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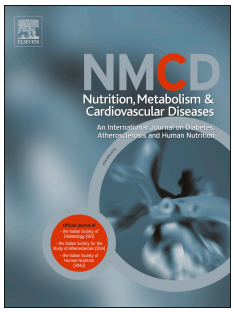

Beneficial Effects of Omega 3 and Vitamin E Co-Administration on Gene Expression of SIRT1 and PGC1α and serum antioxidant enzymes in patients with Coronary Artery Disease

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Beneficial Effects of Omega 3 and Vitamin E Co-Administration on Gene Expression of SIRT1 and PGC1α and serum antioxidant enzymes in patients with Coronary Artery Disease

Running Title: Effects of omega 3 and vitamin E on SIRT1 and PGC1α gene expression and antioxidant enzymes in CAD patients

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Conflicts of Interest
The authors declare no conflict of interest.

Abstract

Background and Aim: SIRT1 and PGC1α are two important genes which play critical roles in regulating oxidative stress and inflammation processes. The aim of this study was to assess the effects of omega 3 and vitamin E supplement co-administration on SIRT1 and PGC1α gene expression and serum levels of antioxidant enzymes in CAD patients.

Methods and Results: Participants of this randomized controlled trial included 60 CAD male patients who received omega 3 (4g/day) and vitamin E placebo (OP), omega 3 (4g/day) and vitamin E (400IU/day) (OE) or omega 3 and vitamin E placebos (PP) for two months. Gene expression of SIRT1 and PGC1α in Peripheral Blood Mononuclear Cells ($PBMC_S$) was assessed using RT-PCR. Furthermore, serum antioxidant enzymes and hsCRP were assessed at the beginning and the end of the intervention. Gene expression of SIRT1 and PGC1α

increased significantly in OE group (P =0.039 and *P*= 0.050, respectively). Catalase and hsCRP increased significantly in OE and OP groups. GPX and SOD did not statistically change in all groups. TAC increased significantly in OE group (*P*=0.009), but not in OP and PP groups.

Conclusion: Combination of omega 3 fatty acids with vitamin E may have beneficial effects on CAD patients by increasing gene expression of SIRT1 and PGC1α and improving oxidative stress and inflammation in these patients.

Keywords: CAD, omega 3, vitamin E, SIRT1, PGC1α, antioxidant enzymes

Introduction

Sirtuins are a class of NAD-dependent histone deacetylase proteins which can transfer acetyl from acetyl-lysin residue of histones to ADP-ribose of NAD[1, 2].Sirtuins deacetylate various substrates such as NFkB, FOXO, PGC-1α and PPARs[3];therefore, they can influence a wide variety of cell pathways such as apoptosis, inflammation, aging process and extension of life span during calorie restriction conditions[4-6].Sir2a or SIRT1 is a member of this family which can regulate oxidative stress via affecting P53 [7, 8].In fact, deacetylation of this tumor suppressor by SIRT1 may have beneficial effects on cellular senescence by inhibiting the expression of growth suppressive genes involved in cellular senescence and hence reducing oxidative stress[8-10].PGC1α is a key regulator of mitochondrial respiration which plays important roles in metabolism and energy homeostasis. Furthermore, it can increase the gene expression of antioxidant enzymes such as SOD, GPX and catalase. Therefore, interventions which increase expression of PGC1α protect body from oxidative stress [11].Studies have shown that inflammation and oxidative stress play essential roles in pathogenesis of cardiovascular diseases[12]. Therefore, strategies which can regulate oxidative stress and inflammation can improve pathologic conditions and overall health of cardiovascular patients.

