The Effect of Vitamin D Administration on Leptin, Adiponectin and mRNA MCP-1 Levels in Adipose Tissue of Obese Female Wistar Rats

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Abstract
In obesity, there is an accumulation of adipocytes which produces adipokine that are pro-inflammatory substance, such as leptin and MCP-1 and anti-inflammatory substance, such as adiponectin, while the bioavailability of vitamin D is decreased. This research aimed to study the effect of vitamin D administration on leptin, MCP-1, and adiponectin levels in adipose tissue rats with obesity. Vitamin D was administered to the obese model of 6-9 months old female Wistar rats. This experiment was a randomized control group design with a post-test group design only. Twenty-seven (27) female obese Wistar rats were included in this study. The animals were divided randomly into 3 groups: 9 rats were given 2400 IU vitamin D (group A), 9 rats were given 800 IU vitamin D (group B) and 9 rats were given a placebo as control (group C). The administration of Vitamin D was given once daily for 8 weeks. The visceral adipose tissue was taken to measure the level of leptin, adiponectin and mRNA MCP-1. Data among groups was analyzed by using one-way ANOVA and followed by LSD test, at a significance level of p <0.05. The lowest level of leptin (1059.15 ± 135.20 pg/ml) and mRNA MCP-1 (2.36 ± 0.75 fg/ml) and the highest adiponectin level (3.43 ± 0.47 ng/ml) were found in group A. In conclusion, oral administration of vitamin D
(2400 IU) decreased pro-inflammatory substances, such as leptin and mRNA MCP-1 and increased anti-inflammatory substances, such as adiponectin, in visceral adipose tissue of obese female Wistar rats.

Introduction
Obesity is caused by long-term imbalance of energy intake and usage. It is a global pandemic all over the world and has been stated as the biggest chronic health problem in adults by World Health Organization.¹

National Basic Health study in 2013 showed that 21.7% of Indonesian adult are obese, where the prevalence is higher in females (26.9%) compared to males (16.3%).² Some risk factors attributed to higher risk of obesity in females, such as pregnancy, lack of physical activity, frequent consumption of sweet snacks and hormonal factors.³ Lower metabolism rates in females are also considered as one of them.

In abdominal obesity, adipose tissue accumulates in abdomen, especially in the visceral part. Adipocytes are important in maintaining energy homeostasis and producing several factors as feedback signal provider in adipose tissue metabolism. Those factors can be identified as adipokines, such as leptin, resistin, adipin, asylation stimulating protein, agouti protein, adiponectin, Interleukin 6, Tumor Necrotizing Factor α, Monocyte Chemoattractant Protein-1 (MCP-1), angiotensinogen and others. Most adipokines are pro-inflammatory which initiate metabolic disorders in obesity, such as leptin and MCP-1. In opposite, there are adipokines which are anti-inflammatory, one of those is adiponectin. The imbalance of adipokines secretion initiates various complications in obesity, especially metabolic disorders and cardiovascular.⁴

Leptin is a peptide produced by adipocyte which is a hormone that regulates body fat reserves and it is related to adipose tissue. This hormone is involved in energy balance homeostasis through central regulation, especially in fat accumulation and metabolic disorder. High level of leptin in circulation is related to the number of adipose tissue, which will give a signal to the central nervous system, that the energy storage in the body has been sufficient. This will provide a response back to body to reduce food intake and increase energy use.⁵ There is a positive association between leptin level and body fat mass, it means more body fat more leptin.⁶

