Indexed by : DOAJ Google Crossref Dimension sinta GARUDA

J.Biomed.Transl.Res
ISSN: 2503-2178

JOURNAL OF BIOMEDICINE AND TRANSLATIONAL RESEARCH

Available online at JBTR website: https://jbtr.fk.undip.ac.id

Original Research Article

The Effect of Liprotide-Encapsulated Vitamin D_3 on MDA and SOD in Rats Deficient Vitamin D and Calcium

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Article Info
History
Received: 27 Oct 2022
Accepted: 07 Feb 2023
Available: 30 Apr 2023

Abstract

Background: Vitamin D deficiency is frequently correlated with elevated malondialdehyde (MDA) levels and decreased superoxide dismutase (SOD) activity. Several studies have demonstrated that vitamin D_3 can reverse intracellular oxidative stress. However, vitamin D is prone to deterioration and instability. Liprotides contain lipids and proteins that can prevent vitamin D from oxidating.

Objective: This study aims to investigate the effects of liprotide-encapsulated vitamin D_3 on MDA concentrations and SOD activity in calcium and vitamin D-deficient rat models.

Methods: The experimental post-test-only control group study used 24 Wistar rats randomly in 4 groups. Groups K(-), K(+), and P were fed a vitamin D and calcium-depleted AIN-93M diet for 14 days. Standard feed AIN-93M was received by normal groups (KN). Groups K- were deficient rats in vitamin D and calcium without intervention. The groups of K+ and P were given vitamin D_3 (180 IU) which was non-encapsulated and liprotide-encapsulated for 28 days. The SOD activity was quantified with Superoxide Dismutase (SOD) Activity Assay Kit, while MDA levels were determined using Thiobarbituric Acid Reactive Substance (TBARS) method. The statistical analysis used One-way ANOVA test with Least Significant Difference follow-up test.

Results: The MDA levels and SOD activity in the K+ and P groups had significant differences ($p<0.05$) against the control group. Liprotides-encapsulated vitamin D_3 significantly reduced MDA levels and enhanced SOD activity compared to non-encapsulated in rats with a deficiency in vitamin D and calcium.

Conclusion: Liprotide-encapsulated vitamin D_3 has the potential to increase SOD activity and decrease MDA levels.

Keywords: *Vitamin D_3; nanoencapsulation; liprotide; MDA; SOD*
Permalink/ DOI: https://doi.org/10.14710/jbtr.v9i1.16289

INTRODUCTION

More than half of people of all ages are affected by vitamin D deficiency in the world.[1] Vitamin D deficiency can occur not only in Western countries but also in tropical countries.[2] Vitamin D deficiency is prevalent in both children and adults. In the United States, 50% of children ages 1–5 and 70% of children ages 6–11 had vitamin D levels < 30 ng/mL.[3] In Jakarta and Kuala Lumpur, 60% of 504 women between the ages of 18 and 40 were vitamin D deficient.[4] The cross-sectional study in North Sumatra, Indonesia, found that 95% of the 156 subject women were vitamin D deficient or inadequate.[5]

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Vitamin D deficiency is linked to several clinical disorders and mortality rates.[6] A lot of chronic conditions, including diabetes mellitus, cardiovascular disease, kidney disease, and autoimmune disease, are associated with the rising incidence of vitamin D deficiency.[7,8] According to several studies, deficiency of vitamin D contributes to oxidative stress and inflammation.[9,10,11] Several factors have been associated with vitamin D deficiency such as inadequate sun exposure, medications, skin pigmentation and low vitamin D consumption.[3]

Vitamin D plays a key role in absorption and homeostasis calcium .[12] Optimal vitamin D levels are necessary to increase the efficiency of calcium absorption.[13] Without adequate vitamin D, the body absorbs no more than 10% to 15% of dietary calcium.[13] Calcium deficiency can cause metabolic or potential pathological changes.[14] There are close connections between oxidative stress and calcium homeostasis.[15] In mitochondria, calcium plays an important role in the formation of ATP and reactive oxygen species (ROS).[16] At the cellular level, a deficiency of vitamin D increases oxidative stress, inflammation indicators, and mitochondrial damage.[17] An imbalance between the production of ROS and antioxidant activities results in oxidative stress.[18] Numerous signaling pathways are activated by oxidative stress, which ultimately increased lipid and protein peroxidation-induced tissue damage.[19,20] High levels of malondialdehyde (MDA) and low levels of superoxide dismutase (SOD) activity are indicators of oxidative stress. The high level of MDA is an indicator of lipid peroxidation initiated by ~~reactive oxygen species (~~ROS~~)~~.[21] Natural defense mechanisms in the body include glutathione enzymes and superoxide dismutase (SOD).[22] The SOD enzyme effectively converts superoxide anions into dioxygen and hydrogen peroxide in physiological conditions.[23] According to biochemical studies, the highest SOD activity was presented in liver and kidney tissue.[22]

