

The Effects of Tocotrienols Added to Canola Oil on Microalbuminuria, Inflammation, and Nitrosative Stress in Patients with Type 2 Diabetes: A Randomized, Double-blind, Placebo-controlled Trial

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Date of Submission: Nov 26, 2012

Date of Acceptance: Feb 14, 2013

How to cite this article: Haghighat N, Vafa M, Eghtesadi S, Heidari I, Hosseini A, Rostami A. The effects of tocotrienols added to canola oil on microalbuminuria, inflammation, and nitrosative stress in patients with type 2 diabetes: A randomized, Double-blind, placebo-controlled trial. *Int J Prev Med* 2014;5:617-23.

ABSTRACT

Background: Tocotrienols (T3) were neglected in the past; today, get attentions due to their antioxidant and none-antioxidant activity. The objective of this study was to evaluate the effects of the daily intake of 200 mg T3 added in canola oil over 8 weeks on microalbuminuria, inflammation, and nitrosative stress in type 2 diabetic patients.

Methods: This study was a double-blinded, placebo-controlled, randomized trial. A total of 50 patients with T2DM and FBS >126 mg/dl treated by non-insulin hypoglycemic drugs were randomly assigned to receive either 15 ml T3-enriched canola oil (200 mg/day T3) or pure canola oil for 8 weeks. Urine microalbumin, volume and creatinine levels, serum hs-CRP, and nitric oxide (NO) levels were measured before and after intervention.

Results: From 50 patients participated in this study, 44 completed the study. There were no significant differences in baseline characteristics, dietary intake, and physical activity between groups. Urine microalbumin and serum hs-CRP were declined significantly in T3-treated group. At the end of the study, patients who treated with T3 had lower urine microalbumin (11 (9, 25) vs. 22 (15, 39.75) nmol/dl, $P = 0.003$) and hs-CRP changes (-10.91 ± 15.5 vs. -9.88 ± 27.5 Pg/ml, $P = 0.048$) than control group. A non-significant decrease was also observed in serum NO level in T3-treated group with no changes in urine volume and creatinine levels.

Conclusions: These findings indicate that T3 leads to ameliorate proteinuria and can protect the kidney against inflammation (hs-CRP) and nitrosative stress (NO).

Keywords: Diabetes mellitus, inflammations, nephropathy, nitrosative stress, tocotrienols

INTRODUCTION

Diabetes mellitus (DM) is a progressive disease, contributed to complications including nephropathy, neuropathy, micro-and macro-vascular damage.^[1] Diabetic nephropathy (DN) is one

of the major mortality factors in diabetics that often contributed to end-stage of renal disease. Approximately 30-40% of patients with type I and 15% with type II DM develop DN.^[2] Currently, immunologic and inflammatory mechanisms have been demonstrated a significant role in development and progression of DN.^[3] Vitamin E is a potent antioxidant and anti-inflammatory agent including eight different isoforms: Four saturated analogues; tocopherols (T) (α , β , γ , and δ) and four unsaturated analogues; tocotrienols (T3) (α , β , γ , and δ). The effectiveness of vitamin E supplementation in preventing or reducing diabetic nephropathy has been demonstrated in several studies.^[2,4-6] While the tocopherols have been investigated widely, little is known about the T3. Previous studies suggest that both the molecular and therapeutic effects of the T3 are different from tocopherols.^[7] Suppression of inflammatory transcription factor NF-kB, which is closely linked to inhibit the releasing of pro-fibrotic cytokines, oxidative stress, chronic inflammation, and apoptosis,^[2] is unique to the T3 and provides a significant reno-protective effects. Although T3 are known to protect more efficiently against some free radical-related diseases than T,^[8] the effects of T3 on renal function in diabetes patients have not been investigated yet. Most data were derived from animal models or *in-vitro* study.^[9] Siddiqui *et al.* demonstrated treatment with PO-TRF palm-tocotrienol rich fraction as well as RBO-TRF rice bran oil-tocotrienol rich fraction significantly improved the glycemic status and renal function in type 1 diabetic rats, but PO-TRF had greater efficiency at similar dose as compared to RBO-TRF.^[10] Other study has confirmed that TRF is effective in preventing K2Cr2O7-induced acute renal injury in rats by improvement in proteinuria, proximal re-absorptive function, glomerular function, and the cellular redox status.^[8] This data indicates that kidney function of diabetic animals can be improved by T3.

