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The Effects of Dark Chocolate Consumption on Oxidative Stress and Blood Pressure in Patients with Metabolic Syndrome: A Randomized Clinical Trial

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ABSTRACT

Background: Consumption of dark chocolate has been often hypothesized to have a role in the prevention of chronic diseases. The objective of this study was to investigate the effects of consumption of dark chocolate (DC) on serum oxidative stress and blood pressure (BP) in patients with metabolic syndrome (MetS). **Methods**: An 8-week parallel randomized clinical trial involving 114 patients with MetS was conducted on stable medication in 2014. Participants were randomly assigned to three groups: 1) consume 40 g/d DC (40G), 2) consume 20 g/d DC (20G), and 3) consume no DC as the control group (CG). BP, radical scavenging activity of 1-diphenyl-2-picrylhydrazyl (DPPH), and malondialdehyde (MDA) were measured at baseline and after 8 weeks of intervention. **Results**: Mean age, weight, and body mass index (BMI) of participants were 51.38 ± 6.95 y, 77.34 ± 12.86 kg, and 28.65 ± 4.4 kg/m^2, respectively. No significant differences were found among the three groups in relation to these variables after the intervention. The mean change of systolic blood pressure in 40G, 20G, and CG were 0.31 ± 1.81, 0.37 ± 1.65, and 0.26 ± 1.56 mmHg (P = 0.3), respectively. These figures for diastolic blood pressure were obtained as 0.08 ± 1.03, -0.02 ± 1.12, and 0.22 ± 1.03 mmHg. No significant changes were observed in MDA and DPPH between three groups. **Conclusion**: Even daily intake of 40 g of DC with 76% purity for an 8-week period had no effect on body weight, BMI, BP, and oxidative stress in patients with MetS.

Keywords: *Cocoa; Dark chocolate; Metabolic syndrome; Oxidative stress biomarkers*

Introduction

Metabolic Syndrome (MetS) - also called syndrome X and the deadly quartet - is a notation for clustering of risk factors for diabetes and cardiovascular diseases (de Rooij et al., 2007, Grundy, 2006). Obesity, glucose/insulin

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disturbance, atherogenic dyslipidemia, and elevated blood pressure are among its clinical manifestations. The prevalence of MetS has enhanced in recent decades which foretells a tsunami of chronic diseases and mortality and thus puts a huge economic burden on the health services(Bremer et al., 2012, Ford, 2004).Many studies have been conducted on the association between free radicals such as reactive oxygen species and the etiology of MetS. Recently, several studies have demonstrated that the consumption of cocoa and dark chocolate (DC) has protective effects against cardiovascular diseases, in particular improvement of vascular endothelium function and blood pressure (BP) (Allen et al., 2008, Azizi et al., 2003, Mohan and Deepa, 2006, Spadafranca et al., 2010). Chocolate contains a number of different compounds such as saturated fat, polyphenols, caffeine, sterols, terpenes, and methylxanthines. Cocoa and its products have also been shown to have antioxidant properties (Mursu et al., 2004). A sparing capacity could be a potential mechanism for such beneficial effects (Spadafranca et al., 2010). Little is known about how DC exerts its effects. Wan et al., have suggested that the daily consumption of cocoa powder and DC can favorably result in improvements in serum total antioxidant capacity (Wan et al., 2001). Spadafranca et al., have also shown that DC can transiently improve DNA resistance to oxidative stress (Spadafranca et al., 2010).

In this regard, we embarked on a clinical trial to evaluate the effects of regular consumption of a 20 g or 40g serving of DC for 8 weeks on malondialdehyde (MDA) concentration, radicals scavenging activity of DPPH, and BP in MetS patients.

Materials and Methods

Design of the trial and participants: This clinical trial was conducted in Diabetic Research Center of Shahid Sadough University of Medical Sciences, Yazd, Iran in 2013-14. A total of 114 patients suffering from MetS were recruited in this intervention. Participants were screened for the following inclusion criteria: Patients with MetS, according to the definition of cholesterol education program adult treatment panel III (NCEP, ATP III) and aged 30-60 y. Exclusion criteria were personal history of cardiovascular, hepatic, and renal diseases, allergic reactions to any cocoa components, being in pregnancy or lactation, and consumption of antioxidant supplements.

After initial screening, participants were randomly categorized by using computer-generated random numbers into three groups: consuming 20g/d (20G), consuming 40g/d (40G) DC, and the third group without getting up DC (CG) for 8 weeks in a parallel design. Each chocolate bar was almost 7g and provided in coded foil wrapped containers. Patients in 20G were instructed to use 1 chocolate bar and 40G to use 2 chocolate bars three times a day, 2 hours after each meal. They were asked to keep their usual diet, drugs, and physical activity throughout the study. Compliance with DC consumption was monitored every 15 days through phone interviews. We also excluded those who consumed less than 80% of the chocolate bars.