Vitamin D supplementation in food is an appealing alternative to elevate the serum level of vitamin D in the population.[24,25] Oxidation, high temperatures, and an acidic environment can easily harm vitamin D.[26,27] Nanoencapsulation is a method for preserving a coated substance by enclosing it in a matrix in its liquid, solid, or gaseous states.[28] Cores are the materials that have been encased, while shells are the materials that have been coated. The substance should be biocompatible and edible for food purposes.[29] Previous research indicates that nanostructured lipid carriers (NLCs) can be controlled to release vitamin D_3 safely and to prevent degradation of vitamin D_3 in simulated stomach fluids, while also releasing more than 90% in simulated intestinal fluids.[30]

Liprotide is a form complex made up of partially denatured protein and lipids that can protect bioactive compounds like vitamin D. To protect the bioactive compound, the protein forms a shell around a fatty acid core.[31] One of the materials used to encapsulate vitamin D_3 is protein-lactoglobulin.[32] Oleic acid and β-lactoglobulin were used to make liprotide in this study, which can increase the bioavailability and protection against acid, heat, and UV light.[31,32] This study was aimed to evaluate vitamin D_3-encapsulated by liprotides on MDA level and SOD activity in the liver of rat models with vitamin D and calcium deficient diets.

MATERIALS AND METHODS

Ethical Statement

This study was approved with ethical approval from the Ethical Commission of Health Research, Medical Faculty, Sultan Agung Islamic University (Ethical clearance number: 29/I/2022/Bioethics Commission).

Chemical and Reagent

Cholecalciferol Vitamin D (≥98%), β-lactoglobulin (≥90% pure), and oleic acid (≥99% pure) were purchased from Sigma Aldrich (Germany). Support materials other such as potassium hydroxide (KOH, 1310-58-3), absolute ethanol (1.00983.2500), concentrate liquid PBS OmniPur® (6506-1LCN), ultra centrifugal filter unit Amicon® (UFC901008), and MiliQ water.

Preparation of Liprotides

The oleic acid (38 mg/mL) was dissolved in 99% ethanol. Six (6) mg/ml β-lactoglobulin, mixed with 1.5 mg/ml oleic acid in 10 mM KOH (pH 10.5) and incubated at 45° C for 30 minutes.[31] Then, the sample mixture was cooled and the pH was adjusted to 7.4 using a PBS solution. Samples were vortexed and centrifuged at 4,000 rpm for 8 minutes.[31] In this study, the liprotides form a clear solution.

Vitamin D Encapsulation

Vitamin D_3 was dissolved in 96% ethanol to a concentration of 115 mM. Then vitamin D_3 was mixed with liprotides that have been formed with a final concentration of 4 mg/ml. The sample was homogenized with a vortex then centrifuged using an ultra centrifugal filter unit for 8 minutes, 30° C, and 4000 rpm.[31] Vitamin D concentration, efficiency, and successful encapsulation were seen using HPLC (Shimadzu corp LC20AD® Serial Number : L20105130725, Kyoto Japan) and SEM (Jeol JSM-6510LA, Japan®). The research was conducted at the Diponegoro University Integrated Laboratory.

Experimental Animals

This was a true experimental study with a post-test-only control group design. Twenty-four male Wistar rats (8-12 weeks) weighing between 150 and 200 grams were acclimatized for 7 days to room temperatures 20-25^0 C, humidity between 60-70%, and 12 hours of the normal cycle with food and water ad libitum. The study was conducted in the Experimental Animal Laboratory of the Center for Food and Nutrition at Gajah Mada University between January and March of 2022. All of the rats were separated into 4 groups: normal group (KN), deficient vitamin D and calcium group with no intervention (K-), non-encapsulated vitamin D_3 (K+), and liprotide-encapsulated vitamin D_3 (P). The normal/healthy groups (KN) only received standard food AIN-93M.[33] For 14 days, rats in groups (K-), (K+), and P were fed modified AIN-93M (vitamin D and calcium depleted) to produce vitamin D and calcium deficient rats. Rats received a normal AIN-93M diet after the vitamin D and calcium depletion phase. Group (K-) was presented as deficient rats without any treatment. The intervention groups were

given liprotide-encapsulated vitamin D_3 (P) and non-encapsulated vitamin D_3 (K+) for 28 days. The doses of vitamin D_3 that were used in previous studies were 180 IU/200g for rats.[34]

Biomarkers Analysis

The rats received 100 mg/kg intramuscular ketamine after 28 days intervention period. The MDA concentration in liver tissue was tested using the TBARS method. Liver tissue with a weight of 1 gram was put into a cold mortar. Then 500 µl 0.9% NaCl was added and homogenized. The homogenate was taken and transferred to an Eppendorf tube. The homogenate was centrifuged at 8000 rpm for 20 minutes and retrieved the supernatant. Then 100 µL supernatants were put into a test tube, add 550 µl NaCl, 100 µl TCA, 100 µL HCL 1 N,100 µL Na-Thio 1 % and homogenized again. After that, the supernatants were centrifuged at 1500 rpm for 10 minutes and heated in a 100^0C water bath for 30 minutes. The sample absorbance was measured with a spectrophotometer at a length maximum wavelength of 532 nm.