Excess adipose tissue in obesity consists of heterogeneous fat cells that respond to excess nutrient intake, such as hypertrophy and hyperplasia adipocyte. With progressive enlargement of adipocyte, blood circulation to adipose tissue will decrease and lead to hypoxia. Hypoxia will initiates necrosis and infiltration of macrophages in adipose tissue which promotes production of pro-inflammatory factors, including TNF α, IL-6 and MCP-1. MCP-1 is produced mostly by macrophages and endothelial cells which are a potent chemotactic factor for monocytes. Several studies have shown the presence of macrophage infiltration into adipose tissue of obese rats. A large number of MCP-1 is also found in adipose tissue of obese mice, suggesting that MCP-1 is also one of the increased adipokines in obesity. This showed that the excess of adipose tissue in obesity as low grade chronic inflammation process is associated to pathogenesis of obesity.⁷ The other adipokine which has anti-inflammatory properties is adiponectin, which is a plasma protein secreted by adipose tissue. Adiponectin has anti-inflammatory, anti-fibrotic, antidiabetic and anti-lipidemic effect. Low levels of adiponectin in plasma are strongly associated with obesity. Plasma adiponectin levels are determined by visceral fat, therefore the distribution of body fat plays a role in the differences of adiponectin levels.⁸ There has been a lot of evidences from research on experimental models which shows that adiponectin is an adipokine that against metabolic dysfunction in obesity. By giving adiponectin systemically, it can reduce hyperglycemia in diabetic rats through increased insulin action.⁹

In obesity, there is not only about imbalance of adipokine, but also the decrease of vitamin D bioavailability.¹⁰ The role of vitamin D in pathophysiology of obesity is still pros and cons
among scientists. Obesity may implicate the vitamin D status because vitamin D is a fat soluble that is readily stored in subcutaneous fat, it may be sequestered in the larger body pool of fat of obese individuals. While body weight and vitamin D status have strong correlation with each other, obesity might be the implications of low plasma vitamin D. This is strongly reported that bioavailability of vitamin D has exhibited a decreased in obese individuals. Although many studies have shown a negative correlations between obesity and serum vitamin D levels, the causes and effects are still unclear. Subjects with hypovitaminosis vitamin D have higher risk of developing metabolic disorders compared to ones without it. Vitamin D is also an important target in adipocytes differentiation process, since it is predicted has a role in inhibiting or stimulating the expression of key molecules involved in differentiation, such as Wingless-related integration site (WNT), CAAT (distinct pattern of nucleotides) and Enhancer Binding Protein β (EBPβ). Vitamin D stimulates human adipocytes differentiation through its activation to 1,25 (OH)2D. Vitamin D will initiate adipogenesis when its expression increases at adipogenic markers, such as CAAT Enhancer Binding Protein α (C/EBPα), C/EBPβ, Fatty Acid Binding Protein 4 (FABP4) protein and Peroxisome Proliferator Activated Receptor Gamma (PPARγ). In rats’ pre-adipocyte cells, the active form of vitamin D which is 1,25 (OH)2D might inhibit adipogenesis by exerting its effect on several targets that attenuate the expression of C/EBPα and PPARγ, especially those in opposite act with PPARγ. Furthermore, it has been observed that the induction of PPARγ was effectively attenuated by 1,25 (OH)2D maintains the expression level of WNT/β that is closely related to adipogenesis and obesity which produce a sustainable β-catenin, an inhibitor of adipogenesis. Therefore, the WNT/β-Catenin plays an essential role in the anti-adipogenic and anti-obesity property of vitamin D.

Although some studies have shown that vitamin D is related to adipogenesis and obesity, there is a lack of study about leptin, adiponectin and mRNA MCP-1 levels in visceral adipose tissue. This research aimed to study the effect of vitamin D administration on leptin, mRNA MCP-1 and adiponectin levels in visceral adipose tissue on rats with obesity.

**Materials and Methods**

**Material**

Vitamin D that is used in this study was Cholecalciferol C9756 Sigma Aldrich, Merck, which is diluted with ethanol and water. Dosage of vitamin D were 2400 IU and 800 IU. One μg Vitamin D = 40 IU, or 1 IU Vitamin D = 0,000025 mg Vitamin D. Therefore, 2400 IU = 0,06 mg Vitamin D, 800 IU = 0,02 mg and converted into rat dosage. In vivo rat experiment was carried out at the Experimental Animal Care Unit, Laboratory of Pharmacology and Therapy, Faculty of Medicine Udayana University from October 2016 – March 2017. This research has been approved by the Health Research Ethics Commission of the Faculty of Medicine, Udayana University/RSUP Sanglah No. 352/UN.14.2/KEP/2016 with protocol number: 1058.02.1.2016.