Cell-culture studies have shown that omega 3 fatty acids can elevate SIRT1 gene expression via increasing expression, phosphorylation and activation of AMPK in macrophages. Furthermore, they can reduce gene expression of pro-inflammatory cytokines [13].Infact, SIRT1 deacetylates NFkB after activation of AMPK and results in inhibition of NFkB signaling and expression of inflammatory genes [14].Vitamin E, as an antioxidant agent, can protect cells from oxidative stress and increase gene expression of antioxidant enzymes [15]. Moreover, Vitamin E can activate AMPK and therefore increase the expression of

sirtuins[16]. Because of the anti-inflammatory and antioxidant effects of omega 3 fatty acids and vitamin E and potential role of sirtuins and PGC1α in protecting cells from oxidative stress and inflammation, this study was designed to assess the effects of omega 3 and vitamin E supplement co-administration on SIRT1 and PGC1α gene expression and serum levels of antioxidant enzymes in CAD patients.

MATERIALS AND METHODS

The participants of this randomized double-blind placebo-controlled clinical trial included 60 male CAD patients with at least 50% stenosis in one coronary artery proven by angiography in the past three months. These volunteers were selected in the Heart Medical Center, Tehran, Iran, between June 2012 and July 2013. An informed consent was signed by them before the study was commenced. The study was approved by the Tehran University of Medical Sciences Ethical Committee (ID: 23605) and registered inwww.clinicaltrial.org by the registry number of NCT02011906.The participants were divided into three randomly groups using random permuted blocks method, including Group 1 received omega 3 and vitamin E group (OE), Group 2 received omega 3 and vitamin E placebo (OP) and Group 3 received omega 3 and vitamin E placebos (PP). The OE group received 4 g/day of omega-3 fatty acids and 400 IU of vitamin E. The OP group received 4 g/day of omega-3 fatty acids and vitamin E placebo. The PP group received both omega-3 fatty acids and vitamin E placebo softgels with the lunch and dinner for two months. Each 1 gram of omega 3 softgels contained 180 mg of eicosapentaenoic acid (EPA) and 120 mg of docosahexaenoic acid (DHA).Omega 3, vitamin E and placebos were produced by Minoo Pharmaceutical, Cosmetic and Hygienic Company, Tehran, Iran. Height, hip and waist circumference were measured before and after the intervention to the nearest centimeter and weight was measured to the nearest kilogram. BMI was calculated as the weight divided by the square of height and waist to hip ratio (WHR) was calculated by dividing the waist circumference to the hip circumference. All patients did not consume omega 3 and vitamin E supplements or fish oil in the past three months before starting the study and we wanted them to not change their dietary patterns during intervention.

In the beginning of the study and after two months of intervention, 15-ml blood samples were collected after12–14 h of overnight fasting. Ten milliliters of the blood were used for PBMC isolation using ficole and the remaining was used for serum separation. Blood serums were separated using centrifuge and stored at -80 ℃ until use.RNA was extracted using RNeasy

Plus Mini Kit and then cDNA was synthesized using Qiagen Reverse Transcriptase Kit (Qiagen, Germany). Real-time PCR were carried out using protocols described in previous studies[17].β-actin was used in real-time PCR as housekeeping gene. Primer sequences used in real-time PCR are described in Table 1.Serum level of TAC was assessed using 2,2'-azino-bis3-ethylbenzthiazoline-6-sulfonic acid (ABTS)[18]. Catalase activity was assessed according to Hego Aebi's method[19]. Serum GPX and SOD were assessed by using paglia et al. and sun et al. respectively[20, 21].Statistical analysis was carried out using SPSS Software v.18. Data were shown as mean ±SE (standard error). The Kolmogorov-Smirnoff test was used for determining normality of the parameters. One-way analysis-of-variance (ANOVA) test was used to compare the mean of the variables between the groups and paired t-test was used for comparison between groups before and after the supplementation. A *P*value of ≤ 0.05 was considered statistically significant.