The Superoxide Dismutase (SOD) Activity Assay Kit from Biovision Inc. was used to quantitatively measure SOD activity in the supernatant of liver tissue samples. A Microplate reader was used to determine the absorbance of the supernatant at a wavelength of 450 nm.

Statistical Analysis

Statistical analysis was performed with SPSS 20 (IBM/SPSS Inc). The Shapiro-Wilk normality test was used to analyze the data research. The data post-test intervention was normally distributed and has the same data variance thus One-way ANOVA test was used to determine the difference between MDA levels and SOD activity. The post hoc LSD test was used to find out in which group there is a significant difference. The result was displayed as a mean ± SD and $p< 0.05$ was considered to be significant.

RESULTS

Effect of Vitamin D_3 Encapsulated by Liprotides on MDA Levels

Table 1 shows lower levels of MDA in the K+ and P groups than K- group after the interventions. There was a significant difference in the mean MDA levels in rat livers after the intervention between all groups using the one-way ANOVA test ($p<0.001$). MDA levels in K+ (3.19±0.55 nmol/g) and P (2.57±0.46 nmol/g) groups are lower than group K- (9.79±0.28 nmol/g). MDA levels in group P were lower than K+ group. While both MDA levels were still higher than the normal control group KN (1.19±0.22 nmol/g). The LSD test results showed that all groups had significantly different MDA levels in liver tissue ($p<0.05$).

Effect of Vitamin D_3 Encapsulated by Liprotides on SOD Activity

The result of the mean in SOD activity is shown in **Table 1** which shows higher SOD activity in the K+ and P groups than K- group after 28 days of intervention. The one-way ANOVA test showed significant differences in SOD activity after intervention in all groups ($p<0.001$). SOD activity in the P group (68.03±4.72 %) is higher than in the K+ group (61.47±4.48 %). While, both SOD activities were still lower than the normal control group KN (79.51±3.06 %). The LSD test showed that all groups had significantly different SOD activity in liver tissue ($p<0.05$).

DISCUSSION

The results of this study showed that liprotide-encapsulated vitamin D_3 could lower MDA levels and increase SOD activity in vitamin D and calcium-deficient rats' livers. In addition, the use of liprotides encapsulation affects the MDA levels and SOD activity. Liprotide-encapsulated vitamin D_3 improved MDA and SOD in rat liver tissue better than non-encapsulated. Liprotides-encapsulated vitamin D_3 has previously been shown to increase stability, bioavailability, and resistance to pH, temperature, and UV light.[31,32]

Deficiency of vitamin D and calcium in rat models by given feeding modified AIN-93M for 14 days (vitamin D and calcium depleted). In this study, there was an increase in MDA levels and decreased SOD activity in deficient vitamin D and calcium diet groups. Nutritional deficiencies such as vitamins and minerals can reduce metabolic status which can increase reactive oxygen species (ROS).[35] Dysregulation of vitamin D and calcium causes decreased mitochondrial respiration which induces reactive oxygen species and contributes to the accumulation of cellular oxidative damage.[16,36,37]

Vitamin D is known to play an essential role in maintaining calcium.[38] Calcium is essential for controlling mitochondrial respiration because it is the main production of cellular ATP and ROS.[16] The deficiency of calcium can cause lower superoxide dismutase (SOD).[39] In this study, adequate vitamin D intake can increase calcium absorption in the intestine.[40] Adequate calcium uptake can maintain the antioxidative capacity (superoxide dismutase, glutathione peroxidase, glutatione) to avoid excessive ROS production.[16]

Oxidative stress due to increased ROS can cause the peroxidation of proteins, nucleic acids, and lipids.[37,41] Lipid peroxidation products discovered in the liver, brain, kidney, lung, and skeletal muscle such as MDA, 4-HNE, and F-2 isoprostanes.[37] Malondialdehyde is a

Table 1. The mean data MDA levels and SOD activity in all groups

Variables	Treatment Groups (n=6)				*p*
	KN	K-	K+	P	
MDA (nmol/g)	1.19±0.22[a]	9.79±0.28[b]	3.19±0.55[c]	2.57±0.46[d]	<0.000*
SOD (%)	79.51±3.06[a]	32.24±3.53[b]	61.47±4.48[c]	68.03±4.72[d]	<0.000*

