

Ascorbic Acid vs Calcitriol in Influencing Monocyte Chemoattractant Protein-1, Nitric Oxide, Superoxide Dismutase, as Markers of Endothelial Dysfunction: In Vivo Study in Atherosclerosis Rat Model

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Introduction: Ascorbic acid and calcitriol were frequently utilized in conjunction as therapy during the COVID-19 pandemic, and individuals with minor symptoms had notable improvements. There have been a few studies, often with conflicting findings, that examine the use of them for endothelium restoration and numerous clinical trial studies that failed to establish the efficacy. The aim of this study was to find the efficacy of ascorbic acid compared to calcitriol on the inflammatory markers monocyte chemoattractant protein-1 (MCP-1), nitric oxide (NO), and superoxide dismutase (SOD), as protective agents which play an important role in the early stages of atherosclerosis formation. This study was an experimental in vivo study.

Methods: The total of 24 male *Rattus norvegicus* strain Wistar rats were divided into 4 groups, namely: control/normal group (N), atherosclerosis group (DL) given atherogenic diet, atherosclerosis group given atherogenic diet and ascorbic acid (DLC), and atherosclerosis group given atherogenic diet and calcitriol (DLD) treatment for 30 days.

Results: Ascorbic acid and calcitriol treatment was significantly effective ($P < 0.05$) in lowering expression of MCP-1 and increasing NO and SOD level. Calcitriol was superior to ascorbic acid in increasing SOD ($P < 0.05$). There was no significant difference between ascorbic acid and calcitriol in decreasing MCP-1 and increasing NO ($P > 0.05$).

Discussion: Both treatments could reduce MCP-1, and increase NO and SOD by increasing antioxidants. In this study calcitriol was superior to ascorbic acid in increasing SOD, but not NO and decreasing MCP-1. According to the theory, it was found that calcitriol through nuclear factor erythroid 2-related factor 2 (Nrf2) causes a direct increase in the amount of SOD. Nrf2 is an emerging regulator of cellular resistance to oxidants.

Conclusion: Ascorbic acid and calcitriol treatment was able to reduce MCP-1 and increase NO and SOD in atherosclerosis rat. Calcitriol was significantly superior in increasing SOD levels compared to ascorbic acid.

Keywords: ascorbic acid, calcitriol, monocyte chemoattractant protein-1, nitric oxide, superoxide dismutase, atherosclerosis

Introduction

Despite the fact that COVID-19 has killed countless populations over the past two years, heart disease is still the leading cause of mortality worldwide. Much research conducted during the COVID-19 pandemic concentrated on vascular inflammation, which turned out to be the pathomechanism for COVID-19 and is similar to the pathogenesis of

atherosclerosis.¹ The potential for the use of various antioxidants as part of the management of atherosclerosis is increased by systemic inflammatory processes, oxidative stress, fat buildup, cell death, and fibrosis in arteries that cause the onset of many systemic disorders, including atherosclerosis.²

Ascorbic acid and calcitriol were frequently utilized in conjunction as therapy during the COVID-19 pandemic, and individuals with minor symptoms had notable improvements.² In theory, ascorbic acid and calcitriol could aid in the process of restoring endothelial function.^{3,4} Endothelial damage is the underlying cause of atherosclerosis.⁵

It has long been understood that ascorbic acid contributes to a number of crucial endothelium processes.⁵ These functions, according to earlier research, include boosting type IV collagen synthesis and deposition in the basement membrane, promoting endothelial proliferation, preventing apoptosis, scavenging free radicals, and encouraging endothelial cells to produce nitric oxide (NO), which regulates blood flow. Another theory describes how ascorbic acid could stop the oxidation of low-density lipoprotein (LDL).⁶

Through the bioactivity of endothelial NO synthase (eNOS), calcitriol promotes NO production.⁶ Although the mechanism is unclear, prior research suggests that vitamin D functions as an anti-inflammatory to impact TNF-cytokines as pro-inflammatory cytokines.⁷ High dosages of vitamin D were found to dramatically lower serum TNF levels in a study on inflammatory bowel disease. Calcitriol can control NO production by stimulating nitric oxide synthase 3 (NOS3) transcription through the binding of vitamin D receptors (VDR).⁸ But it was not clear enough.

There have been a few studies, often with conflicting findings, that examine the use of ascorbic acid and calcitriol for endothelium restoration and numerous clinical trial studies that failed to establish the efficacy. In light of the aforementioned and lack of data, the researcher is eager to compare the effects of ascorbic acid and calcitriol on rat endothelium dysfunction in an atherosclerosis model.

