

Effect of Vitamin D₃ Treatment on Genes Expression of *Corticotrophin Releasing Hormone (CRH)*, *CRH Receptor 1 (CRH-R1)* and *Connexin-43 (CON-43)* in PHM1-41 Cell Line that Induced by Hypoxia

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Abstract

Introduction: Hypothalamic-Pituitary-Adrenal (HPA) axis activity is one of pathophysiologic mechanism that caused preterm labor. Biologic maternal stress, for instance hypoxia condition, is one of the causes that can trigger preterm birth occasion through the activation of HPA axis. It increases Corticotrophin Releasing Hormone (CRH), CRH receptor 1 (CRH-R1), and Connexin-43 (CON-43) as the trigger of the contraction process. Vitamin D as a source of Ca²⁺ ion is needed for myometrium smooth muscle's concentrations and relaxation mechanism. The aim of this study was to determine the effect of vitamin D₃ in PHM1-41 cell line. Materials and Methods: The human smooth muscle uterine myometrium cell line PHM1-41 as an *in vitro* model experimental subject, treated by hypoxia oxidative stress condition and added by vitamin D₃ (5, 10, 50 and 150 nM). The dichlorodihydrofluorescein diacetate (DCFDA) fluorescent was used to measure the level of intracellular reactive oxygen species (ROS). In addition, RNA of treated PHM1-41 cells was isolated for analyzing gene expressions such as CRH, CRH-R1, and CON-43 as a profile of contractility regulation. Results: ROS level effectively decrease in the cells that treated by 150 nM vitamin D₃ group compared to the control hypoxia cell group (7.16 ± 0.23 and 19.49 ± 1.76, respectively). Expression of CRH, CRH-R1, and CON-43 genes are also decrease by treated with 150 nM vitamin D₃ to the cells. Pearson (parametric) correlation analysis evidenced a negative correlation between the vitamin D₃ additional treated to the lower of ROS level, CRH, CRH-R1, and CON-43 genes expression on PHM1-41 cell line that induced by oxidative stress condition. Conclusion: The concentration of 150 nM vitamin D₃ was a prominent potency to prevent the incidence of preterm labor.

Keywords: Corticotrophin releasing hormone, CRH receptor 1, Connexin-43, Oxidative stress, Vitamin D₃

Introduction

Preterm birth (births occurring between 20 and 36 weeks gestation) is one of the leading causes of neonatal morbidity and mortality, and it is still a worldwide problem. [1-3]. According to the data from World Health Organization (WHO), every year approximately 15 million babies are having outcome of preterm birth with an incidence ratio of 1:10 [4]. In 2010, Indonesia was ranked fifth after India, China, Nigeria, and Pakistan, with the number of preterm births attained 675,700 cases and the ratio of preterm birth was 15.5 % per 100 live births [5]. Data of maternal perinatal registration in Indonesia collected from 11 hospitals in West Java showed that during the 6 months period from October 2014 to March 2015 obtained 1,340 of 8,468 (15.82 %) births were preterm.

Preterm birth is a multifactorial process. Based on Lockwood and Kunczyński [6], the activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis is one of the pathophysiologic mechanisms which caused it. The maternal and fetal HPA axis activation is the modification response of the neuroendocrine system to oxidative stress threat. The HPA axis activation will increase the concentration of Corticotrophin Releasing Hormone (CRH) which has a significant role in the physiology of both term and preterm labor

[7]. The HPA axis activation is generally caused by the maternal stress condition. Hypoxia is one of the forms of biological stress metabolism that stimulates the activation of the HPA axis which is characterized by the releasing of CRH [8,9]. Corticotrophin Releasing Hormone will specifically bind to receptor CRH-R1 which indicates the increasing of CRH level will be linearly correlated with the increasing of CRH-R1 receptor activity [10-12].

