ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
February 2017; 16(1):14-20.

Effects of Submaximal Aerobic Exercise on Regulatory T Cell Markers of Male Patients Suffering from Ischemic Heart Disease

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Received: 21 May 2016; Received in revised form: 7 January 2017; Accepted: 17 January 2017

ABSTRACT

There are confirmed beneficiary effects of exercise on atherosclerotic inflammation of ischemia-associated heart diseases. The purpose of this study was to evaluate the effect of aerobic exercise on T-regulatory cell markers of IL-35 as well as FoxP3 and T-helper2 marker of IL-33 in patients with ischemic heart disease (IHD).

This research was performed on 44 asymptomatic male patients with ischemic heart disease. The participants were randomly assigned into two groups of submaximal aerobic exercise and control group. Blood samples were collected before and after the termination of the exercise protocol. Serum levels of IL-35 and IL-33 as well as the amount of FoxP3 gene expression in peripheral blood mononuclear cells were measured by Elisa and Real time PCR, respectively.

Serum levels of IL-35 (p=0.001) as well as the amount of FoxP3 gene expression increased significantly (p=0.012) in exercise group even after controlling the likely confounding effects of age, length of ischemia, duration of the disease, and the amount of such factors before exercise (p≤0.042).

It seems that exercise may yield a better control of atherosclerotic inflammation in patients with ischemic heart disease through the induction of regulatory T cells.

Keywords: Interleukin-35; Interleukin-33; Regulatory T cells; Sub maximal aerobic exercise

INTRODUCTION

Physical inactivity is a well-documented risk factor for coronary heart disease and there is evidence showing regular physical activity reduces the risk of hypertension, type 2 diabetes and helps maintain

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Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

optimal bone mineral density;[1] however, in addition to inactivity, there are other pathophysiologic bases of the disease associated with inflammation. Over the last decades, an abundance of evidence has emerged that inflammation plays a role in all phases of the atherosclerotic disease process, from lesion initiation to progression and, finally, to plaque rupture and the consecutive complications of cardiovascular disease.[2,3] In fact, the inflammatory reactions are a group of the interfering processes in the development of arthrosclerosis and its involved complications.[4] There are numerous reports that show physical activity has significant beneficiary effects on cardiovascular events[5-8] through reducing the risk factors[9-11] as well as processes of atherosclerosis progression.[12,13] The majority of such studies have examined the changes of inflammatory/anti-inflammatory markers such as CRP, VCAM-1, ICAM-1, fibrinogen, IL-6, TNF-a, IL-10, or TGF-β.[14-18]

Regulatory T cells (Tregs), a subset of T cells, suppresse different immune cells such as T and B cells, natural killer cells, natural killer T cells, mast cells, and dendritic cells. The main mechanism of such activity is to produce anti-inflammatory cytokines which control various types of inflammation through the mediatory effect of Fork head box P (Foxp3). Foxp3 is the main transcription factor and phenotypic/functional marker of Tregs.[19,20] Results obtained from animal models of atherosclerosis have shown the protective and fixing role of such cells in atrium plaque.[21] It seems that lack of such protection , which is induced by their anti-inflammatory cytokines of IL-10 and TGF-β, may lead to acute coronary events as well as advanced atherosclerosis.[22,23] IL-35, an IL-12 family cytokine secreted by Tregs may have such anti-inflammatory effects.[24]

The response of Tregs to various types of endurance exercise has been studied in endurance athletes.[25] Perry et al. reported that there was a decrease in both the percentage of Treg lymphocytes and the intensity of FoxP3 expression in CD4+CD25+ Tregs following the participation in endurance exercise in athletes.[26] More specifically, the response of FoxP3 in ischemic heart patients to moderate (submaximal) aerobic endurance exercise has not been examined yet.

IL-33, an IL-1family cytokine secreted by Th2 cells and inducing Th2-associated diseases such as asthma, may have some protective roles in various cardiovascular diseases including atherosclerosis.[26,27] It has been demonstrated that mice deprived of IL-33 receptor, namely IL-33Ra, are afflicted to myocardium inflammation, ventricle dilation and cardiac fibrosis. In addition, the increase in extracellular IL-33Ra level is a predictor of clinical myocardial infarction.[28]

This research was designed, for the first time, to examine the effect of submaximal aerobic instead of intensive aerobic exercise on regulatory T-cells, focusing on two secretory-relevant markers of IL-35 and IL-33 as well as the cell-relevant markers of FoxP3 gene expression in male patients with ischemic heart diseases. Such knowledge may help us to prevent the cardiovascular disorders or to accelerate patients' recovery by promoting anti-inflammatory mechanisms.

