ORIGINAL ARTICLE

Combination of vitamin A and D supplementation for ischemic stroke: effects on interleukin-1ß and clinical outcome

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ABSTRACT

Aim Accumulated evidence suggests that vitamin A and D agonists can alleviate the development of atherosclerosis. Therefore, the aim of this study was to determine the effect of vitamin A and D combination supplement on interleukin-1β (IL-1β) and clinical outcome in ischemic stroke.

Methods A single-blind, randomized controlled trial was conducted on ischemic stroke patients at Adam Malik Hospital between March 2018 to February 2019. The patients were randomized into 4 groups of the treatment consisting of supplementation using vitamin A or D only, combination of vitamin A and D, and placebo group, all given for 12 weeks. Clinical outcome was determined using the National Institute of Health Stroke Scale (NIHSS). At the time of admission and after the treatment was completed, all patients were measured for vitamin A, vitamin D, and IL-1β serum level, and NIHSS score.

Results From the total of 120 patients, in the combination group there were significant increments on both vitamin A ($p=0.04$) and vitamin D ($p=0.01$) serum level after 12 weeks of the treatment, compared to the other groups. In conjunction, IL-1β serum level showed a significant decrement in the combination group ($p<0.001$). Lastly, the biggest improvement of NIHSS could be seen in the combination group, which was marked by the highest decrement of NIHSS score ($p<0.001$).

Conclusion Administration of combination of vitamin A and D supplementation can significantly increase vitamin A and D serum level, decrease IL-1β serum level, and ultimately improve clinical outcome in ischemic stroke patients.

Key words: cerebrovascular disorders, cytokines, nutrients, stroke/drug therapy

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INTRODUCTION

Stroke is one of the vascular diseases of the brain which is categorized as the third leading cause of death after heart disease and malignancy, and is the number one cause of long-term disability in the world (1,2). Mortality and morbidity of cerebrovascular disease are a large socio-economic burden, and also constitute a very large burden for global health services (3). Ischemic or hypoxic injury often causes irreversible brain damage and is a leading cause of disability and death throughout the world (4). Much effort has been made to find pharmacological therapies to reduce brain injury due to cerebral ischemia (5). In recent years, vitamins, minerals and other essential nutrients show a great potential as primary therapies and attract much attention from researchers. This therapy, known as Nutraceutical therapy, can be an excellent choice for the treatment of patients with brain injuries (6,7).

The process of atherosclerosis is characterized by arterial remodelling that causes the accumulation of progressive subendothelic plaques through a series of complex cellular processes that occur in the walls of the arteries with inflammation playing an important role in its various phases (8). In the past decade, a number of studies have examined the relationship between serum vitamin D concentrations and the risk of cerebrovascular event. The results show that hypovitaminosis D can cause atheroma and vitamin D supplementation can prevent the risk of vascular disease (3,9,10).

Laboratory data also show a potential association of vitamin D deficiency as a risk factor for stroke, through systemic and vascular inflammatory stimulation that causes atherogenesis both directly and indirectly (3,11). Studies in experimental animals given a low vitamin D diet for 8 weeks, compared with the control group showed that infarct volume and neurological disorders in the low vitamin D diet group were significantly greater than those in the control group. This occurs due to the up-regulation of ischemia-induced interleukin-6 (IL-6) on a low vitamin D diet (3,11,12).

Vitamin D has roles in a variety of biological actions and most of these actions of vitamin D are considered to be exerted through the nuclear vitamin D receptor (VDR)-mediated control of target genes. Vitamin D Receptor (VDR), a member of the nuclear receptor superfamily, mediates the action of the biological form of vitamin D ($1.25[OH]_2D_3$). This superfamily comprises more than 60 nuclear receptors for lipophilic ligands such as steroid/thyroid hormones, vitamin A and vitamin D (13). The VDR activation by $1.25(OH)_2D_3$ or analogues has been reported to show a protective effect of atherosclerosis in several animal experiments. After activation, VDR binds specifically to the Vitamin D Response Element (VDRE) as a heterodimer with the X Retinoid Receptor (RXR) and then modulates the expression of the target gene (3,14). The retinoid X receptor (RXR) is a type of nuclear receptor that is activated by 9-cis-retinoic acid, a form of vitamin A (retinol) and is a first-generation retinoid (15-18).