Free radicals or commonly known as radical oxygen species (ROS) are derived products of oxygen obtained through various mechanisms such as hypoxia, activation of neutrophils and macrophages during inflammation, and prostaglandin metabolism [13]. A high level of intracellular ROS will induce increasing in myometrium contraction through the activation of PKC and inhibition of BKCa. Activation of PKC will assist the inhibition of BKCa ion channel activity. During the increase of BKCa activity, happened a change in the potential membrane from hiperpolarization to depolarization state. Therefore, happened a signal transduction cascade through the second messenger cause the activation of actin and the changes of myometrium condition from relaxation to contraction [14]. Vitamin D is one of the antioxidant which could be utilized as the therapeutic candidate to prevent preterm birth. Vitamin D is known for its capable to decrease the amount of ROS [15]. Bound Vitamin D₃ which forms vitamin D binding protein (DBP) is associated to the elevation of 1, 25 (OH)₂D₃ in kidney. This was shown as the 1, 25 (OH)₂D level increases up to 2-folds in the 3rd trimester of pregnancy [16].

Many studies on the pathophysiology of preterm labor have been carried out, but studies of the pathophysiology of preterm labor through other mechanisms, such as activation of the HPA axis caused by oxidative stress, are still unknown. The results of the association of oxidative stress and HPA axis activation were still contradictory [17]. Since the number of study related to the pathophysiology of preterm labor through the HPA axis activation caused by oxidative stress were still limited and the results were still contradictive, therefore this study was conducted. PHM1-41 cells were selected as the model to describe the condition of human pregnancy, but the myometrium cells culture could not describe the exact human pregnancy condition since it was a complex process. PHM1-41 is used because PHM1-41 can describe the gene expression level of the *in vitro* cultured.

Materials and methods

Human uterine myometrium smooth muscle, PHM1-41 cell line (ATCC® CRL-3046™) was obtained from American Type Culture Collection (ATCC). The cell line was treated by hypoxia condition and vitamin D₃. This research was conducted at Aretha Medika Utama Biomolecular and Biomedical Research Centre, Bandung, Indonesia. Ethical clearance was obtained from the Health Research Ethics Committee of Padjajaran University, Indonesia (No. 425/UN6.C.10/PN2017).

Human uterine myometrial smooth muscle PHM1-41 cell line cell culture and viability assay

Human uterine myometrium smooth muscle cells line (PHM1-41 cells, ATCC® CRL-3046™) were obtained from Aretha Medika Utama Biomolecular and Biomedical Research Centre, Bandung, Indonesia. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, 11995065) that supplemented with 0.1 mg/mL Geneticin (G-418, Gibco, 10131035), 2 mM Glutamine (Gibco, 25030081), 10 % Fetal Bovine Serum (FBS, Gibco, 26140079) qualified, and 1 % Antibiotic and Antimycotic (ABAM, Gibco, 1772653). Cells have incubated in 5 % CO₂ incubator at 37 °C and 95 % humidity until the cells were 80 - 90 % confluence.

Growth medium was disposed and washed by phosphate buffer saline (PBS, Gibco, 10010023) 1x. Then, the cells were added by trypsin-EDTA (Gibco, 25200072) and incubated at 37 °C for 3 min. Trypsinization was stopped by adding growth medium in equal volume. Cells were suspended and replaced into centrifuge tube, then centrifuged at 1,600 rpm for 5 min. Pellet were resuspended and number of cells were counted by hemacytometer. The cells (5×10³ cell/well) were seeded into 96-well plate and incubated for 24 h at 5 % CO₂ and 37 °C. After cells were 80 - 90 % confluent, growth medium changed with fresh growth medium, and added by Vitamin D₃ (Sigma-Aldrich, C9756-1G) at 300 - 5 nM, incubated in 37 °C, 5 % CO₂ for 24 h. Eventually, 20 μL MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Abcam, ab197010) was added and incubated at 37 °C, 5 % CO₂ for 3 - 4 h. And then, the absorbance was read at 490 nm wavelength (Multiskan™ GO Microplate Spectrophotometer, Thermo Fisher Scientific) [18-20].