Nutritional composition of DC product: The total polyphenolic content of the DCs was determined by spectrophotometry procedure with galic acid equivalent in biochemistry laboratory of Shahid Sadoughi University of Medical Sciences. Other nutritional contents of DC were analyzed by East Azerbaidjan Nobel Laboratory and were supervised by Institute of Standard and Industrial Research of Iran (ISIRI).The nutritional contents of the DC are provided in **Table 1**. We had used 76% purity (Idin Company, Tabriz, Iran) DC in our study. To choose a brand of chocolate in our study, we tried several kinds of chocolates. We looked for a widely available DC containing cocoa; percentage, reasonable price, and small size were also taken into account in our quest.

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Table 1. Macronutrients, and minerals of dark chocolate (Idin company, Tabriz, Iran)

Items	mg in 100 g
Protein	8.50
Carbohydrate	46.70
Fat	31.80
Fiber	1.06
Vitamin B1	0.007
Vitamin A	1.20
Vitamin E	5.60
Sodium	44.80
Calcium (mg)	345.90
Phosphorus	0.05
Iron	9.04
Polyphenols	12.3

Measurements: Waist circumference (WC) was measured at the narrowest part of midriff and hip circumference was measured at the widest circumference around the buttocks using an un-stretched tape measure. Height (cm) was measured using a tape measure to the nearest 0.1 cm without shoes on while shoulders were relaxed. Body weight (kg) was measured to the nearest 0.1 kg using a balance digital scale in light clothing (Seca, Hamburg, Germany). The body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. In addition, BP (mmHg) was measured with a standard mercurial column sphygmomanometer (Model FC-110 Deluxe, Focal Corporation, Japan) after 5 minutes resting in a seated position in a comfortable room. To avoid subjective error, all measurements were made by the same person.

All blood samples were taken after 10-12 hours of fasting at the baseline and end of the study. Plasma oxidant status was assessed immediately after acquisition of venous blood; plasma was separated by centrifugation (3000gat 4 °C for 15 min), heparinized, and then stored at -80°C until assayed.

Plasma oxidant status was evaluated using the two following methods: Circulating MDA concentration was determined by thiobarbituric acid (TBA) assay and expressed as µmol/l. The second method for oxidative stress assessment was radical scavenging activity of 1-diphenyl-2-picrylhydrazyl (DPPH) assay. Briefly, 0.1 ml of deproteinized serum (with adding 100µl acetonitrile solution to serum and centrifuging for 5min) in acetate buffered solution (10µM, pH = 7.8) was incubated in the methanolic solution of DPPH (0.1 mM). After 30 minutes in room temperature, the absorbance at 517 nm was measured. Measurements finally duplicated the free radical DPPH-scavenging activity calculated by the following formula: activity [% of DPPH reduction] = [(A−Ax)/A] × 100%, where A and Ax stand for the absorbance of DPPH solution with methanol and the absorbance of a DPPH solution with serum, respectively.

Data analyses: It was conducted using the SPSS software version 16.0. Descriptive statistics were used for the baseline characteristics of the participants. To evaluate the normal distribution of data, we performed Kolmogrov–Smirnov test. Pre- and post-intervention differences within each group were analyzed applying paired *t*-test. Further, one-way analysis of variance (ANOVA) was conducted for the three-group comparisons. In data with no normal distribution, Kruskal-Wallis test was used for three group comparisons. Within groups' value changes were analyzed by Wilcoxon test. Later, since BMI had different distribution in 3 groups of study, univariate analysis of variance was applied for adjusting its effect.

Chi-square and Fisher's exact *test* were also used to compare the frequency of qualitative variables between the three groups. Continuous normally distributed data were expressed as means ± SD, data with abnormal distribution were reported as 25, 50 (median), 75 percentile, and P-value equal or less than 0.05 was considered as significant.

Ethical considerations: Informed written consents were obtained from all participants. This study protocol was approved by the Research Ethical Committee, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. Furthermore, the study was registered at Iranian website for registry

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of clinical trials with IRCT2013022812122N2 code number (www.irct.ir). The clinical investigator and laboratory staff were blinded to the control and intervention groups.

Results

Of the 114 participants who attend our study, 20 of them declined to participate and were excluded (**Figure 1**). Thus, 94 participants (45 men and 49 women, 51.38 ± 6.95 y) completed the study. Baseline characteristics of the participants (based on three groups) are tabulated in **Table 2**. According to the results achieved through ANOVA, a significant difference was only observed in baseline weight and BMI mean among 3 groups of study, so the effects of these variables were controlled using Univariate Analysis of Variances. No significant differences were observed in other baseline variables.