RESULTS

Sixty-five male CAD patients were initially participated in the current study, five of them discontinued the supplement consumption because of personal reasons and hence were excluded from the study. Therefore, 21, 20 and 19(a total number of 60) patients were distributed into OE, OP and PP groups respectively at the end of intervention. No statistically significant differences were seen between the mean patients' ages and their disease duration within the groups at the beginning of the study ($P = 0.079$ and $P = 0.299$, respectively). Table 2shows the baseline and post-intervention anthropometric parameters of the patients. No significant differences were seen between the anthropometric parameters within the various groups at the beginning of the intervention. Neither omega 3 nor omega 3 and vitamin E supplementation had significant effects on anthropometric parameters. Table 3describes dietary intakes of the study groups at the beginning and the end of the intervention based on their recall analyses. As it is shown, no significant differences in energy and macronutrient intakes were seen between the study groups at the baseline and the end of the intervention. Furthermore, no statistical differences were seen between dietary intakes of vitamin E and fatty acids in the study groups during two months of intervention. Patients in all groups did not change their dietary patterns during the intervention.

Serum antioxidant enzymes and hsCRP

As shown in Table 4, omega 3 alone and a combination of omega 3 and vitamin E supplementations significantly increased serum levels of catalase and decreased hsCRP. Omega 3 and vitamin E supplementation increased serum level of GPX, but it was not

statistically significant (*P*=0.086).Furthermore, TAC increased significantly in OE group (*P*=0.009), but not in OP and PP groups.

Gene expression findings

Results of this study showed that the gene expression of SIRT1 and PGC1α based on $2^{-\Delta\Delta ct}$ calculation were statistically different between the study groups (*P*=0.039 and *P* = 0.050, respectively). Post-hoc analysis (Tukey Test) revealed that there is significant difference between the gene expressions of SIRT1 and PGC1α in OE and PP groups (*P*=0.037 and *P* = 0.043, respectively) but not in OP and PP groups. It seems that omega 3 in combination to vitamin E supplementation can increase the expression of SIRT1 and PGC1α genes in CAD patients (Table 5).

DISCUSSION

The current study was the first one which investigated the effects of nutrients on gene expression of SIRT1 and PGC1α in humans. We used PBMCs of CAD patients for studying the effects of omega 3 and vitamin E on gene expressions because these cells can travel through the blood and enter to various tissues such as the adipose tissue[22]. Furthermore, these cells can reflect the metabolic and immune responses of adipocytes or hepatocytes to dietary interventions at the level of gene transcription[23, 24] and also have critical roles in development of atherosclerosis[25].Results of this study showed that vitamin E and Omega 3 can increase the expression of SIRT1 and PGC1α genes in CAD patients. Previous cell-culture studies have shown that omega 3 fatty acids can increase SIRT1 gene expression by increasing phosphorylation and activation of AMPK and result in suppression of pro-inflammatory genes[13, 14].Increase in SIRT1gene can also modulate endothelial nitricoxide synthase (eNOS) and p53 activity and promote vascular function by affecting smooth muscle cells of the blood vessels[26].Furthermore, omega 3 can increase the expression of genes involving in mitochondrial biogenesis such as PGC1a and increase oxidation of fatty acids via induction of PPARα [27-29]. Fatty acids are the main energy source of heart in adulthood [30, 31]. Expression of PGC1a in cardiac cells can affect a wide variety of enzymes involving in many biology pathways such as Krebs Cycle, fatty acid oxidation and lactate and ketone body metabolisms[32].Therefore, PGC1a may affect cardiac cells by increasing their efficiency of oxygen consumption and ATP production [33].Elevation of ROS levels is a common feature in cardiovascular disease and results in endothelial dysfunction [34, 35]. Over-expression of PGC1a in endothelial cells also decreases ROS by enhancing antioxidant enzymes such as MnSOD, catalase and thioredoxin[36].Vitamin E is a powerful antioxidant

agent and its deficiency may aggravate oxidative stress seen in cardiovascular diseases. Therefore, it is plausible that vitamin E supplementation can affect the level of antioxidant enzymes in CAD patients. In the current study, omega 3 alone and combined omega 3 and vitamin E supplementations resulted in significant increase in serum catalase. However, serum SOD and GPX did not increase. Although omega 3 supplementation did not change the mean level of TAC, co-administration of this substance with vitamin E resulted in statistically significant increase in TAC in OE group. In a research study, supplementation with 400 IU/day of vitamin E for 12 weeks did not change serum SOD in COPD patients [37].Kolahi et al. reported no significant changes in SOD, GPX and TAC in RA patients receiving omega 3 supplements with or without vitamin E[38]. In another study on diabetic patients, Sarbolouki et al. showed that vitamin E alone and a combination of EPA and vitamin E increased significantly serum TAC, but only EPA had significant effects in increasing SOD and GPX; and catalase increased only in group receiving EPA and vitamin E [39].

