Effect of Alfacalcidol on Inflammatory markers and T Cell Subsets in Elderly with Frailty Syndrome: a Double Blind Randomized Controlled Trial

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ABSTRAK

Latar belakang: *Alfakalsidol, suatu analog vitamin D, menunjukkan potensi regulasi imun saat bekerja pada makrofag dan sel T untuk mengontrol peradangan dan disregulasi sel T pada lansia. Saat ini belum diketahui efeknya pada orang tua dengan berbagai keadaan sindrom frailty yang memiliki peradangan kronis tingkat rendah yang berbeda. Penelitian ini bertujuan untuk mengetahui pengaruh alfakalsidol pada sitokin inflamasi (IL-6, IL-10, γ-IFN) dan subset sel T (CD4/CD8 rasio dan CD8 + CD28-) lansia dengan berbagai status sindrom frailty.* ***Metode:*** *selama Januari hingga Juli 2017, uji coba terkontrol acak buta ganda (RCT) dengan penyembunyian alokasi, melibatkan 110 subjek lansia dari Poliklinik Geriatri RS Cipto Mangunkusumo Jakarta, dilakukan untuk mengukur efek pemberian alfakalsidol 0,5 mcg selama 90 hari terhadap inflamasi. Pengukuran sitokin (IL-6, IL-10, γ-IFN) dari supernatan kultur PBMC, serta persentase CD4/CD8 dan CD8+ CD28- menggunakan flow cytometry dilakukan. Analisis statistik menggunakan SPSS versi 20 dilakukan dengan t-test untuk mengukur perbedaan rata-rata.* ***Hasil:*** *dari 110 subjek yang terlibat dalam RCT yang terdiri dari 27 orang sehat, 27 pra-lemah dan 56 orang lanjut usia lemah, 25 (OH) D serum adalah 25,59 (12,2) ng/ml dalam kelompok alfakalsidol dan 28,27 (10.4) ng / ml dalam kelompok plasebo. Alfakalsidol tidak menurunkan IL-6 (p=0,4) dan g-IFN (p=0,001), tetapi meningkatkan IL-10 (p=0,005) dan menurunkan rasio IL6/IL10 (p=0,008). Alfakalsidol meningkatkan rasio CD4/CD8 dari 2,68 (SD 2,45) menjadi 3,2 (SD 2,9); p=0,001 dan penurunan CD8+ CD28- persentase dari 5,1 (SD 3,96) menjadi 2,5 (1,5); p<0,001. Analisis sub kelompok menunjukkan pola yang sama di semua status frailty.* ***Kesimpulan:*** *alfakalsidol meningkatkan penuaan kekebalan dengan bertindak sebagai agen anti-inflamasi melalui peningkatan IL-10 dan penurunan rasio IL6/IL-10 dan juga meningkatkan imunitas seluler melalui peningkatan rasio CD4/CD8 dan penurunan CD8+ CD28-subset pada lansia. Efek ini tidak dipengaruhi oleh status frailty.*

Kata kunci: *Alfakalsidol, inflamasi, sel T, usia lanjut, frailty.*

ABSTRACT

Background: *Alphacalcidol, a vitamin D analog, shows immune regulatory potency as it works on the macrophage and T cell to control inflammation and T cell dysregulation in elderly. None has been known about its effect on elderly with various states of frailty syndrome, which have different level of chronic low grade inflammation.*

This study aimed to determine the effect of alphacalcidol on inflammatory cytokines (IL-6, IL-10, γ-IFN) and T cell subsets (CD4/CD8 ratio and CD8+ CD28-) of elderly with various stages of frailty syndrome. ***Methods:*** *from January to July 2017, a double blind randomized controlled trial (RCT) with allocation concealment, involving 110 elderly subjects from Geriatric Outpatient Clinic Cipto Mangunkusumo Hospital Jakarta, was conducted to measure the effect of 0.5 mcg alphacalcidol administration for 90 days to inflammatory cytokines (IL-6, IL-10, γ-IFN) from PBMC culture supernatant, as well as CD4/CD8 and CD8+CD28- percentage using flow cytometry. Statistical analysis using SPSS version 20 was performed with t-test to measure mean difference.* ***Results:*** *of 110 subjects involved in the RCT consisting of 27 fit, 27 pre-frail and 56 frail elderly, 25(OH)D serum level was found to be as low as 25.59 (12.2) ng/ml in alphacalcidol group and 28.27 (10.4) ng/ml in placebo group. Alphacalcidol did not decrease IL-6 (p=0.4) and γ- IFN (p=0.001), but it increased IL-10 (p=0,005) and decreased IL6/IL10 ratio (p=0.008). Alphacalcidol increased CD4/CD8 ratio from 2.68 (SD 2.45) to 3.2 (SD 2.9); p=0.001 and decreased CD8+ CD28- percentage from 5.1 (SD 3.96) to 2.5 (1.5); p<0.001. Sub group analysis showed similar patterns in all frailty states.* ***Conclusion:*** *Alphacalcidol improves immune senescence by acting as anti-inflammatory agent through increased IL-10 and decreased IL6/IL-10 ratio and also improves cellular immunity through increased CD4/CD8 ratio and decreased CD8+ CD28- subset in elderly. This effect is not influenced by frailty state.*