MATERIALS AND METHODS

Patients

This semi-experimental research included 44 volunteered asymptomatic male patients with ischemic heart disease diagnosed in Shahid Beheshti Hospital of Kashan University of Medical Sciences. All the patients underwent angiography and at least one of the arteries had 75%or more stenosis. They were randomly assigned into two groups of 21 who participated in prescribed submaximal aerobic exercise and 23 patients served as the control group participating in routine regular physical activities. Men were chosen considering the fact that they are more likely to suffer from such ischemic heart diseases at younger age than women; moreover, men at this age are more likely to be able to participate and complete an exercise program. Both groups received their identical regular treatment. Blood samples were collected before and after the termination of the exercise protocol. The exclusion criteria were any clinical or ECG changes during the exercise, any cardiovascular event within the last 3 months, being candidate for revascularization, ventricular tachycardia, uncontrolled hypertension, malignancy, diabetes, autoimmune diseases, allergy, chronic or acute infectious diseases, kidney or liver failure, use of immune-modulating drugs and erythropoietin, peripheral vascular diseases limiting physical activities, valvular heart diseases, and obstructive pulmonary disease. Both control and exercise groups received their identical conventional medication. The ethical committee of the Kashan University of Medical Sciences approved the

project and informed consent was signed by the participants.

Exercise Protocol

The submaximal aerobic exercise was determined based on formula described in most exercise related texts[29] and included three sessions of exercise continued for 12 weeks. An exercise science specialist designed and supervised the exercise protocol. The participants in exercise group performed 5 minutes of mild warm-up exercise prior to performing 40 minutes of submaximal aerobic exercise followed by 5 minutes of cool-down stretching exercises. The target heart rate was monitored by Pollar watch model M300set (China). The health condition of every participant was assessed at the beginning of every exercise session. Weekly general health condition of the participants was evaluated by a cardiologist.

Elisa and Real-time Experiments

24 before and after the termination of the exercise program, 5 cc of venous blood sample was collected from the left arm of the all participants and kept frozen in EDTA-containing tubes until the assays. Serum levels of IL-33 and IL-35 were measured by ELISA sandwich method (eBioscience kit, USA) According to manufacturer's instructions. Total RNA was extracted from peripheral blood mononuclear cells (PBMC) using High Pure RNA Isolation Kit (Cat No: 11828665001, Roche Applied Science, Germany). cDNA synthesized from the extracted RNA using Transcriptor First Strand cDNA Synthesis Kit (Cat No: 04897030001, Roche Applied Science , Germany). The amount of FoxP3 gene expression was measured using Taqman primer probe Comparative CT method) ABI 7300 Real-Time PCR system, USA). β-actin housekeeping gene was used as endogenous control. FoxP3 gene expression in exercise group relative to control group was determined as $2^{\Delta\Delta\text{-CT}}$.

Statistical Analysis

Data were analyzed using descriptive statistics including mean and standard deviations. The measurements were compared before and after the exercise using paired and in both groups by independent T-tests. Analysis of covariance (ANCOVA) was employed to control the confounding variables and baseline differences. The entire significant test was performed by alpha level set to 0.05. SPSS 16.0 (Chicago, USA) and STATA software 11, (Stata Corp., College Station, TX, USA), were used to analyze the data.

RESULTS

Basic and clinical characteristics of the patients have been included in Table 1.

There was no significant difference between the two groups regarding to serum levels of IL-35 and IL-33 as well as the amount of FoxP3 gene expression before exercise.

The changes of both IL-35 serum levels and FoxP3 gene expression in exercise group were significantly higher after the exercise in comparison with those of before exercise ($p\leq0.012$) (Table 2). The amount of FoxP3 gene expression increased significantly in exercise group compared to control group in post-test condition ($p=0.012$) (Table 2). There were differences in IL-35 and IL-33 serum levels as well as the amount of FoxP3 gene expression between the groups in the primary condition of the protocol. Therefore, we compared the groups at the post-test state using related factors as the covariates in pre-test state. There was a significant difference in the serum level of IL-35 and the amount of FoxP3 gene expression between both groups at the end of the protocol ($p\leq0.019$) (Table 3).