Treatment hypoxia oxidative stress and vitamin D₃

Based on viability cell assay result, concentrations of vitamin D₃ for treating PHM1-41 cell line were 150 - 5 nM that dissolved in 1 % alcohol. There were 3 groups of treatments, first group was non-

hypoxia control (negative control) that cells cultured in normal condition (37 °C, 5 % CO₂) and was not treated with Vitamin D₃. Second group was hypoxia control (positive control) that cells which cultured in hypoxia condition (37 °C, 5 % CO₂, 2 % O₂) and was not treated with vitamin D₃. The last group was treated group that cells cultured in hypoxia condition and treated with various vitamin D₃ concentrations (150, 50, 10 and 5 nM). The cells (2.5×10⁵ cells/well or flask) were seeded into 6 well-plate (for intracellular ROS measurement) and into a T25 flask (for RNA isolation) in growth medium and incubated in 37 °C, 5 % CO₂ until the cells were 80 – 90 % confluent. After confluent, the cells were washed by PBS 1x, then replaced by fresh growth medium that has been added with the various concentrations of vitamin D₃ (150, 50, 10 and 5 nM) and incubated for 24 h [18]. Then, the cells that had been treated according to the group were then isolated the RNA and the intracellular of treated cells ROS level can be measured.

Measurement of ROS level

The intracellular ROS levels were measured using a DCFDA Cellular Reactive Oxygen Species Detection Assay Kit (Abcam, Ab113851) protocol with slight modification by flow-cytometry (MacsQuant Analyzer 10, Miltenyi). The growth medium of PHM1-41 cells was disposed and the cells were washed by PBS 1x. Cells were trypsinized with trypsin-EDTA 0.25 % for 3 min in 37 °C. Trypsinization was stopped by added growth medium in equal volume. The cells were suspended and replaced into 15 mL centrifuge tube, centrifuged at 1,600 rpm for 5 min. The cells pellet was resuspended with 1 mL growth medium, and then cells were counted. Centrifuged cells changed recent growth medium by 500 µL DCFDA working buffer, and resuspended cells in 5 mL falcon round bottom tube. Stained cells with 25 µM DCFDA, then incubated for 30 min in 37 °C, 5 % CO₂. ROS levels were analyzed with MacsQuant Analyzer 10 Flow Cytometer, Miltenyi [21].

RNA Extraction and cDNA synthesis

RNA extraction was performed using Aurum Total RNA Kit (Bio-Rad, 7326820) according to the manufacturer's instructions. After the extractions, the quality and quantity were measured using Microdrop plate and Multiskan Go Spectrophotometer Thermo Fisher Scientific. The RNA then used for cDNA synthesis using Mix iScript cDNA Synthesis Kit (Bio Rad, 1708890) at 25 °C for 5 min, 46 °C for 20 min, with a final step of at 95 °C for 1 min. The product was stored at –20 °C [22].

Quantification of genes expression by real-time qPCR

The Corticotrophin Releasing Hormone (CRH), CRH receptor 1 (CRH-R1), and Connexin-43 (CON-43) genes expression with housekeeping gene that is β-actin gene was analyzed using real-time quantitative polymerase chain reaction (qPCR). The primers used in this study are shown in **Table 1**. The RT-qPCR was conducted by real-time PikoReal (Thermo Scientific Inc.) with condition pre-denaturation cycle at 95 °C for 5 s, then followed by 40 cycles of denaturation at 95 °C for 1 s, annealing at 50 °C for 40 s, and elongation for at 72 °C for 10 s.

Table 1 Sequence of primers that used in RT-qPCR.