**OneWay ANOVA test* $p<0.001$

[a,b,c,d] *Post Hoc LSD Test, significantly different $p<0.05$*

peroxidation product of polyunsaturated fatty acids, such as phospholipids and triglycerides.[42,43,44] In animal studies showed that deficiency of vitamin D and calcium caused increased MDA levels in liver tissue.[39,45] Increased ROS leads decrease endogenous antioxidants such as glutathione, superoxide dismutase, catalase, and peroxiredoxin. [42,46] Previous studies demonstrated that deficiency of vitamin D decreased the activity of glutathione peroxidase, catalase, and superoxide dismutase in the liver.[45]

The administration of non-encapsulated and liprotides-encapsulated vitamin D_3 increased SOD activity and decreased MDA levels in liver rats. Vitamin D can enhance natural antioxidant defenses. Numerous studies have shown that vitamin D_3 increases SOD activity in the serum and liver tissue of experimental animals.[45,47,48] The administration of vitamin D will maintain calcium homeostasis in the body.[49] Calcium controlled redox regulation and oxidative stress in mitochondrial.[16] Vitamin D was activated enzyme glucose-6-phosphate-dehydrogenase produces glutathione which reduces nitrogen oxides and SOD that is responsible for transforming O_2^- into H_2O_2.[44,50,51] The other research shows that supplementing with vitamin D_3 can lower MDA concentrations in liver tissue.[45,48,52] However, several studies have shown that vitamin D supplementation does not significantly lower MDA levels.[53,39]

Active vitamin D bound to VDR (vitamin D receptors) can alter the activity of multiple signaling pathways both non-genomically and genomically.[54] Vitamin D_3 administration has been shown in some studies to increase the production of the transcription factor Nrf2 which can increase antioxidant capacity through binding to the antioxidant response elements (ARE).[50,55] Vitamin D_3 markedly increased protein expression of Nrf2. It means that Vitamin D_3 can activate the expression of Nrf2 and in turn promote the synthesis of antioxidant-associated proteins. Consequently, the damage caused by oxidative stress to the liver can be attenuated.[47] This is supported by the results of other studies which state that crosstalk occurs between the NF-κB- and Nrf2 pathways.[56] NF-κB (p65) can interact with Keap1 to inhibit activation of the Nrf2-ARE pathway. Meanwhile, the activation level of NF-κB phosphorylation (p65) is positively associated with the secretion of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α and negatively correlated with the secretion of the anti-inflammatory cytokine IL-10.[47,56]

In this study, groups of liprotides-encapsulated vitamin D_3 improved MDA levels and SOD activity better than non-encapsulated groups. In line with other studies, the administered vitamin D encapsulated had significantly higher levels of vitamin D in their blood than non-encapsulated recipients.[29,32,57] This is associated with the provision of encapsulation can increase the bioavailability of vitamin D in the blood.[31] Vitamin D_3 may be bound in a hydrophobic region that shields it from oxidative damage, high temperatures, and UV rays, which improves its stability and bioavailability.[31]

Liprotides have good interaction with the membranes. In the vitamin D_3 transfer assay to the phospholipid membrane, it was shown that >80% of the hydrophobic liprotide components (vitamin D_3 and oleic acid) transferred well to the vesicles.[31] The β-lactoglobulin protein is an effective carrier encapsulant for vitamin D because of the van der waals and hydrogen bond involved in the binding of fatty acid to these proteins.[58] Other studies have shown that vitamin D_3 cress seed mucilage-β-lactoglobulin nanocomplexes can affect the speed of content delivery which in vitro tests using SGF (simulated gastric fluid) and SIF (simulated intestinal fluid) showed that the release in the stomach was small and would increase in the intestine.[59] The limitations of this study show that the results have been able to reduce MDA levels and increase SOD activity, but it has not been able to reach normal conditions.

CONCLUSION

The administration of both non-encapsulated and liprotide-encapsulated vitamin D_3 can improve MDA levels and SOD activity. In this study, it can be concluded that the administration of liprotide-encapsulated vitamin D_3 can reduce MDA levels and increase SOD activity in the liver better than non-encapsulated. Studies of longer duration may be needed to determine how long the MDA levels and SOD activity to return to normal.

ACKNOWLEDGMENTS

This research was part of “The Study Bioavailability of Vitamin D_3 Encapsulated Liprotides in Rats on a Vitamin D and Calcium Deficiency Diet.” It was supported by Indonesian Ministry of Education, Culture, Research, and Technology with Grant Number: No.187-13/UN7.6.1/PP/2022.

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