**Sample Preparation**

The test diet is composed of 200 grams standard diet (CP-594), 100 grams wheat flour, 8 grams cholesterol, 40 ml pork tallow and 50 ml water. These components were mixed until homogeneous. The mixture is formed into pellets and the pellets were dried in an oven at 150°C for 4 hours. The test diet was formulated by following the method of Murwani and Muliartha. The pellet of high-fat diet contains of 30% fat, 55% carbohydrate, 13% protein and 2% cholesterol.

**Diet Induce Obese**

Thirty five six-week-old rats with 120-140 grams in weight, were obtained from Laboratory of Pharmacology and Therapy, Faculty of Medicine Udayana University. The rats were kept in a laboratory with temperature 24°C and humidity 40±5%, 12 hours dark and light, with ad-libitum diet induced obese and free access of drinking water. The rats were fed with the formulated diet for 4 months until weight gain of 225 grams (BMI > 0.68) was achieved, according to a standard method. The weight of rats was recorded every 2 weeks using an analytical scale.

**Sampling and Data Collection**

The accessible population of this study is 6-9 months old obese Wistar rats, with weight more than 225 grams. Inclusion criteria is 6-9 months old obese female Wistar rats with weight more than 225 grams, without any physical deformities and able to feed...
normally. Exclusion criteria is sick female Wistar rats. Drop out criteria is dead female Wistar rats during experimental period. The sample size used in this study was determined by following the procedures described by Federer.\textsuperscript{18}

Using the formula of $(t-1)(r-1) > 15$

Where:
$t$: number of experimental groups
$r$: repetitions

In this study, $t = 3$, written as: $(3-1) (r-1) > 15$. From the formula obtained $r = 9$, therefore the minimum sample size for this study was $9x3 = 27$ rats.

Twenty-seven rats with weight more than 225 grams are randomly distributed into 3 groups of treatment. Group A were given 2400 IU vitamin D, Group B were given 800 IU, and Group C were given placebo as a control. After 7 days of acclimatization, rats were given standard diet (CP-594) and administered with vitamin D 2400 IU, 800 IU and placebo once daily for eight weeks. At the end of 8 weeks of administration, the animal were sacrificed and visceral adipose tissue were taken. The adipose tissue was extracted and centrifuged at 12000 rpm for 10 minutes to get a supernatant of the adipocyte.

Level of Leptin and Adiponectin in visceral adipose tissue were determined by using the Elisa kit (Bioeureaux, Quantikine). Meanwhile, Level of mRNA MCP-1 in visceral adipose tissue was determined by using highly sensitive reverse transcription and polymerase chain reaction (RT/PCR) technique (MyGo Mini Realtime PCR, IT-IS Life Science, UK, 2016).

**Statistical Analysis**

The normality data analysis is using the Shapiro-Wilk test ($p <0.05$). In determining the difference of average between groups, it was analyzed by using One Way ANOVA with significance level $p<0.05$. When there is a significant difference post hoc Least Significant Difference (LSD) was done. The statistical analysis was carried out using SPSS.

Table 1 shows that the average of rats’ weight in Group A and B which each were given 2400 IU and 800 IU vitamin D were $251.67 \pm 20.2$ grams and $248.22 \pm 17.4$ grams, while the average of rats’ weight in Group C, as the control group was $243.33 \pm 11.8$ grams. The significance analysis by using One Way ANOVA shows that the value of $F = 0.559$ and $p = 0.580$. It means that average of rats’ weight between the three groups before treatment is not significantly different ($p>0.05$).