Gene	Forward	Reverse	Reference
CON-43	5'-GCGTGAGGAAAGTACCAAAC-3'	5'-GGGCAACCTTGAGTTCTTCC-3'	[23]
CRH	5'-TCCGAGGAGCCTCCCATC-3'	5'-AATCTCCATGAGTTTCCTGTGTC-3'	[24]
CRH-R1	5'-CAAACAATGGCTACCGGGAG-3'	5'-ACACCCCAGCCAATGCAGA-3'	[25]
β-Actin	5'-TCTGGCACCACACCTTCTACAATG-3'	5'-AGCACAGCCTGGATAGCAACG-3'	[26]

Statistical analysis

Statistical Package for the Social Sciences (SPSS) statistics version 20.0 software was used to perform the statistical analysis. Normality test Shapiro-Wilk was conducted, followed by homogeneity variance and one way ANOVA. Then, also followed by Tukey HSD and Dunnett post hoc test and $p < 0.05$ was considered to be significant. The correlation of all data was analyzed using Pearson's correlation coefficient. Data are presented as mean ± standard deviation (SD).

Results and discussion

In general, preterm birth has the same main mechanism as term birth, which begins with the increase of uterine contractions, cervical ripening, and rupture of the amnion which causes fetal expulsion [6]. Some of the pathophysiological mechanisms which cause preterm birth were including inflammation and infection, activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis in the mother and fetus, decidual bleeding, and pathological stretching of the uterus [6,12,27,28].

There have been many studies on the pathophysiology of preterm birth. However, research on the pathophysiology of preterm birth through other pathways, including through the HPA axis activation pathway due to oxidative stress was still rare. The studies about the effect of oxidative stress on the HPA axis activation still present contradictory results. Oxidative stress has long been associated with preterm birth because of its ability to interfere with maternal and fetal physiology, especially the circulatory system [8]. ROS interferes with the vasodilation of blood vessels. Vasodilatation mechanisms are needed to increase maternal blood flow as well as in the development of the fetus and uterus [29]. The disruption of the circulatory system causes stress to the mother and fetus. This stress condition could activate the HPA axis, increase the expression of CRH, CRH receptors, and cause preterm birth [30,31].

The activation of the HPA axis is generally caused by stressful conditions in the mother and fetus. The stress that occurs could be manifested as psychological, physiological, physical, nutritional, or metabolic stress. Hypoxia is a form of metabolic stress. Stress triggers the activation of the HPA axis which is characterized by the release of the CRH hormone. The CRH hormone then stimulates adrenocorticotrophic hormone (ACTH) production and stimulates an increase in cortisol production. Cortisol will then bind to glucocorticoid receptor (GR) and trigger an increase in metabolism and gene transcription mediated by glucocorticoid-response elements (GRE). An increase in the metabolic rate will trigger the production of ROS so that it exceeds its elimination capacity and causes oxidative stress [32]. ROS in preterm delivery could modulate myometrial contraction through PKC activation, inhibit BKCa activity, and increase Connexin-43 expression. ROS activates Protein Kinase C (PKC) which plays a role in the contraction of actin and muscle cell myosin myometrium [33]. Apart from actin contraction, PKC activation by ROS also affects the ion channel large-conductance voltage - and Ca^{2+} - activated K^+ channel (BKCa). BKCa is an important ion channel that plays a role in regulating the potential of the myometrial cell membrane. In the active state, BKCa keeps the potential membrane from changing so that the myometrium remains in a quiescence state. PKC activation helps inhibit BKCa ion channel activity. When BKCa activity decreases, the potential membrane changes from hyperpolarization to depolarization state. The result is a cascade of signal transduction via second messengers, resulting in activation of actin and changes in myometrial condition from relaxation to contraction. Vitamin D has also been shown to reduce the number of ROS and prevent oxidative stress [34].