Previous studies have shown that AMPK can impose inhibitory effects on NFkB signaling through the induction of deacetylase activity of SIRT1 and subsequent suppression of pro-inflammatory genes expression [14]. Xue et al. have shown that SIRT1 is required for the anti-inflammatory effects of omega 3 fatty acids in antagonizing NFkB signaling in macrophages [13].Several studies have shown that omega 3 fatty acids are anti-inflammatory agents and can reduce inflammation through inhibiting the production of cytokines such as IL1, 1L2 and TNFα [40-42].Moreover, they can reduce serum CRP level [43, 44]. Vitamin E can decrease the release of interleukins such as IL-6 and reduce serum CRP by lowering pro-inflammatory cytokines such as IL-1β [45-47].Recently, Saboori et al. have revealed that supplementation with vitamin E in the form of either α-tocopherol or γ-tocopherol can reduce serum CRP significantly [48]. In the present study, serum hsCRP decreased significantly in OP and OE groups, but OE group experienced more decline in serum CRP than OP group did. Similarly, Ramezani et al. have reported that omega 3 alone and combined omega 3 and vitamin E significantly decrease hsCRP in CAD patients[49]. So, it seems that omega 3 fatty acids in combination to vitamin E have powerful effect in reducing inflammatory processes via decreasing CRP. To the best of the authors' knowledge, this is the first human study which investigates the effects of nutrients on gene expression of SIRT1 and PGC1α in CAD patients. However, further studies are needed to reveal the exact mechanisms; by which, omega 3 and vitamin E influence these key genes in humans. In conclusion, results of the current study showed that omega 3 fatty acids and vitamin E supplementation increased gene

expression of SIRT1 and PGC1α in PMBC with beneficial effects on some antioxidant enzymes such as catalases and may relieve inflammation via decreasing hsCRP.

Conflicts of Interest

The authors declare no conflict of interest.

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Table 1. Primers used in the current study

Primer	Sequence
SIRT-1 Forward	GCCGGAAACAATACCTCCAC
SIRT-1 Reverse	ACACCCCAGCTCCAGTTAG
PGC-1A Forward	CTTGGCAGAGTATGACGATG
PGC-1A Reverse	TAGTGCAAGTAGAAACACTGC
β-actin Forward	CCTGGCACCCAGCACAATGAAG
β-actin Reverse	CTAAGTCATAGTCCGCCTAGAAG

Table 2. Anthropometric parameters of the study groups before and after the intervention