Keywords: *Alphacalcidol, inflammation, T cell, elderly, frailty.*

INTRODUCTION

Although the term of immune senescence has been widely accepted to describe immune dysregulation in elderly, not much has been known about immune dysregulation in frail elder people.[1-5] Frailty syndrome not only will lead to impairment of specific but also non specific immunity, which will be more severe than immunity dysregulation on healthy elderly.[6] Two substantial immune senescence concepts in patient with frailty syndrome are inflammaging and immune risk profile (IRP). Inflammaging corresponds to the increasing of various pro inflammatory cytokines and the low production of anti-inflammatory cytokines in respond to infection stimulus while specific immunity system dysregulation described by IRP corresponds to the lower CD4/CD8 ratio besides the decreasing amount of T CD8+CD28+ cells.[7]

The benefit of vitamin D in improving the immune system dysregulation on elderly is achieved through its mechanism of inhibiting the excessive production of pro inflammatory cytokine by monocyte and macrophage through MAP phosphokinase 1 (MAPK-1) pathway.[8] Moreover, vitamin D directly and indirectly works on T cells.[9] Systematic review assessing the effect of vitamin D on infectious disease shows conflicting result, but none of the trials involved frail elderly as subjects.[10] The objective of this study was to determine the benefit of alphacalcidol, a vitamin D analog on frail elderly immune system dysregulation. Alphacalcidol, a vitamin D analog was chosen in this study because besides having lower hypercalcemia risk, it does not need hydroxylation in kidney thus should be safer for elderly patients with kidney impairment, and was expected to be more potent on immune cells.

METHODS

A total of 134 elderly subjects aged >60 years old who visited Geriatrics Outpatient Clinic in Cipto Mangunkusumo National Hospital Jakarta Indonesia from January to July 2017 were recruited into this double blind placebo-controlled alphacalcidol intervention study. Inclusion criteria was patient aged >60 year who agreed to be involved in the study. Exclusion criteria was hypercalcemia, consumption of NSAID, steroid, hydroxychloroquine, presence of acute inflammation (based on medical history, physical or laboratory examination), autoimmune disease and malignancy.

Ethics

All subjects provided written informed consent before joining the trial. This trial was conducted according the principles of the Helsinki Declaration and had received ethical approval from Faculty of Medicine Universitas Indonesia (No 706/UN2.F1/ETIK/2016). This

trial was registered on clinicaltrials.gov (ID NCT0392744).

Intervention

Subjects were randomized to either intervention group receiving alphacalcidol 0.5 mcg daily for 90 days or the placebo group receiving equivalent number of identical placebo capsules for 90 days. All subjects were instructed to consume the capsule once daily and maintain their usual diet and physical activity habits. Randomization was performed by an independent researcher using a computerized random sequence generation program. Subjects, investigators and outcome assessors were blinded and allocation concealment was performed. Caregivers were involved to ensure drug consumption by marking a tick on the drug logbook. Compliance was assessed by evaluating drug consumption log book, counting the drugs left in the drug container returned by the subjects every 30 days and measuring Parathyroid Hormone (PTH) concentration on day 0 dan 90. Good compliance was indicated by taking 90% of the prescribed drugs as shown in drug log book.

Data Collection and Analysis

Medical history and physical examination, including frailty state assessment using Cardiovascular Health Study criteria were obtained from eligible subjects. Frailty was defined as clinical syndrome in which three or more of the following criteria were present: unintentional weight loss, self-reported exhaustion, weakness, as well as slow walking speed and low physical activity. Subjects with 1 or 2 criteria were considered pre-frail and those without any criteria were considered fit. All participants were assessed with Barthel Activity of Daily Living to evaluate independency: food recall for assessment of dietary pattern and Mini Nutritional Assessment for nutritional status assessment. Sign and symptoms of acute condition that may change the immune response were recorded every 2 weeks.