Cell viability or cell cytotoxicity assay is a preliminary assay before the main assay of this study was conducted. The results of this test will become the baseline of concentration variation that will be selected, which is 5 - 300 nM, according to the literature of Thota *et al.* [35]. According to **Table 2**, the lowest cell viability happened on the concentration of 300 nM vitamin D, otherwise, the highest cell viability happened on the concentration of 10 nM vitamin D, which was $96,21 \pm 2,13$. The results of this cell viability assay in all vitamin D concentrations that will be tested will be useful for the other further assessment. However, in ROS levels and gene expression assessment for CRH, CRH-R1, and CON-43 only concentrations of vitamin D₃ 5, 10, 50 and 150 nM have been used since they had better cell viability compared to vitamin D₃ 300 nM, with the result of cell viability more than 90 %. The material used was said to be non-toxic if it shows cell viability results of more than 90 % in the MTS toxicity test.

Table 2 Effect of various concentrations of Vitamin D₃ toxicity towards PHM1-41 cell viability.

Treatment	Number of cells (Cells)	Viability (%)	Inhibition (%)
Negative Control	11,574 ± 416 ^e	100.00 ± 2.09 ^e	0.00 ± 2.09 ^a
Ethanol Control	9,047 ± 992 ^a	87.31 ± 4.98 ^a	12.69 ± 4.98 ^e
Vitamin D ₃ 5 nM	10,481 ± 418 ^{cde}	94.51 ± 2.10 ^{cde}	5.49 ± 2.10 ^{abc}
Vitamin D ₃ 10 nM	10,891 ± 424 ^{de}	96.21 ± 2.13 ^{de}	3.79 ± 2.13 ^{ab}
Vitamin D ₃ 50 nM	10,266 ± 324 ^{bcd}	93.44 ± 1.62 ^{bcd}	6.56 ± 1.62 ^{bcd}
Vitamin D ₃ 150 nM	9,639 ± 464 ^{abc}	90.29 ± 2.33 ^{abc}	9.71 ± 2.33 ^{cde}
Vitamin D ₃ 300 nM	9,298 ± 893 ^{ab}	88.57 ± 4.48 ^{ab}	11.43 ± 4.48 ^{de}

Data are presented as mean ± standard deviation (SD). Different small letters (a, ab, abc, bcd, cde, de, e) in the same row showed significant at $p < 0.05$ (Tukey HSD post hoc test).

Testing of intracellular ROS levels was carried out to determine the levels of ROS contained in intracellular PHM1-41 cells that had been treated with hypoxia and normal (non-hypoxia), as well as groups of cells with the addition of vitamin D₃ which experienced hypoxic oxidative stress. ROS levels had a significant relationship with increased expression of contractility regulation profile genes in PHM1-41 cells. **Table 3** shows that the percentage value of effective intracellular ROS levels was significantly lower at the 150 nM vitamin D₃ concentration (7.16 ± 0.23) compared with hypoxia control (19.49 ± 1.76). At 5 - 50 nM vitamin D concentrations the ROS levels were higher. **Figure 1** shows that the cell groups with the addition of vitamin D₃ 150 nM showed a shift in the fluorescence of ROS levels in a more negative direction, namely at 15.30 ± 0.34 MFI. The percentage and MFI values indicated that the ROS levels were lower with the addition of vitamin D₃ 150 nM.

Table 3 Reactive oxygen species levels in the intracellular PHM1-41 cell line treated with vitamin D₃ under conditions of hypoxic oxidative stress.

Treatment	ROS Level (%)	ROS Level (MFI)
Hypoxia Control	19.49 ± 1.76^a	18.54 ± 0.65^a
Vitamin D ₃ 5 nM	14.37 ± 0.45^b	19.35 ± 0.03^a
Vitamin D ₃ 10 nM	12.26 ± 0.12^{bc}	15.93 ± 0.46^{bc}
Vitamin D ₃ 50 nM	10.67 ± 0.05^{cd}	16.43 ± 0.22^b
Vitamin D ₃ 150 nM	7.16 ± 0.23^e	15.30 ± 0.34^c
Non-hypoxia control	8.74 ± 0.84^{de}	16.33 ± 0.39^{bc}

Data are presented as mean \pm standard deviation (SD). Different small letters (a, b, bc, c, cd, de, e) in the same row showed significant at $p < 0.05$ (Tukey HSD post hoc test).