Treatment group		**OP (*n* = 20)**	**OE (*n* = 21)**	**PP (*n* = 19)**	***P*.value***
Height (cm)	Baseline	169.04 ±1.36	170.32 ±1.19	170.92 ±1.58	0.623
Weight (kg)	Baseline	79.95 ±2.68	78.54 ±2.17	78.35 ±1.87	0.864
	Post-intervention	80.13 ±2.70	78.85 ±2.14	79.23 ±1.82	0.916
	difference	0.18 ±0.33	0.30 ±0.30	0.88 ±0.40	0.322
	P.value#	0.591	0.335	0.139	
BMI (kg/m^2)	Baseline	27.95 ±0.83	27.08 ±0.70	26.85 ±0.61	0.530
	Post-intervention	28.00±0.81	27.17±0.66	27.14±0.58	0.616
	difference	0.05±0.12	0.09±0.10	0.29±0.13	0.318
	P.value#	0.687	0.370	0.170	
Waist circumference (cm)	Baseline	98.72 ±2.11	95.76 ±1.58	96.18 ±1.88	0.479
	Post-intervention	98.30 ±2.01	95.64±1.45	96.42±1.88	0.556
	difference	-0.42±0.45	-0.12±0.53	0.24±0.39	0.618
	P.value#	0.359	0.827	0.548	
Hip circumference (cm)	Baseline	101.12 ±1.59	100.33 ±1.15	99.63 ±0.80	0.701
	Post-intervention	100.75±1.42	100.78±1.23	99.37 ±0.83	0.644
	difference	-0.37±0.52	0.45±0.54	-0.26±0.45	0.455
	P.value#	0.481	0.411	0.567	
WHR	Baseline	0.97 ±0.01	0.95 ±0.01	0.96 ±0.01	0.463
	Post-intervention	0.97 ±0.01	0.95 ±0.01	0.97 ±0.01	0.214
	difference	-0.001 ±0.005	0.005 ±0.005	0.005 ±0.004	0.304
	P.value#	0.850	0.325	0.191	

OP, omega-3 fatty acid and placebo; OE, omega-3 fatty acid and vitamin E; PP, placebo and placebo; BMI, body mass index; WHR; waist hip ratio*mean±SE; *ANOVA analysis; #paired T test

Table 3. Dietary intakes of the study groups before and after the intervention

Treatment group		**OP (*n* = 20)**	**OE (*n* = 21)**	**PP (*n* = 19)**	***P*.value***
Energy (Kcal)	Baseline	1450.74 ±114.37	1469.10 ±93.33	1528.49 ±111.25	0.867
	Post-intervention	1684.55 ±131.39	1649.48 ±122.47	1508.20 ±130.59	0.596
	difference	233.81 ±169.99	180.38 ±162.53	32.36 ±173.82	0.688
	P.value#	0.185	0.281	0.854	
Carbohydrate (g)	Baseline	231.47 ±22.93	228.44 ±16.93	264.01 ±18.52	0.800
	Post-intervention	271.56 ±25.45	259.51 ±21.96	238.00 ±21.37	0588
	difference	40.09 ±26.02	31.06 ±25.68	-8.01±30.04	0.426
	P. value#	0.140	0.241	0.793	
Protein (g)	Baseline	62.25 ± 7.75	69.64 ±8.76	62.27±7.71	0.770
	Post-intervention	60.44 ±5.74	66.16 ±6.98	59.30 ±6.49	0.722
	difference	-1.81 ±10.58	-3.48 ±11.49	3.42 ±10.64	0.992
	P.value#	0.866	0.765	0.751	
Fat (g)	Baseline	33.05 ±2.79	33.45 ±2.96	35.08 ±3.82	0.895
	Post-intervention	42.88 ±3.75	41.80 ±4.10	37.01 ±3.86	0.538
	difference	9.82 ±4.97	8.34 ±5.28	1.93 ±5.53	0.538
	P. value#	0.063	0.131		
Vitamin E (mg)	Baseline	2.70 ±0.55	2.72 ±0.74	2.29 ±0.65	0.427
	Post-intervention	4.19±1.04	4.04 ±0.97	2.77 ±0.45	0.311
	difference	1.48 ±1.29	1.33 ±1.35	0.48 ±0.78	0.971
	P.value#	0.265	0.338	0.456	
Omega-3 fatty acids (g)	Baseline	0.12 ±0.03	0.13 ±0.04	0.11 ±0.04	0.963
	Post-intervention	0.21±0.10	0.11±0.05	0.10 ±0.03	0.464
	difference	0.09±0.11	-0.01±0.06	-0.01 ±0.05	0.570
	P.value#	0.428	0.821	0.781	
Omega 6 fatty acids (g)	Baseline	11.47 ±0.93	10.80 ±1.17	10.89 ±1.76	0.926
	Post-intervention	13.76 ±1.95	13.58 ±2.15	12.87 ±2.19	0.148
	difference	2.29 ±2.21	2.78 ±2.59	1.98 ±2.91	0.534
	P.value#	0.273	0.380	0.506	
Saturated fatty acids (g)	Baseline	8.17 ±6.28	9.73 ±1.05	10.33 ±1.19	0.333
	Post-intervention	9.16 ±0.80	8.98 ±0.77	10.11 ±1.13	0.649
	difference	0.99 ±1.07	-0.75 ±1.28	-0.22 ±1.65	0.643
	P.value#	0.367	0.566	0.897	