Adjusting the effects of total duration of exercise activity, duration of ischemia history, and age of the participants, we performed further multivariate analysis of covariance on IL-35 and IL-33 serum levels and the amount of FoxP3 gene expression to compare the exercise group versus the control group after the termination of the exercise program. The results of analysis indicated that none of those covariates contributed significantly to such difference. This indicates that after the exclusion of the covariate effects, there was still a significant difference of IL-35 and FoxP3 expression between the groups ($p\leq0.042$). The level of increase after adjustment for those variables remained significant (Table 4).

Table 1. Basic and clinical characteristics of the ischemic heart disease patients in submaximal exercise and control group

Variants	controls (n=23) (Mean±SD)	exercise (n=21) (Mean±SD)	*p* value
Age (years)	64.78±12.21	58.5±7.9	0.035
Duration of disease (months)	11.43±4.51	7.71±2.57	0.002
Duration of exercise (hours)	9.61±27.31	27.29±22.84	0.026
Pre-test IL-33 (pg/mL)	75.04±29	89.69±43.75	0.194
Pre-test IL-35(pg/mL)	51.33±22.89	41.53±23.87	0.172
Pre-test FoxP3	12.52±2.85	11.81±2.68	0.4

Table 2. Comparing the levels of IL-35, IL-33, and FoxP3 expression in the ischemic heart disease patients in submaximal exercise and control groups in pre- and post-test condition

Marker	Group	Before	After	Difference	*p* value
	control	51.33 (4.77)	50.42 (4.45)	-0.9 (2.4)	0.714
IL-35(pg/mL)	exercise	41.53 (5.21)	57.79 (6.86)	16.3 (4.3)	0.001
Mean (SE)	difference	-9.79	7.36	-	-
	p value	0.172	0.365	-	-
	control	75.04 (6.05)	76.84 (7.84)	1.80 (7.33)	0.808
IL-33(pg/mL)	exercise	89.69 (9.55)	89.95 (7.90)	0.26 (14.70)	0.986
Mean (SE)	difference	14.65 (10.11)	13.10 (12.37)	-	-
	p value	0.194	0.269	-	-
	control	12.52 (0.6)	11.87 (0.46)	-0.65 (0.54)	0.243
FoxP3 (pg/mL)	exercise	11.81 (0.58)	9.33 (0.87)	-2.48 (0.90)	0.012
Mean (SE)	difference	0.71	-2.5	-	-
	p value	0.4	0.012	-	-

Table 3. Analysis of covariance (ANCOVA) for the serum levels of IL-35, IL-33, and the amount of FoxP3 gene expression in the ischemic heart disease patients in submaximal exercise and control groups using related covariates

Model		Unstandardized coefficients		Standardized coefficients	t	*p* value
		B	Standard error	β		
IL-35	constant	3.405	6.389		0.353	0.957
(pg/mL)	IL-35 before	0.916	0.106	0.814	8.640	0.00
	group	16.335	4.995	0.11	3.279	0.002
IL-33	constant	70.633	15.629		4.519	0.00
(pg/mL)	IL-33before	0.083	0.174	0.075	0.477	0.636
	group	11.892	12.747	0.146	0.933	0.365
FoxP3	constant	6.872	2.196		3.128	0.003
(pg/mL)	FoxP3before	0.399	0.168	0.325	2.375	0.22
	group	2.252	0.019	-0.325	-2.450	0.019

Table 4. Multivariate analysis of covariance (ANCOVA) for the serum levels of IL-35 and IL-33 and the amount of FoxP3 gene expression in the ischemic heart disease patients in submaximal exercise and control groups