Blood Sample Analysis

Blood were drawn on baseline (day 0) and after intervention (day 90) for measurement of 25(OH), calcium and PTH concentration in

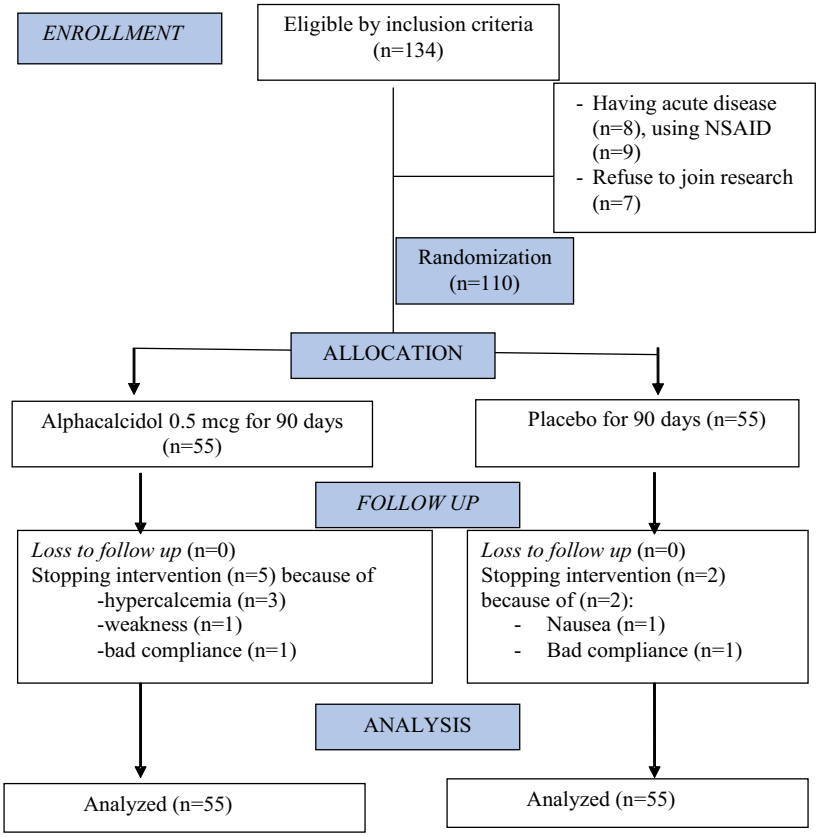

Figure 1. Flowchart of the subjets allocation

serum, whole blood were taken for peripheral blood mononuclear cells (PBMC) isolation and culture. Blood serum were drawn also on day 30 and 60 for calcium measurement. Serum 25(OH)D were measured using ELISA with inter- and intra assay coefficients of variation of <10% and <4%. IL-6, IL-10 and γ-IFN from culture supernatant were measured using R&D commercial ELISA kits and percentage of CD4, CD8, CD8+ CD28- T cell was measured from culture pellet with flow citometry.

PBMC Culture and Isolation

PBMC were isolated by centrifuging fasting whole blood samples collected in BC Vacutainer cell preparation tubes with heparin. The harvested PBMC pellet was re-suspended in RPMI medium with 10% dimethyl sulphoxide (DMSO) and stored at -80 C. Immediately prior to analysis, PBMC samples were thawed and washed in excess phosphate buffered saline (PBS) and centrifuged (400 g, 5 minutes 4^0C) to remove residual RPMI and DMSO. The supernatant was discarded and the pellet re-suspended in 100 mcl of triple detergent buffer.

Statistical Analysis

Analysis were performed on an intention to treat basis using SPSS version 20. Shapiro-Wilk test and visual inspection on histograms and scatterplots were used to assess normality. Baseline characteristics are presented as mean + SD and frequencies (%) or median (interquartile range (IQR) for skewed variables. Independent t test was used to examine the mean difference among variables. Efficacy of the intervention on the outcomes (between-group differences) were analyzed using changes in outcome variables. All tests were two sided and statistical significance was set at $p < 0.05$.

RESULTS

From January to July 2017, 134 elderly subjects were screened, and there were 110 patients eligible for the research. These subjects were randomized into 2 group. (**Figure 1**)

Mean age of the subjects were 73.5 (SD 5,5) year. Most (73,6%) were woman and frail (52%). Patients with vitamin D insufficiency was 85%. Baseline demographic, clinical and immunological characteristics of the subjects is shown in **Table 1**, After 90 days, cytokines from PBMC culture were measured as shown at **Table 2**. No subjects were showing signs and or symptoms of acute infection since 2 week before the end of the study (day 90).

