delivery to term delivery incidence being 1:10.[1] Preterm labor could cause complications that result in mortality in infants, with the mortality rate reaching up to 1.3 million children. For those who still survive, lifetime disability probably could occur.[1,2]

Indonesia is one of the countries which contributes the most abundant cases of preterm labor incidence. In 2010, the incidence of preterm labor in Indonesia was ranked 5th globally after India, China, Nigeria, and Pakistan, with preterm labor incidences of approximately 675,700 cases and a preterm birth ratio of up to 15.5% per live birth.[2] At Dr. Hasan Sadikin General Hospital, a referral hospital in West Java, there were 491 cases of premature babies during the period January–December 2015, and it was ranked third in causes of death.[3]

Many factors have been associated with an increased risk of preterm labor, such as a history of preterm labor, multiple pregnancies, shortening of the cervix, smoking, infections, chronic conditions such as high blood pressure, diabetes, autoimmune and depression, polyhydramnios, presence of fetal birth defect, age of mother, and also problems with the uterus or placental, including the abnormalities in uterus morphology or muscle strength.[4]

Vitamin D is one of the important nutrition for pregnancy. Vitamin D plays a role in calcium metabolism and bone health for both

mother and fetal,[5] as well as in improving the nerve, immune and vascular systems, and expressing the specific genes.[6–8] Several mechanistic studies have also provided some insight into the possible role of vitamin D activity in injured muscle. In vitro and in vivo rodent studies show that vitamin D mitigates reactive oxygen species (ROS) production, augments antioxidant capacity, and prevents oxidative stress, a common antagonist in muscle damage.[9,10]

Uterine myometrium smooth muscle cells pregnant human myometrial (PHM) 1-41 is a subculture of primary human myometrium,[11] and is expected to have the same characteristic as in vivo human myometrium. Cell culture is still used for gene expression research because the gene expression level response in cells cultured in vitro is relatively the same as cells in natural condition with the same treatment.[12] This study aimed to determine whether vitamin D can increase muscle cell viability.

Methods

The study was an experimental study using an in vitro model of uterine cell or PHM 1-41 conducted at the Culture and Cytogenetic Laboratorium, Faculty of Medicine Universitas Padjadjaran and the ARETHA Medika Utama Research Laboratory, Biomolecular and Biomedical Research Center with ethical approval from the Health Research Ethics Committee No. 425/UN6.C.10/PN/2017. PHM1-41 cells obtained from the American Type Culture Collection (product code ATCC® CRL-3046™) were treated with a thawing process and cultured with Dulbecco's Modified Eagle's Medium (DMEM) which had been supplemented with 0.1 mg/mL Geneticin (G-418), 2 mM Glutamine, 10% fetal bovine serum (FBS) qualified, and 1% antibiotic and antimycotic (ABAM).

Cells were cultured and grouped into three treatments; negative control group, which was a group of cells that were not administrated vitamin D; group treated with hypoxia without administering vitamin D3, and group administered vitamin D3 dissolved in ethanol with various concentrations. In cell viability test or cell cytotoxicity test, the variables concentration used were based on literature review and repeat viability test. This was the basis for determining the variation in concentration used (5 nM–300 nM). The ethanol control group was a group of cells treated with 1% ethanol solution as a solvent of vitamin D. This was to ensure whether the ethanol solution would be a cytotoxic solvent.

The cell viability test was then conducted to determine the safest concentration of Vitamin D3, so that it does not cause cytotoxicity in further treatment. The method of cell viability test used the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) (MTS) assay.

Data was analyzed by one-way ANOVA to compare the control and treatment groups. Kolgomorov-Smirnov was used to determine data normality, and Levene's test was used to determine data homogeneity. If the data was normally distributed then proceeded with the one-way ANOVA test, whereas if the data was not normally distributed then proceeded with the non-parametric Kruskal Willis Test. Post Hoc test was carried out to determine significant differences between each group of cells with different treatments. The Post Hoc test conducted was the Tukey HSD test. One way ANOVA data was then completed with a 95% confidence interval and a significance level of $p<0.05$.

Results

The results of the PHM1-41 cells viability test administered with various concentrations of

Table PHM1-41 Cell Viability Test Result on Vitamin D Administration

Treatment	Number of Cells (Cells)	Viability (%)	Inhibition (%)	p-value
Negative Control	11,574±416^{e}	100±2.09^{e}	0±2.09^{a}	
Ethanol Control	9,047±992^{a}	87.31±4.98^{a}	12.69±4.98^{e}	
Vitamin D3 300 nM	9,298±893^{ab}	88.57±4.48^{ab}	11.43±4.48^{de}	0.069
Vitamin D3 150 nM	9,639±464^{abc}	90.29±2.33^{abc}	9.71±2.33^{cde}	0.70
Vitamin D3 50 nM	10,266±324^{bcd}	93.44±1.62^{bcd}	6.56±1.62^{bcd}	0.81
Vitamin D3 10 nM	1,089±424de	96.21±2.13^{de}	3.79±2.13^{ab}	0.231
Vitamin D3 5 nM	10,481±418^{cde}	94.51±2.10^{cde}	5.49±2.10^{abc}	0.54

Note: Data was presented with an average±deviation standard. Different superscripts on viability results indicated a significant difference. ($p<0.05$, Turkey HSD post hoc test on the same column).

vitamin D (300, 150, 50, 10, and 5 nM) were still considered safe. The cell viability values of all the concentration variations tested still showed an average of above 90%.