Model		Unstandardized coefficients		Standardized coefficients	t	*p* value
		B	Standard error	β		
IL-35(pg/ml)	(constant)	-20.527	19.201		-1.079	0.287
	IL-35before	0.905	0.107	0.803	8.444	0.00
	group	12.176	5.775	0.231	2.108	0.042
	length of ischemia	-1.985	1.114	-0.294	-1.7	0.097
	duration of exercise	0.096	0.097	0.096	0.994	0.327
	age	0.699	0.393	0.0284	1.778	0.083
IL-33(pg/ml)	(constant)	71.360	48.925		1.495	-0.153
	IL-33 before	0.075	0.179	0.067	0.416	0.680
	group	20.302	15.124	0.250	1.342	0.187
	length of ischemia	2.790	2.973	0.280	0.938	0.354
	duration of exercise	-0.066	0.263	-0.043	-0.253	0.802
	age	-0.484	1.057	-0.128	-0.458	0.649
FoxP3(pg/ml)	(constant)	5.366	3.611		1.486	0.146
	FoxP3 before	0.301	0.174	0.245	1.730	0.092
	group	-2.493	1.064	-0.371	-2.343	0.024
	length of ischemia	-0.370	0.218	-0.450	-1.697	0.098
	duration of exercise	-0.026	0.018	-0.202	-1.408	0.167
	age	0.111	0.077	0.355	1.438	0.159

DISCUSSION

This study was conducted to determine the effect of 12 weeks of submaximal aerobic exercise on the serum levels of IL-35 and IL-33 in patients with symptom-free ischemic heart disease. This exercise training program significantly improved the level of regulatory T cell-mediated markers of IL-35 and FoxP3 gene expression.

Our study confirmed the anti-inflammatory role of submaximal aerobic exercise on serum level of IL-35 in patients with ischemic heart disease. These properties of IL-35, at least partly, are mediated through the proliferation of Tregs and inhibition of Th17 cells that play a significant role in recruitment of neutrophils, which in turn, intensify inflammatory processes.[29] In the case of inflammation associated with ischemic heart disease, other studies have introduced IL-35 as a significant bio-marker in the incidence of coronary artery disease[30] in a way that its decrease may be an indicator of ischemia.[31] Such protective effect has also been shown in other inflammatory conditions.[32]

The findings of this study is in agreement with some other investigations that demonstrated the anti-inflammatory effects of Tregs, where moderate aerobic exercise increased the suppressive function of CD4+CD25+Foxp3+ cells on Th2 inflammatory responses including airway eosinophilia and allergen-induced asthma.[33,34]

According to our results, sub maximal aerobic does not make any significant changes in serum levels of IL-33. However, one-session of an aerobic exercise made significant increases in the level of serum IL-33 in healthy young men and women.[35] These contradictory findings to our study may be attributed to the different types of physical activities and populations employed. Considering blocking effects of Th2 cells on Th1 ones, we emphasize that more physical activity in severity and/or time may also change serum levels of IL-33 and confirm its protective roles in heart ischemia conditions.

The amount of submaximal aerobic exercise applied in this research was sufficient to produce significant changes in regulatory T-cell markers of patients with

ischemic heart disease. However, the response to moderate level of aerobic physical activity may differ from that of intense and prolonged periods of endurance exercise. A recent report in mice, contradictory to human being, suggested that while intensive exercise increased Treg levels, moderate exercise had no effect on murine Tregs.[36] The effects of exercise regarding its intensity have also been the focus of studies in which moderate aerobic exercise reduced the incidence of upper respiratory tract problems, while high intensity and prolonged course of aerobic exercise led to more upper respiratory tract infections in elite athletes.[36]

It is likely that, other than exercise, some clinical features of our patients affect the production of Tregs. Considering a multivariate regression analysis, we confirmed that exercise was the only explanation for the observed results in our patients.

Our study had some limitations. Firstly, there were no longitudinal follow-up measurements to evaluate the changes of selected markers during the time. This limitation allowed just a cross-sectional analysis with only a limited robustness. Secondly, functional assays which provide further information on the immune-inhibitory effects of the molecules were not performed.

It seems exercise may yield a better control of atherosclerotic inflammation in patients with ischemic heart disease through the induction of regulatory T cells. These findings may be used for both diagnostic approaches of stroke severity and therapeutic strategies.

ACKNOWLEDGEMENTS

This study was supported by Deputy of Research, Kashan University of Medical Sciences (KAUMS), Grant No. 9238. The researchers wish to thank the patients for their sincere and generous contribution to the research protocol.

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