On the other hand, changes in immune function are related to a variety of age-related diseases such as diabetes that have been associated with increased production of nitric oxide (NO). Proteasome is a basic regulator of inflammation that induced pro-inflammatory cytokines and nitric oxide (NO) in response to stimulation. Proteasome inhibitors can inhibit NO production by macrophages. δ -tocotrienol is a natural

proteasome inhibitor, which reduces TNF- α and NO levels.^[11]

To our knowledge, the effects of tocotrienols on nitrosative stress, inflammation and microalbuminuria in diabetic patients have not been investigated. Therefore, this study was undertaken to investigate if the treatment with tocotrienols could improve the microalbuminuria, hs-CRP, and NO levels in type 2 diabetic patients by the virtue of their anti-inflammatory activities.

METHODS

Participants

The present study was performed between November 2010 and April 2011 in Endocrinology and Metabolism Center of Tehran University of Medical Sciences. The inclusion criteria were as follows: Non-insulin type 2 diabetics aged 35-60 years, had a body mass index (BMI) of less than 40 kg/m², had fasting blood sugar (FBS) \geq 126 mg/dl, diagnosed diabetes at least 1 year before the study, free of known liver, thyroid, cancer, inflammatory and infectious disease. Participants were excluded from the study if they were pregnant or lactating women, treating with insulin, anti-inflammatory or anti-coagulant drugs, smoking, taking alcohol, nutritional supplements. The medication had not modified over the last month, and there were homogeneity regarding their treatments (Metformin: 1-1.5 gr/d, Gliclazide: 160-240 mg/d). A total of 50 participants who were followed in endocrine clinic and met the above inclusion criteria were recruited into a double-blind clinical trial through a convenience sampling. The objectives and protocol of the study were explained to the participants and written informed consent provided from them. The study was approved by the ethics committee of the School of Public Health of Tehran University of Medical Sciences (1421/18.7.2010), and was registered at the Iranian registry of clinical trials (IRCT) as IRCT201008092365N2.

Preparation of tocotrienol-enriched canola oil

Palm-based mixture of vitamin E tocotrienol was prepared commercially (Tocomas, PT.MUSIM MAS manufacturer, Malaysia, and it contained approximately 51% tocotrienols (total tocotrienols and tocopherols). The mixture consisted of approximately

38.4% total T3, 13.2% α -T3 and 16.6% γ -T3 and 16% α -tocopherol. Tocomas remaining ingredients includes 23.5% monoglycerides, 8.8% diglycerides, 0.7% triglycerides, 4.7% squalene, 9.4% phytosterols, Co Q10 and 1% fatty acids, respectively. In lab, 29400 mg tocomas added to 810 gr canola oil. Thus, a table spoon of canola oil (15 ml) contained approximately 525 tocomas, 200 mg total T3, 69.3 mg α -T3, and 87.15 mg γ -T3. Adding tocomas to canola oil did not change its appearance, odor, or taste. The bottles of T3-enriched canola oil and pure canola oil provided unlabelled, and then, an independent co-ordinator labeled these bottles with subject numbers (1-50) using the randomization list.

Design

Participants were assigned into two groups randomly by using a random number table. For this, an independent co-ordinator created the randomization list assigning participants to the T3-enriched canola oil or pure canola oil group. The participants and all investigators were blinded to the treatment allocation, except the independent co-ordinator who provided the randomization list. One group received 15 ml/day of T3-enriched canola oil ($n = 25$), containing 200 mg/day total T3, and the other received same amount of pure canola oil for 8 weeks. Two bottles (500 ml bottles) of oil were provided to each participant and consuming during the four weeks, and the bottles were returned at the end of 4-weeks duration. Participants ingested 15 ml/day of the oil after lunch or dinner, as preferred, by adding one table spoon of the oil to their cooked foods or salad. Since vitamin E isomers are sensitive to heat and oxidation, they were requested to avoid using the oil in cooking process.

Dietary intakes of energy, carbohydrate, fat, vitamin A, C, E, selenium, and zinc were monitored by 24 hour food recall, including 2 week days and 1 weekend day, at entry and end of the study. A nutritionist completed the questionnaires by a direct interview. These daily nutrient intakes were determined using nutritionist 4 (N-squared Computing, San Bruno, CA). Physical activity levels were assessed by the International Physical Activity Questionnaire (IPAQ) at beginning and end of the study.