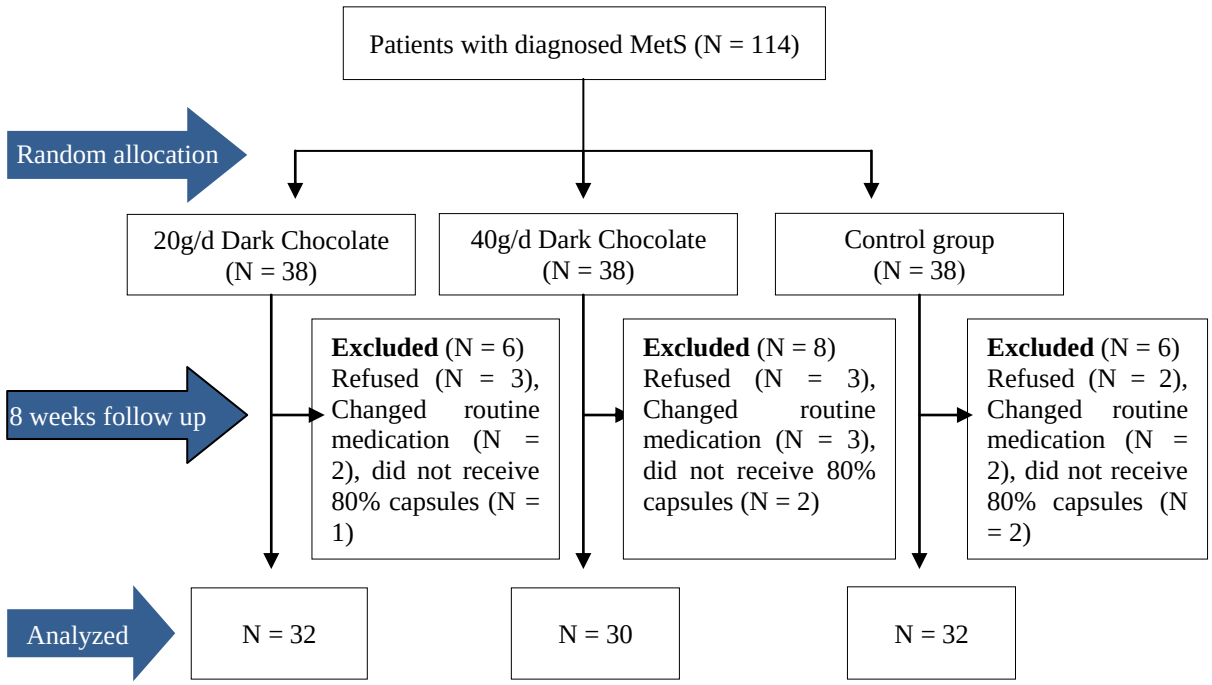

Figure 1. Study flow diagram.

Table 2. Baseline characteristics of patients in three groups' characteristics of patients in three groups

Variables/Groups	Control	20 g/d DC[c]	40 g/d DC	P-value[b]
Age (year)	52.78 ± 6.80[a]	49.62 ± 6.80	51.77 ± 7.04	0.10
Weight (kg)	75.32 ± 12.75	74.68 ± 13.11	82.13 ± 11.72	0.04
Height (cm)	164.49 ± 8.03	164.87 ± 11.53	164.00 ± 9.29	0.93
Body mass index (kg/m2)	28.01 ± 4.76	27.48 ± 4.14	30.50 ± 3.70	0.01
Waist circumferences (cm)	102.18 ± 22.95	100.81 ± 9.80	108.44 ± 7.51	0.11
Hip circumferences (cm)	110.95 ± 18.71	105.87 ± 9.56	111.72 ± 10.49	0.18

[a]:Mean ± SD; [b]: One-way ANOVA test; [c]:Dark chocolate

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The means of BP and their changes are represented in **Table 3**. According to which, there were significant differences systolic blood pressure (SBP) mean scores before and after the intervention among the three groups ($P < 0.05$). No significant differences were observed in mean change of differences ($P = 0.30$). Furthermore, no significant changes were observed within the three groups of study ($P > 0.05$). Regarding the different baseline distribution of weight and BMI among the three groups, the effects of these variables were controlled using Univariate Analysis of Variances. DC effect on SBP was not significant ($P = 0.23$). Additionally, according to **Table 3**, there were no significant differences in diastolic blood pressure (DBP) before the intervention among three groups ($P = 0.11$). The differences in DBP level were significant after the intervention among groups ($P = 0.04$). Besides, neither the mean change of differences in DBP ($P = 0.55$, nor the changes found in DBP within groups ($P > 0.05$) was significant.