OP, omega-3 fatty acid and placebo; OE, omega-3 fatty acid and vitamin E; PP, placebo and placebo; *mean±SE; *ANOVA analysis; #paired T test

Table 4. Serum antioxidant enzymes, TAC and hsCRP values of the study groups before and after the intervention

Treatment group		**OP (*n* = 20)**	**OE (*n* = 21)**	**PP (*n* = 19)**	***P*.value***
Catalase (mg/dl)	Baseline	71.35±2.22	64.43 ±2.16	65.00 ±2.67	0.073
	Post-intervention	76.00 ±2.13	71.19 ±2.62	66.58 ±2.83	0.042
	difference	4.65 ±1.74	6.76 ±1.94	2.44 ±2.16	0.303
	P.value#	0.015	0.002	0.273	
SOD (mg/dl)	Baseline	175.05 ±25.97	139.47 ±7.46	194.85 ±26.63	0.183
	Post-intervention	167.48 ±18.31	134.68 ±6.40	183.34 ±25.35	0.164
	difference	-7.55 ±13.11	-4.16 ±6.04	-8.94 ±7.55	0.936
	P.value#	0.571	0.499	0.252	
GPX (mg/dl)	Baseline	1.74 ±0.65	1.70 ±0.045	1.85 ±0.056	0.163
	Post-intervention	1.81 ±0.068	1.81 ±0.045	1.79 ±0.057	0.959
	difference	0.068 ±0.072	0.12 ±0.065	-0.052 ±0.078	0.243
	P.value#	0.353	0.086	0.514	
TAC (mg/dl)	Baseline	112.26 ±23.04	113.91 ±21.15	79.40 ±3.13	0.342
	Post-intervention	116.30 ±22.81	119.32 ±21.07	81.88 ±3.50	0.316
	difference	4.04 ±2.61	7.58 ±2.62	2.45 ±3.15	0.418
	P.value#	0.138	0.009	0.446	
hsCRP (mg/dl)	Baseline	2.76 ±0.48	3.12 ±0.55	3.34 ±0.45	0.720
	Post-intervention	1.80 ±0.18	1.60 ±0.23	3.57 ±0.64	0.001
	difference	-0.96 ±0.46	-1.21 ±0.48	0.23 ±0.64	0.132
	P.value#	0.050	0.008	0.716	

OP, omega-3 fatty acid and placebo; OE, omega-3 fatty acid and vitamin E; PP, placebo and placebo; hsCRP, high-sensitivity C-reactive protein; *mean±SE; *ANOVA analysis; #paired T test

Table 5. Gene expression of SIRT1and PGC1α in the study groups[#]

	OP (*n* = 22)	**OE (*n* = 20)**	**PP (*n* = 20)**	***P* value[*]**
Gene expression of SIRT1	1.44 ±0.31	2.77 ±0.79	0.95 ±0.16	0.039
Gene expression of PGC1α	5.28 ±1.58	10.81 ±3.71	2.24 ±0.98	0.050

OP, omega-3 fatty acid and placebo; OE, omega-3 fatty acid and vitamin E; PP, placebo and placebo; #mean±SE; [*]ANOVA analysis; the values were reported based on $2^{-\Delta\Delta ct}$ calculation

Highlights

For the first time the effects of nutrients on gene expression of SIRT1 and PGC1α in humans was investigated. Vitamin E plus Omega 3 supplementation increased the gene expression of SIRT1 and PGC1α genes in CAD patients. It seems that the increase in these genes can improve the overall health of CAD patients.