Anthropometry measurements

Body weight and height were measured on a digital scale (model 763; Seca GmbH and Co, KG)

with participants wearing light indoor clothing. BMI was calculated by dividing weight (kg) by height (m²). All anthropometric indices were measured by following the WHO standard procedures.^[12]

Biochemical investigations

Blood samples were collected before and after the intervention after 10-12 h overnight fast and before taking the hypoglycemic drugs. Serum was obtained by centrifugation at 3000-4000 rpm for 10 min. hs-CRP was measured by particle-enhanced turbid-metric immunoassay (Roche Products, Germany). Serum nitric oxide (NO) was determined by calorimetry (calbiochem co.kit, Germany). Besides, urine excretion for 24 hours was then calculated to measure urinary albumin by immunoturbidimetry on a Cobas Bioanalyzer (Roche Products, Switzerland). Urine volume and creatinine were measured as an index of glomerular filtration rate (GFR) to confirm the microalbuminuria.

Compliance and safety

No adverse events were reported during the study. To monitor adherence to intervention, a close supervision of all participants was carried out through personal contact weekly. Compliance, assessed by measuring the remaining volume of oil in the returned bottles, was more than 95%.

Statistical analysis

The Kolmogorov-Smirnov test was applied to assess normality of data. Normally distributed data within groups were compared using paired-samples T-test and between groups by independent samples T-test. Comparison of non-normally distributed data was conducted using Wilcoxon signed-ranks and the Mann-Whitney U-test. Data are presented as mean \pm SD, unless stated otherwise. Statistical analyses were performed by SPSS version 15 (SPSS Inc., Chicago, IL). A two-tailed $P \leq 0.05$ was considered significant statistically.

RESULTS

Baseline characteristics of the 50 participants who completed the study are shown in Table 1. Six patients, 2 in T3-enriched canola oil group (one person due to immigration and the other due to changing in treatment protocol) and 3 in pure

canola group (first one due to inability to walk to center, second due to unavailable 24 hr urine collection, and third due to unwilling to end the study) withdraw the study. There were no significant differences between the groups in regard of age, sex, weight, height, BMI, disease duration, type of drug consumption, and physical activity. Likewise, no significant changes were observed for weight, BMI, and physical activity levels throughout the study. The hypoglycemic agents used were metformin ($n = 10$) or gelibenclamid alone ($n = 16$) or in combination ($n = 19$) from

the beginning of the study, in doses that remained unchanged during the study.

Dietary intake of energy, carbohydrate, fat, vitamin A, C, E, selenium, and zinc, as determined by 24 hr food recall, were not significantly different between the 2 groups before and after intervention [Table 2].

Effects of T3 on microalbuminuria and nitrosative stress and hs-CRP are presented in Table 3. Baseline variables did not differ between the 2 groups of the study entry. T3 intake significantly reduced microalbuminuria

Table 1: Baseline characteristics of the participants in the two groups

Variables	T3-enriched canola oil $n=23$	Pure canola oil $n=22$	P value ¹
Age years	55.9±5.9	55.2±5.6	0.709
Female no. (%)	18 (78.3)	15 (68.2)	0.445
Weight (kg)	64.5±17.1	68.5±12.0	0.374
Height (cm)	160.82±8.04	161.68±10.14	0.756
BMI (kg/m ²)	25.1±6.9	26.1±3.3	0.533
Duration of disease, years	4.8±4.1	4.7±2.9	0.923
Hypoglycemic medications, no. (%)			
Metformin	7 (30.4)	3 (13.6)	0.303
gelibenclamid	6 (26.1)	10 (45.5)	
Metformin+gelibenclamid	10 (43.5)	9 (40.9)	

¹ P values refer to comparisons between groups (independent t -test and Chi-square test as appropriate), BMI= Body mass index

Table 2: Dietary intakes of participants before and after 8 weeks of intervention

Variables	Group	Week 0	Week 8	Changes
		$\bar{X} \pm SD$	$\bar{X} \pm SD$	$\bar{X} \pm SD$
Energy (Kcal)	Intervention	1391±1136.68	3540±1455.67	349.32±1835.3
	Control	2928±1051.04	2867±1734	-60.70±1559.0
Carbohydrates (gr)	Intervention	415.66±159.37	507.39±148.69	91.27±208.9
	Control	400.39±173.45	410.35±216.54	9.96±287.36
Protein (gr)	Intervention	87.95±32.40	102.62±53.84	14.66±40.71
	Control	88.28±32.52	94.74±32.55	6.18±43.98
Fat (gr)	Intervention	139.54±59.22	118.61±41.88	-20.92±5.25
	Control	114.71±55.72	110.98±37.62	-7.09±56.31
Zinc (gr)	Intervention	10.24±3.64	10.83±3.82	0.598±4.53
	Control	9.63±3.30	9.00±2.81	-0.622±4.65
Vitamin A (μgr)	Intervention	1204.6±590.27	1697.5±1126.65	492.85±1224.3
	Control	1321.4±1418.28	1755.56±1924.6	434.19±2549.2
Vitamin C (mg)	Intervention	199.59±85.55	192.16±142.53	-7.43±764.84
	Control	156.77±56.33	174.26±95.68	17.49±123.66
Vitamin E (mg)	Intervention	17.27±17.04	20.35±41.18	3.07±4.25
	Control	9.46±9.01	19.46±37.63	9.74±40.48
Selenium (mg)	Intervention	0.11±0.035	0.11±0.037	0.002±0.044
	Control	0.10±0.053	0.1±0.052	0.002±0.84