Recent evidence suggests that vitamin A (retinol) is also a risk factor for cardiovascular disease and mortality (19). Plasma retinol levels are inversely proportional to the risk of cardiovascular disease mortality (19). Research has shown that patients with coronary heart disease or atherosclerosis show significantly lower levels of retinol than controls (19,20). There is increasing evidence that the retinoic acid (RA) signalling pathway provides an important mechanism for regulation of the blood brain barrier (BBB) in the neurovascular system. The protective effect of RA on the BBB further reduces cerebral damage in ischemic stroke (21). The development of future clinical experiments can identify RA as a potential target for the treatment of neurovascular disease (21,22).

Retinol and its derivatives carry out their biological actions via specific nuclear receptors that regulate gene transcription. The RA receptors can also interact with other nuclear receptors that have neuroprotective effects such as vitamin D_3, which is neuroprotective against stroke (5,23,24).

Overall, accumulated evidence suggests that VDR and RXR agonists can alleviate the development of atherosclerosis through inhibition of oxidative and inflammatory stress (24,25). Nevertheless, the role of combined vitamin A and D as beneficial nutrients in health as well as disease such as ischemic stroke is still a controversial topic. In addition, there have been no studies that have examined the effect of combined vitamin A and D supplementation on ischemic stroke patients.

The aim of this study was to determine the effect of the combination of vitamin A and D supplementation on changes in IL-1β and also clinical outco-

me using the National Institutes of Health Stroke Scale (NIHSS) in ischemic stroke patients.

MATERIALS AND METHODS

Patients and study design

This was a prospective cohort, single-blind, placebo-controlled, pre and post-test study. The patients were selected from the Stroke Unit of the Neurology Department, Adam Malik General Hospital in Medan - Indonesia, in the period March 2018 to February 2019. The study was conducted in accordance with the Declaration of Helsinki (1964), and the protocol was approved by the Health Research Ethical Committee, Medical Faculty, Universitas Sumatera Utara. Informed consents were taken from the patients prior to participation to ensure complete satisfaction.

The inclusion criteria were the patients with acute ischemic stroke diagnosed clinically and evidenced by neuroimaging examination, admitted to the Hospital on day ≤3 after onset and were >18 years of age. The exclusion criteria were the patients with impaired liver function (26), impaired renal function (serum urea concentration > 2 mg/dL, nephrolithiasis) (27), patients taking supplements containing vitamin A and D at the time of the study (27,28), hypercalcemia, cardioembolic stroke, and patients with sepsis and diabetes mellitus.

As the ischemic stroke patients got admitted in the Neurology Department, every consecutive patient was assessed for inclusion and exclusion criteria and was randomized by a simple randomization method, where random numbers were computer generated using Microsoft Excel Spreadsheet Software.

One hundred and twenty three ischemic stroke patients were assessed for the study, 120 were found to be eligible and were randomized, and assigned to four groups: supplementation using vitamin A only, using vitamin D only, combination of vitamin A and D, and also placebo group of 30 patients in each group.

Methods

The patients in the vitamin A group were given vitamin A 50,000 IU per week (5,000 IU vitamin A per tablet, grinded and inserted into a capsule to make 50,000 IU dose), while the vitamin D group patients were given vitamin D_3 50,000 IU per week, using vitamin D_3 soft capsule (50,000 IU vitamin D_3 per capsule). The combination group was given 50,000 IU vitamin A combined with 50,000 IU vitamin D, also given once a week, and the placebo group was given capsules containing saccharose lactis as the placebo. Both the supplement and placebo were given in capsules of the same size, colour, and weight to ensure that patients were blinded toward the treatments. All of the treatments (vitamin D supplementation and placebo) were given upon admission until 12 weeks. The standard medical treatment for ischemic stroke and physiotherapy were administered in both groups. No lost to follow-up was found until the end of this study on 3 months.

At the time of admission, all patients were measured for NIHSS score, vitamin D and vitamin A serum level, and IL-1β serum level. After 12 weeks, all of the measurements were repeated once again to evaluate the differences.

Initial stroke severity and clinical outcome were determined using the National Institute of Health Stroke Scale (NIHSS). The scale consists of five score sections: score 0 (no stroke symptoms), score 1–4 (minor stroke), score 5–15 (moderate stroke), score 16–20 (moderate to severe stroke), and score 21–42 (severe stroke) (29).