Vitamin D insufficiency in obesity is an established theory, that vitamin D is stored in adipose tissue, and its metabolism is influenced by adipose tissue. Excess body fat causes vitamin D sequestration which reduces vitamin D bioavailability.\textsuperscript{19-21}

<table>
<thead>
<tr>
<th>Subject Group</th>
<th>N</th>
<th>Mean of Body Weight (gram)</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (2400 IU)</td>
<td>9</td>
<td>251.67+20.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group B (800 IU)</td>
<td>9</td>
<td>248.22+17.4</td>
<td>0.559</td>
<td>0.580</td>
</tr>
<tr>
<td>Group C (Placebo)</td>
<td>9</td>
<td>243.33+11.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adipose tissue also produces leptin, it means that more adipose will produce more leptin that may has inhibitory effects on 25(OH)D synthesis via their receptors. These hypothesis has explained that vitamin D administration does not show any significant effects to reduce bodyweight.\textsuperscript{22} The complex biochemical interactions between adipose tissue and vitamin D \textit{in vitro} raises the question whether hypovitaminosis D contributes to obesity or inhibit weight loss in vivo. A few studies has shown that vitamin D, with or without calcium, appears has no definite effects on weight, but that it may affect fat mass and distribution.\textsuperscript{23} This study is also supported by other meta-analyses and trials that have shown
no evidence of effect in vitamin D supplementation on the reduction of obesity or bodyweight.24

The lowest mean of leptin and mRNA MCP-1 were found in group A, followed by group B and group C. The highest mean of adiponectin was also found in group A, followed by group B and C (Table 2).

Those results were significantly different which was p = 0.001 (p<0.05). It was also proved with Least Significant Difference / Post hoc test that showed the differences among three groups (A, B and C) with three parameters (Leptin, Adiponectin and mRNA MCP-1 (Table 3).

Table 2: The Average Comparison of Leptin, Adiponectin and mRNA MCP-1 Level between Groups of Obese Female Wistar Rats’ Adipose Tissue after Administration of Vitamin D

<table>
<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>Mean Level</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (2400IU)</td>
<td>B (800 IU)</td>
<td>C(Placebo)</td>
</tr>
<tr>
<td>Leptin (pg/ml)</td>
<td>9</td>
<td>1059.15 + 135.20</td>
<td>1382.98 + 265.14</td>
</tr>
<tr>
<td>Adiponectin(ng/ml)</td>
<td>9</td>
<td>3.43 + 0.47</td>
<td>2.43 + 0.27</td>
</tr>
<tr>
<td>mRNA MCP-1(fg/ml)</td>
<td>9</td>
<td>2.36 + 0.75</td>
<td>3.04 + 0.66</td>
</tr>
</tbody>
</table>

*Significance at p <0.05

Table 3: Multiple Comparisons of Leptin, Adiponectin and Mrna MCP-1 Among Three Groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>Groups</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin Level</td>
<td>A</td>
<td>B</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>A</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>A</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>0.000</td>
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<tr>
<td>Adiponectin Level</td>
<td>A</td>
<td>B</td>
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<tr>
<td></td>
<td>C</td>
<td></td>
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<td>B</td>
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<td></td>
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<td>A</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>0.005</td>
</tr>
<tr>
<td>mRNA_MCP _1 Level</td>
<td>A</td>
<td>B</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>A</td>
<td>0.042</td>
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<tr>
<td></td>
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<td>C</td>
<td>0.000</td>
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<tr>
<td></td>
<td>C</td>
<td>A</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Significance at p<0.05
Table 3 showed the variable differences among 3 groups. The mean of leptin level in group A was significantly different with group B and C. It means that leptin level in group A was lower than group B, and in group B was lower than group C, and definitely the leptin level in group A was lower than in group C. Moreover, the mean of adiponectin level in group A showed a significant difference compared to group B and C. This shows that the adiponectin level in group A was higher than group B, and in group B was higher than group C, and definitely the adiponectin level in group A was higher than group C. It was similar with the mRNA MCP-1 level that was significantly different among the three groups. It could be stated that the differences among the three groups were significant after the administration of vitamin D, which was (p <0.05) for all groups. (Table 3).