Data are mean±SD, *Significantly different from baseline ($P<0.05$), **Significantly different between groups ($P<0.05$)

Table 3: The effects of T3-enriched canola oil vs. pure canola oil on urine microalbumin, inflammation and nitrosative status

Variables	T3-enriched canola oil (n=23)	P ₁ ¹	Pure canola oil (n=22)	P ₁ ¹	P ₂ ²
Serum NO (pmol/ml)					
Week 0	147.30±58.02	0.846	162.84±55.84	0.219	0.078
Week 8	142.84±59.58		179.70±68.11		
Changes	-3.00±66.2		16.85±6.08		
Urine microalbumin (nmol/dl)					
Week 0	20 (13, 37)	0.001	20 (15, 32)	0.067	0.847
Week 8	11 (9, 25)		22 (15, 39.75)		
Changes	-8 (-29, -2)		0.5 (-1.25, 10.5)		
Serum Hs-CRP (Pg/mL)					
Week 0	25.02±20.93	0.004	26.50±32.87	0.125	0.710
Week 8	14.11±11.36		15.85±18.34		
Changes	-10.91±15.5		-9.88±27.5		
Urine volume (mg/dL)					
Week 0	2055.2±710.42	0.122	1777.1±462.09	0.979	0.141
Week 8	1846.5±555.41		1780.0±549.49		
Changes	-208.68±621.60		2.85±481.82		
Urine creatinine (mg/dl)					
Week 0	1.06±0.33	0.560	1.21±0.43	0.230	0.107
Week 8	1.08±0.32		1.27±0.39		
Changes	-0.261±0.211		0.05±0.27		

Data are mean±SD or median (interquartile range, IQR), ¹P values refer to comparisons between week 0 and week 8 within groups (Paired *t*-test or Wilcoxon as appropriate), ²P values refer to comparisons between groups (independent *t*-test or Mann-Whitney as appropriate), *P* < 0.05 is significant

compared to baseline (*P* = 0.003), but volume levels and urine creatinine did not affect. Microalbuminuria was significantly lower in T3-enriched canola oil group compared to pure canola oil after the intervention (*P* < 0.001); the change was also significant when compared with canola oil group (*P* < 0.001). In T3 group, hs-CRP levels decreased significantly compared to baseline (*P* = 0.004). Hs-CRP changes in T3 group were also significant compared to canola group (*P* = 0.048). There was no difference in nitric oxide (NO) concentration between the groups and during the intervention.

DISCUSSION

Diabetic nephropathy is the most common single cause of renal damages in the world that often progresses to end-stage renal disease.^[13] The proteinuria that develops after induction of diabetes is mainly due to an increased excretion of low molecular weight proteins. Increased urinary excretion of low molecular weight proteins generally is because of decreased tubular

re-absorption of filtered plasma proteins.^[14] This is important because elevated microalbumin in urine is indicator of renal disease, especially in diabetics.^[11]

In our study, 200 mg/day T3 for 8 weeks reduced microalbuminuria in individuals with T2DM; microalbuminuria levels were also significantly lower in T3 group compared to control at end of the study (*P* = 0.001). No significant changes were observed in urine volume and creatinine levels. However, no changes in creatinine and urine volume does not imply that there are no improvement in renal function, and further analyses is needed to assign the effects of T3 on diabetic renal disease.^[13] These findings are in line with previous experimental studies, which indicated that T3 have reduced proteinuria in different doses. It has been shown that T3 supplementation for 21 days in potassium dichromate (K₂Cr₂O₇)-induced acute renal injury in rats caused an improvement in proteinuria. In another study, the effects of T3 supplementation at different doses of 25, 50, or 100 mg/day and 100 mg/kg α-tocopherol for 2 months were investigated in streptozotocin (STZ)-induced