Serum vitamin D was evaluated using enzyme-linked immunosorbent assay (ELISA). Serum was extracted from venous blood, and quantitative measurement of total 25-OH vitamin D_3 in serum was performed using a monoclonal antibody that binds to 25-OH vitamin D_3 (30). Serum vitamin A was measured by high-pressure liquid chromatography (HPLC). Serum was extracted from venous blood and protected from light and chilled until centrifugation (31-32). Serum IL-1β was measured using the Quantikine® HS ELISA for Human IL-1β (R&D System, a Biotechne brand) according to the manufacturer's protocol (33).

Statistical analysis

To analyse baseline characteristics across the treatment groups, one-way ANOVA and $\chi 2$ tests were used. To determine the differences of serum vitamin A and D levels, Serum IL-1β, and NIHSS between before and after treatment paired t-test was used.

Table 1. Baseline characteristics of 120 patients with ischemic stroke

Parameter	Reference values	Treatment groups				p
		Vitamin A + D	Vitamin A	Vitamin D	Placebo	
Age (Year; Mean ± SD)		65.85 ± 4.23	66.85 ± 4.62	62.65 ± 5.66	66.35 ± 5.30	0.066
Gender (No)						0.122
Male		13	15	8	17	
Female		17	15	22	13	
Smoking (No)						0.100
Yes		11	14	8	13	
No		19	16	23	17	
Alcohol drinking (No)						
Yes		9	11	8	14	
No		21	19	22	16	0.150
NIHSS on admission (Mean ± SD)		13.25 ± 1.61	12.10 ± 1.92	13.20 ± 1.24	13.15 ± 1.39	0.067
Systolic blood pressure (Mean ± SD) (mmHg)	120 mmHg	152.05 ± 9.85	151.15 ± 9.72	157.85 ± 8.78	155.65 ± 9.48	0.097
Blood sugar level (Mean ± SD) (mg/dL)	70 – 130 mg/dL	121.10 ± 13.21	127.75 ± 10.76	123.10 ± 12.22	127.70 ± 19.27	0.352
Dyslipidaemia (No)						
Yes		16	15	13	14	0.848
No		14	15	17	16	
Vitamin A serum level (Mean ± SD) (µg/L)	200 – 800 µg/L	462.90 ± 246.78	476.85 ± 201.79	487.90 ± 107.18	473.80 ± 273.49	0.720
Vitamin D serum level (Mean ± SD) (ng/mL)	> 30 ng/mL	19.71 ± 7.15	20.42 ± 7.58	18.19 ± 6.90	20.69 ± 7.41	0.038
IL-1β serum level (Mean ± SD) (pg/mL)	< 0.70 pg / mL	0.49 ± 0.17	0.47 ± 0.42	0.30 ± 0.19	0.43 ± 0.39	0.170

NIHSS, National Institute of Health Stroke Scale;

RESULTS

From the total of 120 patients, the baseline data on admission showed no significant difference across the four treatment groups. The overall mean of vitamin D level of the patients on admission showed insufficiency level (20.75±7.53 ng/mL) (Table 1). The overall mean vitamin A level was normal (422.90 ± 214.45 **µg**/L). Initial mean of IL-1β serum level was above the normal value (0.75 ± 1.72 pg/mL).

In the vitamin A group and combination group, serum vitamin A level after the treatment was increased comparing to initial levels (p=0.21 and p=0.04, respectively), whereas in the vitamin D group and placebo groups a slight (no significant) decrease was found (p=0.92 and p=0.18, respectively) (Table 2).

Table 2. Changes in serum vitamin A level before and after treatment

Treatment group	**Vitamin A serum level** (Mean ± SD) (µg/L)		p
	Before	After	
Vitamin A	476.85 ± 201.78	498.55 ± 204.59	0.21
Vitamin D	487.90 ± 107.18	486.70 ± 126.55	0.92
Vitamin A+D	462.90 ± 246.78	511.95 ± 282.45	0.04
Placebo	473.80 ± 463.95	463.95 ± 266.34	0.18

In the vitamin D group and vitamin A+D supplementation group, serum vitamin D level was increased significantly (p=0.02 and p=0.01, respectively) after the treatment, whereas in the placebo group and vitamin A group, serum vitamin D decreased slightly (no significant) (p=0.08 and p=0.07, respectively) (Table 3).