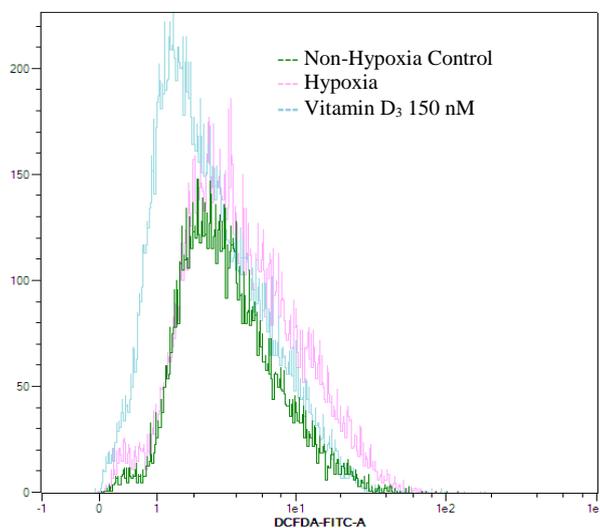


Figure 1 Comparison of intracellular reactive oxygen species level histogram in non-hypoxia cells group, hypoxia cells group and treated by vitamin D₃ cells group.

It is expected that the decrease in ROS levels will prevent various reactions that trigger an increase in myometrial contractility. In addition to utilize endogenous antioxidants that are already present in the body such as superoxide dismutase (SOD), catalase (CAT), and glutathione (GHS), decreasing ROS levels can also rely on secondary antioxidants that can be obtained through supplementation of vitamins and other antioxidants from outside the body. Preterm birth is expected to reduce CRH levels as well. This is evidenced by the lower levels of intracellular ROS in PHM1-41 cells, it turns out that the levels of CRH gene expression are lower as well. The best result was to reduce the level of CRH gene expression

by adding vitamin D₃ with a concentration of 150 nM, which was 0.18 ± 0.01 (**Figure 2**). This value was not significantly different from the level of gene expression in the non-hypoxic normal cell group. This shows that the addition of vitamin D₃ 150 nM can return the expression level almost similar to the level of gene expression in normal cells.

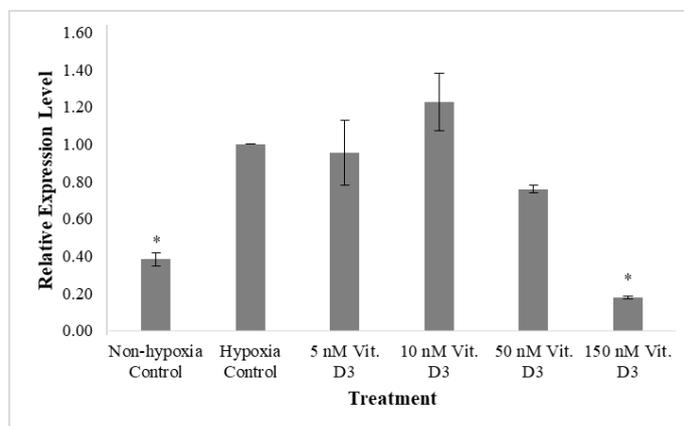


Figure 2 Relative expression of CRH in PHM1-41 cell line that induced by oxidative stress condition. Data are presented as the mean \pm SD value of at least 2 independent experiments. * $p < 0.05$ vs. hypoxia control.