diabetic rats. In that study, the 100 mg T3 group showed a significant improvement in polyuria and in urinary albumin excretion. In addition, in that study, T3 (100 mg/kg) was demonstrated to be more effective than α -tocopherol (100 mg/kg).^[2] Also, in agreement with our study, Siddiqui *et al.* demonstrated that administration of 200 mg/kg PO-TRF for 8 weeks significantly improved the proteinuria in diabetic rats in comparison to RBO-TRF group and control group.^[10] In the present study, palm tocotrienols have been used, and based on our search, this is the first study, which investigated the effect of tocotrienols on renal parameters in humans. Future studies on the other renal parameters such as BUN and serum creatinine are needed to confirm the nephroprotective action of T3.

Several studies have established that T3 have greater antioxidant activity than tocopherols.^[15,16] Both reduce the serum levels of C-reactive protein (CRP) and advanced glycation end products, and expression of cell adhesion molecules. The CRP-lowering effects of T3 are greater than T. Tocotrienols reduce inflammatory mediators, δ -tocotrienol being more potent, followed by γ - and α -tocotrienol.^[17] It has been shown to be antioxidant and anti-inflammatory in terms of decreasing C-reactive protein (CRP) and release of pro-inflammatory cytokines, the chemokine IL-8 and PAI-1 levels, especially at high doses.^[18] In the present study, we observed a significant reduction of hs-CRP after 8 weeks of treatment in both groups, but it was not significant in control group.

Proteasome is a basic regulator of inflammation that induced pro-inflammatory cytokines and nitric oxide (NO) in response to stimulations. Proteasome inhibitors can inhibit NO production by macrophages. δ -tocotrienol is a natural proteasome inhibitor that reduced TNF- α and NO levels.^[11] Tan *et al.*, in 2011, studied the inhibitory effects of palm alpha, gamma- and delta-tocotrienol on lipopolysaccharide-induced nitric oxide production in BV2 microglia. Microglia is an immune cell in central nervous system. Microglia were treated with varying doses of tocotrienols for 24 h and stimulated with lipopolysaccharide (LPS). All tocotrienol isoforms reduced NO release by LPS-stimulated microglia, with 50 μ M being the most potent tocotrienol dose. Of the isoforms tested, δ -tocotrienol lowered NO

levels the most, reducing NO by approximately 50% at 48 h post-LPS treatment ($P < 0.05$).^[19] In present study, T3 supplementation decreased NO levels, but it was not significant that may be due to lower concentration of δ -tocotrienol compared to other isoform of tocotrienols in tocomas. Also, Kuhad and Tiwari demonstrated that 100 mg/kg tocotrienols administration in 8 weeks can reduce nitrite levels in kidney and brain of wistar rats.^[2] In these studies, NO levels were measured in kidney and brain tissue, but in present study, NO levels have been measured in serum. Qureshi's study in 2011 was the only study, which carried out in serum levels of NO in female chicken in response to 50 ppm δ -tocotrienol supplementation. In that study, δ -tocotrienol reduced NO serum levels by 45%.^[20] In Qureshi's study, the most effective isoform of tocotrienols in reducing NO levels was assessed, but in our study, we investigated total tocotrienols. Also, the amount of δ -tocotrienol in tocomas (8.6% tocotrienols equal 17 mg) was not sufficient in humans to reduce serum NO levels. The composition of T3 isomers may modify the effects of T3 supplementation, which must be considered in future studies. In this study, we added T3 to canola oil in order to improve its compliance and absorption. Because of the low content of T3 in edible natural sources, it does not seem that the dietary sources can provide sufficient amounts of T3.^[21] Thus, promoting intakes of T3 through supplements is reasonable, especially in diabetic patients, and fortification of oil with this vitamin is an appropriate way to increase its intake.

CONCLUSIONS

In conclusion, our study shows that in type 2 diabetes patients, the intake of tocotrienols leads to ameliorates proteinuria and can protect the kidney against inflammation (hs-CRP) but does not improve nitrosative stress, urine volume, and creatinine.

ACKNOWLEDGEMENTS

We thank all the volunteers who participated in this study. This study was supported by grants from Vice Chancellor of Research, Tehran University of Medical Sciences, M/994 (Tehran, Iran). This study was a part of MSc thesis in Tehran University of Medical Sciences.

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Source of Support: Tehran University of Medical Sciences,
Conflict of Interest: None declared.