Table 1. Baseline characteristics of study subjects

Variables	Alfacalcidol (n=55)	Placebo (n=55)
Age (year), mean (SD)	73.4 (4.5)	73.7 (4.9)
Gender, n (%)		
- Male (n=29)	14 (35)	15 (28)
- Female (n=81)	41 (65)	40 (72)
Education		
- Primary school	0 (0)	0 (0)
- Secondary school	13 (23)	8 (14)
- High school	42 (76)	47 (85)
Functional status		
- Independent	2 (3)	5 (9)
- Low dependent	6 (10)	7 (12)
- Intermediate dependent	35 (63)	30 (54)
- Highly dependent	12 (21)	13 (23)
Body Mass Index (kg/m^2), mean (SD)	21.7 (3.3)	22.3 (4.5)
Frailty state, n (%)		
- Fit (n=27)	13 (16)	14 (14)
- Pre-frail (n=27)	12 (29)	15 (34)
- Frail (n=56)	30 (54)	26 (50)
Serum 25(OH)D1 (ng/ml), mean (SD)	27.59 (12.2)	28.27 (10.4)
Daily intake, mean (SD)		
- Energy (kkal)	1366.13 (76)	1202 (48.4)
- Protein (gram)	46 (2.5)	49 (2.2)
- Vitamin D (IU)	188 (21)	184 (22)
- Calsium (mg)	302.5 (22)	387.5 (15.2)
Daily Sun exposure, n (%)		
- None	5 (9)	7 (12)
- 0-30 min	28 (50)	29 (52)
- 30-60 min	22 (41)	19 (34)
IL-6,(pg/ml), mean (SD)	36 (24.9)	35.2 (27.9)
IL-10 (pg/ml), mean (SD)	5.2 (2.0)	4.7 (2.2)
IL-6/IL-10 ratio, mean (SD)	4.7 (0.8)	3.2 (1.1)
γ–IFN (pg/ml), mean (SD)	2.37 (1.1)	2.35 (0.7)
CD4 (%), mean (SD)	20.3 (0.8)	20.6 (1,0)
CD8 (%), mean (SD)	11.3 (0.7)	8.8 (0.3)
CD4/CD8 ratio, mean (SD)	2.66 (1.6)	2.68 (2.45)
CD8+ CD28- (%), mean (SD)	4.5 (2.2)	5.1 (3.9)
CD8+ CD28+ (%), mean (SD)	7.5 (0.4)	5.1 (0.2)

Table 2. Cytokine and T cell level on day 90

Immune parameter	Alfacalcidol	Placebo	P value
IL-6 control (pg/ml)			
- Control	38.2 (6.1)	33.0 (7.4)	0.4
- +LPS	213 (7.2)	174 (7.2)	0.005
IL-10			
- Control	28.3 (13)	16.2 (1.9)	0.005
- +LPS	70.3 (19)	55.0 (4.5)	0.02
γ–IFN			
- Control	6.9 (3.4)	5.2 (3.4)	0.001
- +LPS	7.3 (3.4)	11.7 (3.8)	0.001
IL-6/IL-10 ratio	3.56 (0.2)	3.87 (0.2)	0.008
CD4/CD8 ratio	3.2 (2.9)	1.81 (1.07)	0.001
CD8+ CD28- (%)	2.5 (1.5)	6.0 (3.72)	<0.001

As shown on **Table 2**, alphacalcidol only increased IL-10 but it showed no effect on IL-6 and γ-IFN. Ratio of IL-6//IL-10 was decreasing in Alphacalcidol group. Similar pattern was found on subgroup analysis based on frailty state, as shown on **Table 3**. The decrease of CD4/CD8 ratio was also clinically significant while its effect on CD8+ CD28- reduction percentage was modest. Adverse events were mild hypercalcemia in 3 subjects after 30 days of alphacalcidol use and one subject reported weakness with high normal calcium level. No serious adverse event was found during the research.