Table 3. Changes in serum vitamin D levels before and after treatment

Treatment group	**Vitamin D serum level** (Mean ± SD) (µg/L)		p
	Before	After	
Vitamin A	20.42 ± 7.57	19.67 ± 8.02	0.08
Vitamin D	18.19 ± 6.91	20.37 ± 8.66	0.02
Vitamin A+D	19.71 ± 7.15	25.70 ± 10.39	0.01
Placebo	20.69 ± 7.41	20.62 ± 7.10	0.07

There was a significant difference between serum IL-1β level before and after the treatment, in the vitamin A+D group (p<0.001), and also in the placebo group (p<0.001) (Table 4).

Table 4. Changes in serum IL-1ß levels before and after treatment

Treatment group	**IL-1β serum level** (Mean ± SD) (pg/mL)		p
	Before	After	
Vitamin A	0.47 ± 0.42	0.49 ± 0.29	0.14
Vitamin D	0.30 ± 0.19	0.27 ± 0.17	0.19
Vitamin A+D	0.49 ± 0.17	0.21 ± 0.14	<0.001
Placebo	0.43 ± 0.39	0.79 ± 0.26	<0.001

Although the result of this study indicated a significant decrease in the NIHSS value after the treatment in all treatment groups (p<0.001), the biggest decrement was found in the combination group (Table 5).

Table 5. Changes in NIHSS (National Institute of Health Stroke Scale) before and after treatment

Treatment group	**NIHSS** (Mean ± SD)		p
	Before	After	
Vitamin A	12.10 ± 1.92	10.30 ± 1.59	< 0.001
Vitamin D	13.20 ± 1.24	10.40 ± 1.23	< 0.001
Vitamin A+D	13.25 ± 1.61	6.00 ± 1.52	< 0.001
Placebo	13.15 ± 1.39	11.75 ± 1.29	< 0.001

DISCUSSIONS

The results of the study found that the baseline characteristics of the study patients did not differ significantly between the treatment groups. This indicates that the initial conditions of the patients were quite homogeneous when the study begins. In all groups, the mean of serum vitamin D level at the beginning of the study were below normal values (insufficiency). This is consistent with research on vitamin D level (insufficiency) also conducted in Indonesia by Sari et al. on 2017 (34) and with the study of Sari et al. (2014), which was also carried out in the North Sumatra region (17.71 ng/mL). There is an influence of the type of work and also the source of food on the adequacy of vitamin D (35). This finding is consistent with research conducted in Indonesia (36).

In the vitamin A and combination A+D supplementation groups, serum vitamin A levels after supplementation showed an increase compared to other groups. In the combination A+D group, a significant increase in serum vitamin A level was seen. In the vitamin D and placebo groups, there was a slight decrease in serum vitamin A level, but it was not significant. This shows that, without adequate intake from outside, serum vitamin A level can become even lower. Likewise, supplementation containing vitamin A can increase serum vitamin A level. It turns out that a combination of vitamin A+D provides the highest increase among the other 3 groups. This supports the theory that the action of vitamins A and D work together, involving nuclear receptors (37-41).

After the treatment, in the vitamin D and vitamin A+D supplementation groups, serum vitamin D levels were found to increase significantly. Whereas in the vitamin A and placebo groups, serum vitamin D levels showed a slight decrease. This is consistent with research conducted in the North Sumatra region in 2017 (34). The similarity of these findings is possible because, in general, the population of the two studies was the same, and also the areas where they were carried out. Thus, it can be concluded that different types of supplementation will affect changes in serum vitamin D levels (34).

The increased initial IL-1β serum levels were probably due to the initial examinations carried out in the acute phase of stroke (<3 days after onset), where the inflammatory process in stroke was taking place, so that pro-inflammatory cytokine levels would also increase (42). This is consistent with the theory of increasing serum IL-1β, the pro-inflammatory cytokines; in a healthy brain, IL-1 is not detected, but will be expressed after an injury or during an illness (43). For example, in animal stroke models (induced by occlusion of cerebral artery media), a rapid increase in IL-1α, and IL-1β expression is observed in microglia within a few hours of damage and in the brain area, which experiences cell death (42,43).