Method

The study was an experimental study. The study used 24 male white rats (*Rattus norvegicus*) aged 4 weeks, with body weights ranging from 50 to 100 g, divided into four treatment groups. Each treatment unit contained six white male rats. The treatment groups were: N (rats given standard diet or control), DL (atherogenic diet), DLC (atherogenic and ascorbic acid), and DLD (atherogenic and calcitriol). The treatment was carried out for 90 days and 30 days of intervention. After an acclimatization period for 2 weeks, 6 rats were given normal diet while 18 rats were chosen randomly and given atherogenic diet (containing 0.2% cholic acid, 2% egg yolk, 5% goat fat, and 92.8% corn rice) ad libitum for 8 weeks.⁹

The treatment phase was started in the ninth week.¹⁰ In this phase, the rats were fed according to the research plans as described above. Particularly in the treatment groups, the rats were given calcitriol dosage for rats (0.009 µg/day) equivalent to 0.25 µg calcitriol dosage recommended for humans, and ascorbic acid (9 mg/day) equivalent to 500 mg ascorbic acid dosage recommended for humans, given for 30 days. At the end of treatment phase, euthanasia was performed by researchers and laboratory staff using intraperitoneal ketamine (Ilium ketamil; Troy Laboratories, Australia) and xylazine (Xyla; Interchemie, Netherlands). Blood was taken from the heart, then placed in venoject. Blood plasma samples were centrifuged using microcentrifuge (MC-12; Benchmark, Sayreville, New Jersey, United States) at 3000 rpm for 10 minutes. It was stored immediately at -80°C. Plasma would be used for examination of NO, MCP-1, and SOD concentration.

NO, MCP-1, and SOD Concentration Measurement

Measurement of NO, MCP-1, and SOD concentration in blood plasma samples of rat was conducted by enzyme-linked immunosorbent assay (ELISA) method. NO concentration was measured with Rat NO ELISA kit (Cat. No. E-BC-K035-M; Elabsience, Houston, Texas). MCP-1 concentration was measured with Rat MCP-1 ELISA kit (Cat. No. E-EL-R0633; Elabsience, Houston, Texas), and SOD concentration was measured with Rat T-SOD ELISA kit (Cat. No. E-BC-K019-M; Elabsience, Houston, Texas).

ELISA for MCP-1 was begun with standard and sample of 100 µL inserted into the well. The sample (except blank well) was incubated at 37°C for 1 hour. Then 90 µL substrate reagent was added and incubated for 15 minutes at 37°C (away from light). The reaction was stopped by adding 50 µL stop solution. After 5 minutes, the sample was read using ELISA reader (xMark™ Microplate Absorbance Spectrophotometer; Bio-Rad, Hercules, California, USA) at 450 nm.

ELISA for NO was begun with standard and sample of 100–300 μL . Add 200 μL of reagent 1, and mix fully with a vortex mixer. Add 100 μL of reagent 2, and mix fully with a vortex mixer. Stand for 15 min at room temperature, centrifuge at 3100g for 10 min. Take 160 μL of supernatant to the corresponding wells of microplate for chromogenic reaction. Add 80 μL of chromogenic reagent to each well, oscillate for 2 min, and stand at room temperature for 15 min. The sample was read using ELISA reader (xMarkTM Microplate Absorbance Spectrophotometer; Bio-Rad, Hercules, California, USA) at 550 nm.

ELISA for SOD was begun with sample of 90 μL inserted into the well of non-enzyme working solution, control enzyme solution, and sample enzyme solution. Shake for 10 s with microplate reader, and cover the plate with sealer. Incubate for 50 min at 37°C. Add 180 μL of chromogenic agent. Shake for 10 s, and stand for 10 min in room temperature. The sample was read using ELISA reader (xMarkTM Microplate Absorbance Spectrophotometer; Bio-Rad, Hercules, California, USA) at 550 nm.

Ethic Statement

The research was conducted at the Animal Laboratory, Faculty of Veterinary Medicine, Universitas Syiah Kuala, following adapted guidelines for the care and use of laboratory animals by Institut Teknologi Bandung and National Research Council of The National Academies. The implementation of this research has been approved by Veterinary Ethics Committee, Faculty of Veterinary Medicine, Universitas Syiah Kuala, reference 160/KEPH/VIII/2022.

Statistical Analysis

The normality of data distribution and equal variances in all groups was tested using the Shapiro–Wilk test (P value >0.05), and one-way ANOVA test was used to determine the effect of ascorbic acid and calcitriol administration on the concentration of MCP-1, NO, and SOD. Furthermore, analysis was performed using post hoc test to understand the differences of each parameter in each group. Independent T -test was used to compare ascorbic acid and calcitriol. This statistical test was conducted using SPSS 20 (IBM Cooperation, New York, NY).

Results

All of the rats survived till euthanasia. The Shapiro–Wilk test showed normally distributed data (P value >0.05).

Effect of Ascorbic Acid in MCP-I, NO, and SOD Levels

Table 1 shows that ascorbic acid could reduce MCP-1 level ($P=0.006$), increase NO level ($P=0.01$), and increase SOD level ($P=0.000$). As shown in Table 2, the average decrease in MCP-1 levels in the DLC group was 128 ng/mL ($P=0.005$). The average increase in NO levels in the DLC group was 33.2 $\mu\text{mol/L}$ ($P=0.01$). The average increase in SOD levels in the DLC group was 72 U/mL ($P=0.000$).