The Relationship of Vitamin D and Level of Leptin, Adiponectin and mRNA MCP-1 in Adipose Tissue

In this study, it is found that the lowest level of leptin is group A, followed by group B and C (Table 2). The result, regarding serum leptin, are similar to the other study that reported by Belenchia, which state that the administration of high dose vitamin D 2000 IU in twice daily on obese adolescents rats for 3 months has reduced the level of leptin significantly, compared to other obese groups which do not receive the treatment.25 This indicates that the higher level of vitamin D affects the reduction level of leptin in the obese group. The reduction level of leptin shows a modulation activity on adipinsualar axis by 1,25(OH)2D, but the exact mechanism remains unclear.25 This result is also supported by other studies which explain the change of lifestyle including dietary patterns and physical activity for a year increase plasma levels of vitamin D and reduce the volume of adipose tissue as well as reduce leptin levels (p <0.001).26 In this study, the change of dietary habit is done, where the rats were given high fat diet before administration of Vitamin D, and were given standard diet during administration of Vitamin D without changing the physical activity.

The possible mechanism explains that the positive relationship between the administration of vitamin D and serum adiponectin might be caused by the increase of adiponectin gene expression or the changes in activity of renin-angiotensin-aldosterone system.27 The activation of vitamin D is considered as renin-angiotensin-aldosterone system inhibitor. The increased activity of renin-angiotensin-aldosterone system is related to the increased of angiotensin production, leads to the decreased of serum level of adiponectin. Based on that, vitamin D administration increases adiponectin level by inhibiting angiotensin production.28 It is proved in this study, that the highest average of adiponectin level was found in group A which was given the highest dose of Vitamin D (2400 IU) (Table 2). The other mechanism that could explain the relationship of vitamin D and adiponectin, there is a regulation from adiponectin gene by the active form of vitamin D, which the receptor is on pre-adipocyte cells.29 The active form of vitamin D also plays an important roles in the regulation of production of TNF-α which is involved in adiponectin synthesis.30

In this study, level of mRNA MCP-1 is found decreasing by increasing the dose of vitamin D (Table 2). The relationship between vitamin D and inflammation is still controversial. Some hypotheses state that inflammation will reduce concentration of 25 (OH) D levels,31 while the others state that the increasing vitamin D status will reduce inflammation.32 Furthermore, with the second hypothesis, where vitamin D can reduce inflammation, it can be explained through in vitro research which shows 1,25 (OH) 2D3 has a strong anti-inflammatory ability.33 This result is also supported by the research from Tripathi, Pandey, and Gao, where there is a protein which caused stress in endoplasmic reticulum that will initiate inflammatory pathway of NF-κβ and c-Jun N terminal kinase.34,35 Activation of NF-κβ pathway is very important in signaling transduction of pro-inflammatory cytokines in adipocytes and various other cell types. The breakdown of Iκβ protein will initiate NFκβ activation, leading to movement of NFκβ in nucleus as transcription regulator. The active form of vitamin D, that is 1,25 (OH)2D3, can increase Iκβ protein in human pre-adipocyte cells, and therapy by using 1,25(OH)D3 initiates the reduction of IL-6 and MCP-1 release in human pre-adipocyte cells, same as pre-adipocyte cells stimulate macrophage migration.35

Leptin and adiponectin are two types of adipokines from many other cytokines produced by adipose tissue. Leptin is a pro-inflammatory adipokine and
adiponectin is an anti-inflammatory adipokine. The levels of leptin and adiponectin which have changed after the administration of vitamin D in this study, also contributes in reducing MCP-1 mRNA levels as a marker of inflammation.

**Conclusion**

Oral administration of vitamin D (2400 IU) decreased pro-inflammatory substances, such as leptin and mRNA MCP-1 and increased anti-inflammatory substances, such as adiponectin, in visceral adipose tissue of obese female Wistar rats. This result is strengthen the anti-inflammatory theories of vitamin D.

**Acknowledgements**

This research was financially supported by the Research and Development Department, Faculty of Medicine, Udayana University, with number of grant: 231.5/UN14.2.2/PD/2017

**Funding**

The author(s) received no financial support for the research, authorship, and/or publication of this article.

**Conflict of Interest**

The authors declare no conflict of interest.

**References**