The CRH-R1 receptor acts as a major regulator of CRH hormone activity in humans. The CRH hormone has a high affinity for the CRH-R1 receptor but has a low affinity for the CRH-R2 receptor. In **Figure 3**, the expression level of the CRH-R1 gene was lower in the group of cells given additional vitamin D₃ with concentrations of 50 and 150 nM, with values of 0.58 ± 0.11 and 0.60 ± 0.09 , respectively.

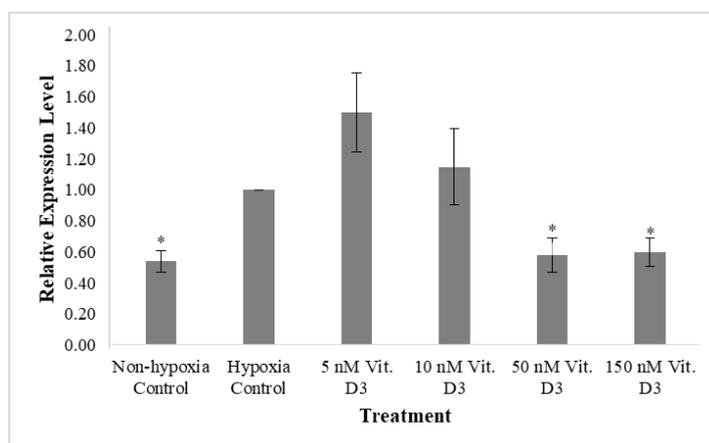


Figure 3 Relative expression of CRH-R1 in PHM1-41 cell line that induced by stress oxidative condition. Data are presented as the mean \pm SD value of at least 2 independent experiments. * $p < 0.05$ vs. hypoxia control.

CRH is regulated by both internal stress and external stress. The stress that occurs can be in the form of systemic stress or metabolic stress. When a stress stimulus occurs, sensory information can be transmitted directly to the paraventricular nucleus (PVN) portion of the hypothalamus or transmitted to CRH neurons via the peptidergic neural pathway. The presence of this stimulus transmission will then trigger an increase in CRH gene transcription and CRH hormone secretion. The CRH hormone works by binding to the CRH receptor which is part of the G-protein coupled receptor superfamily (GPCR). There

are 3 types of CRH receptors, namely Type 1 (CRH-R1), Type 2 (CRH-R2) and Type 3 (CRH-R3). CRH-R1 and CRH-R2 were identified in mammals while CRH-R3 was only identified in catfish. In humans, CRH-R1 is actively expressed in the central nervous system, pituitary, heart, adrenal glands, ovaries, and placenta whereas CRH-R2 is actively expressed in the brain and heart. In addition to the CRH receptor, in mammals, the CRH hormone is also regulated by the presence of CRH Binding Protein (CRH-BP). The CRH hormone has a higher affinity for CRH-BP when compared to CRH-R1. Under normal conditions, the amount of CRH hormone is low and the hormone binds to CRH-BP so that its work is inhibited. However, in stressful conditions, the high level of CRH exceeds the amount of CRH-BP so that the CRH hormone will bind to the CRH-R1 receptor and actively work to affect various systems [36,37].

ROS can trigger myometrial cell contractility. ROS together with CRH increase the expression of CON-43 gene through the activation process of transcription factor 1 (AP-1) c-Jun and c-Fos. Increased expression of CON-43 gene will lead to a well-coordinated increasing of myometrial contraction which ultimately leads to the birth process [33,38]. The expression level of the CON-43 gene was lower in the group of cells was given vitamin D₃ in the concentration range of 10 - 150 nM. All treatments with the addition of vitamin D₃ had lower gene expression levels than those in the hypoxic cell group without the addition of vitamin D₃ (**Figure 4**). However, the best vitamin D₃ concentration to reduce the level of expression of the CON-43 gene is by giving vitamin D₃ at a concentration of 150 nM and vitamin D₃ 50 nM