DISCUSSION

There were 2 pro-inflammatory cytokines studied in this trial. First was IL-6 which acts as anti inflammatory cytokine in acute inflammation and pro inflammatory cytokine in chronic inflammation. Second was γ-IFN, the main pro-inflammatory cytokine secreted by T helper-1. Alphacalcidol did not decrease IL-6 in chronic and acute inflammation setting. To our knowledge, this study was the first reporting effect of alphacalcidol in inflammation profile in elder people of various frailty state. Other studies using different vitamin D preparations showed inconsistent results. Meta analysis from Calton of 12 trials showed basal IL-6 reduction after vitamin D intervention for minimum 12 weeks.[11] Other trial by Yusupov using 2000 IU Vitamin D3 for 3 months concluded that vitamin D3 did not change inflammatory profile including IL-6.[12] Alphacalcidol could not reduce IL-6 in chronic and acute inflammation probably due to several possible reasons. First, the duration and dose of alphacalcidol could not improve cytokine secretion impairment in most subjects on this study who were mostly in pre-frail and frail state. Second, based on research by Ojaimi, IL-6 reduced occurs while serum 25-(OH)D increased above 40 ng/ml.[13] In this trial considering alphacalcidol could not directly increase 25(OH)D as it would be very rapidly converted into 1.25 (OH)2D3, the 25(OH)D sufficiency state could be reflected by the PTH level. PTH level on day 90 shows that the vitamin D insufficiency state had not been fully recovered. Third, difference in nutrient and vitamin D intake of Indonesian elderly which showed poor intake pattern probably also contributed to this immune profile. There was no effect on γ-IFN found in this study, consistent with Yusupov who also found no effect on γ-IFN using vitamin D3 preparation.[13] Trial in Multiple Sclerosis patients by Mahon also did not find any benefit in term of γ-IFN production.[14]

Alphacalcidol increased IL-10 in elderly. This finding is consistent with research by Yusupov[13] in healthy adults and Schleithoff[15] in 123 patients

Table 3. Subgroup analysis of inflammatory cytokines based on frailty state

Category	Intervention	IL-6 (pg/ml), mean (SD)		IL-10 (pg/ml), mean (SD)		γ-IFN (pg/ml), mean (SD)	
		Control	LPS	Control	LPS	Control	LPS
Fit (n=27)	Alphacalcidol	14.7 (6.4)	135 (3.0)	8.1 (0.2)	56.5 (2.8)*	3.1 (0.6)	7.0 (2.2)
	Placebo	14.6 (7.6)	143 (7.1)	9.7 (1.1)	13.2 (0.6)	5.3 (0.92)	8.3 (0.50)
Pre-frail (n-27)	Alphacalcidol	50.1 (3.3)	356.5 (18.7)	8,1 (16)	88.5 (4.5)*	2.4 (0.36)	6.9 (0.14)
	Placebo	49.1 (6.1)	266 (8.3)	13.3 (0.7)	42.2 (2.87)	2.4 (0.3)	2.6 (0.92)
Frail (n=56)	Alphacalcidol	30.1 (27.0)	247.6 (12.9)	11.9 (1.0)	87.2 (8.3)*	9.1 (0.55)	13.8 (0.54)
	Placebo	40.6 (24.1)	43 (10.8)	24.2 (4.1)	59 (4.1)	4.6 (0.62)	5.3 (0.89)

with chronic heart failure (CHF) using vitamin D for 9 months. Low anti-inflammation activity in elderly will cause prolonged inflammation leading to more severe impact on host. Increase of anti-inflammatory cytokine will improve the recovery from inflammation process. IL-10 increased by vitamin D was related with activation of Vitamin D Receptor (VDR) which will further suppress IL-10 promotor gene and enhance upregulation of IL-10 production.[16]

Interestingly, this finding showed that alphacalcidol only influenced IL-10, did not seem to be influenced by difference of frailty state. Alphacalcidol could not improve IL-6 and γ-IFN, showing that the pro-inflammatory state can not be affected through 3 months supplementation of alphacalcidol. However, decreased IL-6/ IL-10 ratio caused by alphacalcidol showed domination of anti-inflammatory property of alphacalcidol. Because in immune senescence low grade pro-inflammatory status exist with defect on anti-inflammatory response, this study might support the use of alphacalcidol for elderly in any frailty state.

CD4/CD8 ratio, which is also a predictor of negative health outcomes, was improved in alphacalcidol group. Vitamin D is known to have direct and indirect effect on T cells, through improvement of γ-IFN production by T helper 1. Percentage of CD8+ CD28- was also decreased by alphacalcidol. However, as the mean percentage at baseline was still below 30%, this effect still had no clinical implication. CD8+ CD28- T cell have negative impact in immune response through several mechanisms which will cause defect on general cellular response in elderly. CD28- T cell will expand oligoclonally occupy available space thus other subset will be decreased in number. CD8+ CD28- decrease means lower immune suppressive condition and better response to vaccination.[17]

This study, to the extent of authors knowledge, was the first to examine the effect of alphacalcidol in immune response involving frail elder subjects, in which in many trials, frail elderly were generally excluded. Limitation of this study was that cytokine measurements were only conducted before and after intervention thus the kinetics of cytokine secretion could not be observed.

CONFLICT OF INTEREST

Authors declare no conflict of interest for this study.

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