Effect of Calcitriol in MCP-I, NO, and SOD Levels

As shown in Table 1, calcitriol could reduce MCP-1 level ($P=0.007$), increase NO level ($P=0.02$), and increase SOD level ($P=0.000$). Table 2 shows that the average decrease in MCP-1 levels in the DLD group was 117 ng/mL ($P=0.006$). The average increase in NO levels in the DLD group was 46.1 $\mu\text{mol/L}$ ($P=0.02$). The average increase in SOD levels in the DLD group was 96 U/mL ($P=0.000$).

Comparison of Ascorbic Acid Vs Calcitriol in Decreasing MCP-I, and Increasing NO and SOD

Table 3 shows that there is no significant difference between ascorbic acid and calcitriol in decreasing MCP-1. There was no significant difference between ascorbic acid and calcitriol in increasing NO. It showed calcitriol was superior to ascorbic acid in increasing SOD.

Table 1 Effect on MCP-I, NO, and SOD Levels in All Group

Serum	Group	n	Mean (SD)	P value
MCP	N	6	346 (52.8)	0.006
	DL	6	413 (77.1)	
	DLC	6	284 (38.5)	
	N	6	346 (52.8)	0.007
	DL	6	413 (77.1)	
	DLD	6	295 (13.6)	
NO	N	6	55.7 (16.3)	0.001
	DL	6	37.6 (10.2)	
	DLC	6	70.9 (9.1)	
	N	6	55.7 (16.3)	0.02
	DL	6	37.6 (10.2)	
	DLD	6	83.7 (41.1)	
SOD	N	6	432 (9.7)	0.000
	DL	6	195 (9.4)	
	DLC	6	91.6 (10.1)	
	N	6	43.2 (9.7)	0.000
	DL	6	19.5 (9.4)	
	DLD	6	115.6 (5.2)	

Table 2 Post Hoc Analysis of MCP-I, NO, and SOD Levels in DLC and DLD

Serum	Group	Mean Difference	CI 95%		P value
			Max	Min	
MCP	DL vs DLC	128	37	218	0.005
	DL vs DLD	117	32	202	0.006
NO	DL vs DLC	33.2	14.1	52.4	0.001
	DL vs DLD	46.1	5.3	86.8	0.02
SOD	DL vs DLC	72.0	56.8	87.2	0.000
	DL vs DLD	96.0	82.9	109.1	0.000

Table 3 Comparison of Ascorbic Acid vs Calcitriol in Decreasing MCP-I, and Increasing NO and SOD

Serum	Group	Mean (SD)	P value
MCP-I	DLC	284 (38)	0.544
	DLD	295 (13)	
NO	DLC	70.9 (9.1)	0.472
	DLD	83.7 (41.1)	
SOD	DLC	91.6 (10.1)	0.000
	DLD	115.6 (5.2)	

Discussion

In DLC group there was decreasing of MCP-1. This result is the same as research in 2022 that found that there is a relationship with ascorbic acid in the decrease in MCP-1. Ascorbic acid not only contains benefits as antioxidant but as an anti-inflammatory suppresses MCP-1 through inflammatory mechanism.¹¹

The DLC group had an increase in SOD. This is in accordance with previous studies that state that ascorbic acid works as an antioxidant that could increase SOD.¹² The DLC group had an increase in NO. This is in accordance with previous studies which stated that ascorbic acid has a direct effect on increasing NO and prevents NO from oxidizing, which will eventually produce free radicals.¹³

The DLD group had a decrease in MCP-1. This is in accordance with previous studies conducted that found a decrease in MCP-1 through vitamin D receptor (VDR) so that the inflammatory process could be inhibited.¹⁴ The DLD group had an increase in SOD. This is in accordance with previous studies that proved that there is a significant increase in SOD in mice with renal impairment.¹⁴

The DLD group had an increase in NO. Calcitriol could increase the production of eNOS, which is an enzyme that increases the formation of NO. This is in line with a study conducted before in rats with arterial stiffness.¹⁵ In this study, they found a significant increase in NO. The other research observed that nitric oxide (NO) production in ovariectomized rat aortic endothelial cells was restored following a 12-h treatment with calcitriol.¹⁶ Another research showed that calcitriol induced a very rapid increase in endothelial NO production via interaction with VDR.³

In this study calcitriol was superior to ascorbic acid in increasing SOD, but not NO and decreasing MCP-1. There has been no prior research comparing the two. According to the theory, it was found that calcitriol through nuclear factor erythroid 2-related factor 2 (Nrf2) causes a direct increase in the amount of SOD. Nrf2 is an emerging regulator of cellular resistance to oxidants.¹⁷ Nrf2 regulates the basal and induced expression of an array of antioxidant response element dependent genes to regulate the physiological and pathophysiological outcomes of oxidant exposure.¹⁸

Conclusion

This is a novel finding in atherosclerosis rat. The administration of ascorbic acid and calcitriol was able to reduce MCP-1 and increase NO and SOD in atherosclerosis rat. Calcitriol was superior in increasing SOD levels compared to ascorbic acid.

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Disclosure

The authors declare no conflicts of interest in this work.

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