In the negative control group, cell viability showed a viability value of 100%. The lowest cell viability results were found at a vitamin D concentration of 300 nM (88.57±4.48), meanwhile the highest cell viability result appeared at a vitamin D concentration of 10 nM (96.21±2.13) (Table). However, there was no significant difference with the negative control group. This was shown by the results of the cells amount, cell viability percentage, and cell inhibition percentage, which showed that all three still had the same superscript on the same row.

Discussion

This study has explored whether vitamin D can increase muscle cell viability and determine the safest concentration, which would not cause cytotoxicity with further treatment. The lowest cell viability result was found at a vitamin D concentration of 300 nM (88.57±4.48), whereas the highest was at a vitamin D concentration of 10 nM (96.21±2.13).

In such pathophysiological situations, oxidative stress plays a dominant role in cellular damage in which the production of reactive oxygen species (ROS) suppresses the antioxidant defense system of the cells, which consequently causes cellular destruction. Under physiological conditions, the antioxidant defense system maintains the oxidant-antioxidant balance by adjusting to changes in oxidants levels. The antioxidant defense systems includes enzymes such as glutathione peroxidase, catalase, superoxide dismutase, and other compounds (albumin, GSH). Furthermore, different nutrients, such as vitamin D can also affect the antioxidant balance.[13]

The cell viability test is an initial test that must be done before carrying out further tests. Determining the safe dose (concentration) is very essential to ensure the concentration reaches a lethal dose. Therefore, the vitamin D concentration determined from the literature review needs to be double-checked to ensure the safety of each dose (concentration) of vitamin D for the tested cells. In this study, the result of the PHM1-41 cells viability test administered vitamin D at various concentrations (300, 150, 50, 10, and 5 nM) were still considered safe. Cell viability for all vitamin D concentrations still shows an average value above 90%.

Previous research has shown that administering vitamin D at concentration between 5–300 nM (significance for 150–300 nM) could decrease estrogen and Connexin-43 receptor.[5] Vitamin D has been proven to decrease the amount of ROS and prevent the oxidative stress.[14] A study investigated hypoxia-induced inflammatory gene expression in PHM1-41 cell cultures such as ROS levels and gene expression (CRH, Cx43, MMP-9, IL-6, and NF-κB). The cell groups with the addition of 150 nM vitamin D3 showed a significant decrease in intracellular ROS levels, especially at 150 nM.[15] Meanwhile administration of more than 10 nM vitamin D3 in our study caused a decrease in cell viability compared to the negative control. These results suggest that administering high concentrations of vitamin D3 may not be as effective as 10 nM, but is still considered safe with an average of above 90%.

A study demonstrated that high-dose cholecalciferol treatment of the CaSki cell lines inhibited cell viability, as indicated by reduced cell count, in addition to inducing apoptotic cell death.[16] Studies have shown that vitamin D-induced apoptosis occurs through various intracellular mechanisms. Vitamin D-induced apoptosis is mediated by downregulation of the anti-apoptotic proteins B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra-large (Bcl-XL),[17] and the upregulation of pro-apoptotic protein Bcl-2-associated X protein (Bax), Bcl2-antagonist/killer 1 (Bak), Bcl-2-associated death promoter (Bad), G0-G1 switch 2 (GOS2), death-associated protein (DAP-3), Fas-associated death domain (FADD), and caspases.[16,18] These findings indicate that although vitamin D is mandatory for cell viability, it is still a micronutrient that has the optimal dose to consume.

Vitamin D is one of the key controllers of systemic inflammation, oxidative stress and mitochondrial respiratory function, and thus, the aging process in humans. Vitamin D significantly decreased iNOS and COX-2 expressions in brain areas, such as hippocampus and prefrontal cortex, and inhibited degranulation of activated neutrophils by reducing ROS production and myeloperoxidase release.[19] In turn, the molecular and cellular action of forming 1,25(OH)2D slows down oxidative stress, cell and tissue damage, and the aging process. On the other hand, hypovitaminosis D impairs mitochondrial function, and enhances oxidative stress and systemic inflammation.

The interaction of 1,25(OH)2D with its intracellular receptors modulates vitamin D–dependent genes transcription and the activation of vitamin D-responsive elements, which triggers multiple second messenger systems. Thus, it is not surprising that hypovitaminosis D increases the incidence and severity of several common age-related diseases, such as metabolic disorders linked to oxidative stress.[14]

Our data suggest that vitamin D and downstream receptor signalling play a key role in the ability of macrophages and other immune cells to enhance host antimicrobial defence and coordinate variety of biological responses, including immune and inflammatory activities.[11] A study showed the association of inflammatory cytokines with 25-hydroxy vitamin D, indicating that 25-hydroxy vitamin D is an immune modulator. The ROS and cytokines further play an important role in disease pathogenesis and are responsible for increasing the severity of disease.[20] The use of vitamin D3 as an antioxidant could be a potential candidate in preventing preterm birth by reducing ROS levels.[15]

However, this study is limited due to it only uses simple in vitro functional assays for cell viability. A follow up study is needed to analyze the antioxidant activity of vitamin D by determining the molecular mechanism of vitamin D in preterm labor. To conclude, vitamin D at a specific dose can improve cell availability and in this study the optimal dose to improve cell viability is 10 nM.

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