Vitamin D is one of the nutraceuticals that has a major role in modulating the inflammatory process in ischemic stroke, through its anti-inflammatory and antioxidant properties, especially in modulation of interleukin. In this research, groups that received vitamin D supplementation (vitamin D only and vitamin A+D) showed a decrement in serum IL-1β levels, whereas the decrement was more significant in the combination group. This could be attributed to the fact that giving vitamin A alongside vitamin D will produce a synergism effect which can improve the efficacy of both vitamins **(**3,12,25, 43-45).

All of the findings of this study support the theory that vitamin A can increase the effectiveness of vitamin D. Optimal vitamin D activation requires vitamin A, so that the administration of vitamin A together with vitamin D as supplementation can maximize the expected anti-inflammatory and neuroprotective effects. The study results show that co-administration of VDR and RXR agonists synergistically can reduce atherosclerosis and correlate with endothelial protection through suppression of oxidative stress and inflammation. The combination of treatment with VDR and RXR agonists is a promising strategy for the prevention and treatment of atherosclerosis (25,46,47). When examined more deeply, among the 4 treatment groups, the highest decrease in NIHSS was seen in the combination of vitamin A and D supplementation; it seems to have the most inflammatory effect, characterized by an increase in serum vitamin D and A levels, as well as the most significant decrease in serum IL-1β level in this supplementation group compared with the other 3 groups.

This study showed that type's supplementation had a significant relationship with clinical outcomes (NIHSS) where the combination of vitamins A and D provided the most significant improve-

ment in clinical outcomes. Vitamins A and D can affect inflammation in a number of ways, such as antioxidant/anti-inflammatory mechanisms, inhibition of nitric oxide synthase, regulation of neuronal calcium, and detoxification pathways (48). Over time, it appears that vitamin D deficiency often occurs throughout the world and is associated with several chronic health problems, including cardiovascular, musculoskeletal, infection, autoimmune, and malignancy (4,11,39). *In vitro* and animal studies, vitamin D deficiency affects the activity/expression of macrophages and lymphocytes in atherosclerotic plaque, which causes chronic inflammation of the artery walls. Also, vitamin D modulates the immune system by regulating the production of inflammatory cytokines and inhibits pro-inflammatory cell proliferation, both of which are important in the pathogenesis of vascular inflammation that causes atherogenesis (3,4,49,50).

Vitamin A is a nutrient that influences the immune response through multiple mechanisms, such as helping the production of B cells from immunoglobulin A (IgA) by up-regulating IL-6 (51). Retinoic Acid (RA), a bioactive derivative of vitamin A and an important differentiation factor during vertebrate development, has been found to participate in neuron and vascular development. In animal models, it has been proven that RXR agonists inhibit the development of atherosclerosis (25,46,47,52). Retinoic Acid receptors can also interact with other nuclear receptors that have neuroprotective effects such as vitamin D_3, which is neuroprotective against stroke (5, 22–24).

Vitamins A and D reduce the production of T cells, increase the production of anti-inflammatory T cell mediators and promote regulation of T cell differentiation. Vitamin A also reduces IL-6 and IFN-γ production and limits the toxic effects of Reactive Oxygen Species (ROS) released during inflammation (53–55). Nevertheless, factors that influence stroke outcomes certainly do not only depend on changes in serum vitamin levels and changes in cytokines, but there are still other factors that also play a role in clinical outcomes of stroke, such as age and location of lesions (56,57).

Some limitations in this study were that, other factors that affect the metabolism of vitamins A and D such as daily food intake, were not investigated in more details. To ensure that the metabolism of vitamins A and D in the body goes well, an examination should be made of several things such as the content of food intake, intestinal absorption and genetic factors. However, the main purpose of this study was not to determine the nutritional status of vitamins in the subjects, but rather to see the effect of vitamin supplementation on clinical outcomes, through cytokine modulation. Furthermore, serum vitamin A and D levels after supplementation, can be used as an overall representation of the success of supplementation to improve vitamin levels in the body, and thus also affect changes in cytokine levels, and ultimately, clinical outcomes.

Ultimately, the findings of this study suggested that administration of combination of vitamin A and D supplementation can significantly improve clinical outcome in ischemic stroke when compared to patients without supplementation.

In conclusion, administration of the combination of vitamin A and D supplementation significantly increased vitamin A and D serum level, decreased IL-1β serum level, and ultimately improved clinical outcome in ischemic stroke patients.

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CONFLICTS OF INTEREST

Competing interests: None to declare.

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