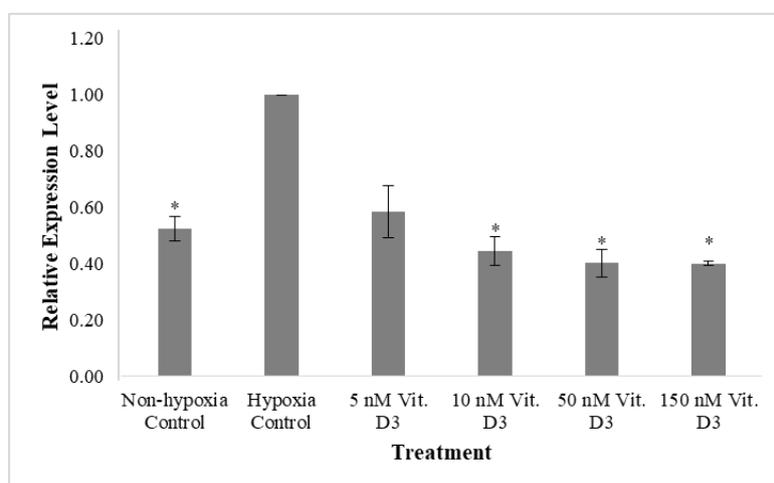


Figure 4 Relative expression of CON-43 in PHM1-41 cell line that induced by stress oxidative condition. Data are presented as the mean \pm SD value of at least 2 independent experiments. * $p < 0.05$ vs. hypoxia control.

The CON-43 gene is upregulated by the AP-1 promoter. Activation of the AP-1 promoter can occur when transcription factors from the c-Jun and c-Fos families bind to the promoter sequence of the AP-1 site. The transcription factors c-Jun and c-Fos are specific transcription factors expressed primarily by human uterine smooth muscle cells. The expression of the transcription factors c-Jun and c-Fos was influenced by signal transduction due to PKC activation. PKC activation occurs when there are diacylglycerol molecules in cells. Diacylglycerol molecules are molecules that can act as activators and are the result of the breakdown of inositol phospholipids. The breakdown of inositol phospholipids, which are part of the phospholipids, occurs when the uterus is distended. Uterine distension is caused by a progesterone withdrawal event, an increase in the level of the hormone estrogen so that it is at a high level, and an increase in the level of the hormone prostaglandin just before birth [39].

Pearson correlation analysis showed that there was a negative relationship between the addition of vitamin D₃ with levels of ROS and the expression of the CRH, CRH-R1, and CON-43 genes (**Table 4**). Vitamin D has been shown to reduce the number of ROS and prevent oxidative stress. In addition, it is known that the addition of vitamin D with a concentration of 5 - 300 nM (significant at 150 - 300 nM) can reduce the expression of estrogen receptors and the expression of CON-43 gene.

Table 4 The association of vitamin D₃ addition to lower levels of ROS, CRH gene expression, CRH-R1 gene expression, and CON-43 gene expression in PHM1-41 line cells under the conditions of oxidative stress.

Correlation of Vitamin D ₃ addition to parameters	Correlation coefficient	p-value
ROS level	-0.743	< 0.001
CRH gene expression	-0.773	< 0.001
CRH-R1 gene expression	-0.719	0.001
CON-43 gene expression	-0.688	0.002

Conclusions

The addition of vitamin D₃ can reduce levels of ROS and the expression of genes related to contractility regulation. The most optimal level of addition of vitamin D₃ to prevent the contractility process in the human uterine myometrium smooth muscle PHM1-41 cell line experiencing hypoxic oxidative stress is the addition of 150 nM vitamin D₃. The use of vitamin D₃ as an antioxidant can be a potential candidate in the prevention of preterm birth through reduce ROS levels in the *in vitro* model of myometrial cells at the time of the incidence of preterm birth. However, this is only the basis for further research. Vitamin D₃ is not the only related element that can reduce ROS levels in cells, there are several other factors that influence the decrease in ROS levels in cells, namely endogenous antioxidants and other metabolic precursors in the human body.

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