Table 3. Changes in blood pressure of 3 study groups through the intervention.

Variables/Groups		40 g/d DC[c]	20 g/d DC	Control	P-value[b]
Systolic blood pressure (mmHg)	Before	13.15 ± 1.75[a]	12.07 ± 1.58	13.00 ± 1.26	0.01
	After	13.46 ± 1.43	12.38 ± 1.67	12.83 ± 1.54	0.02
	Change	0.31 ± 1.81	0.37 ± 1.65	0.26 ± 1.59	0.30
	P-value[d]	0.34	0.22	0.39	
Diastolic blood pressure (mmHg)	Before	8.35 ± 0.77	7.90 ± 1.02	8.25 ± 0.08	0.11
	After	8.43 ± 0.85	7.87 ± 0.81	8.09 ± 0.95	0.04
	Change	0.08 ± 1.03	-0.02 ± 1.12	0.22±1.03	0.55
	P-value	0.66	0.88	0.23	

[a]:Mean ± SD; [b]: One-way ANOVA test; [c]:Dark chocolate; [d]: Paired *t*-test.

Table 4. Changes of oxidative stress biomarkers among the 3 groups through the intervention.

Variables/Groups		40 g/d DC[a]			20 g/d DC			Control			P-value[c]
		25[b]	50	75	25	50	75	25	50	75	
DPPH (mg/dl)[e]	Before	16.78	46.71	80.17	26.97	42.78	90.62	26.00	62.98	104.29	0.55
	After	44.02	54.48	96.95	37.62	60.61	75.43	33.23	54.76	69.46	0.68
	Change	-17.85	23.27	49.36	-44.15	05.19	43.92	-48.94	06.57	41.67	0.49
	P-value[d]		0.12			0.92			0.99		
MDA (µmol/l)[f]	Before	51.29	68.38	109.23	40.84	67.43	104.48	47.49	73.13	278.3	0.84
	After	58.89	92.13	132.02	34.19	89.28	126.32	56.99	77.88	151.97	0.83
	Change	-25.17	15.20	61.26	-58.89	24.69	63.63	-49.27	19.95	128.23	0.90
	P-value		0.49			0.71			0.49		

[a]:Dark chocolate; [b]: Data expressed as 25, 50 (median) and 75 percentile; [c]: Wilcoxon test ; [d]: Kruskal-Wallis test; [e]: 1-diphenyl-2-picrylhydrazyl ; [f]: Malondialdehyde

The means and the mean changes of oxidative stress biomarkers among the 3 groups through the intervention are shown in **Table 4**. According to this table no significant difference was observed neither

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in MDA concentration nor in DPPH assay's results among and within the three groups ($P > 0.05$).

Discussion

In the present study, we demonstrated that a 2 months consumption of 20 g/d or 40 g/d DC with purity of 76% didn't have any significant effect on BP, MDA level, and radical scavenging activity of DPPH, which partially conflicts with the previous findings.

In contrast with present study, in Almoosawi et al.'s (Almoosawi et al., 2010) study administration of two doses of DC (500mg or 1000mg polyphenols) for a period of two weeks was helpful in reducing BP. Similarly, in Grassi et al.'s (Grassi et al., 2005) study, a daily intake of 100 g of DC for 15 days was associated with a powerful reduction of SBP and DBP. Higher intakes of DC (22g), in a study conducted by Allen et al. (Allen et al., 2008), was associated with greater reductions in SBP. Generally, the beneficial effects in these studies have been attributed to polyphenolic compounds in cocoa that are commonly abundant in DC (Mursu et al., 2004, Spadafranca et al., 2010).

However, consistent with our results, in a cohort study conducted on university graduates; no protective effect of chocolate consumption was observed on BP(Alonso et al., 2005). Furthermore, in Muniyappa et al.'s (Muniyappa et al., 2008) clinical trial, administration of cocoa drink (900 mg flavonols per day) to a group of patients with essential hypertension for two weeks did not lead to any change in BP. These different effects might be related to the intervention period and the different target populations in different studies.

Till now, several clinical trials with various designs have investigated the effects of cocoa and DC on oxidation. Contrary to our results, Fraga et al. (Fraga et al., 2005) reported a decrease in serum MDA levels after 15 days of consuming milk chocolate in young healthy adults, while those who ate white chocolate showed higher levels of oxidative stress. Rein et al. (Rein et al., 2000) and Wang et al. (Wang et al., 2000) both observed an inverse association between different amounts of flavanol-rich DC and plasma thiobarbituric acid reactive substances (TBARS) concentrations in healthy subjects 2 hours after injection. However, in agreement with our results, in a study conducted by Taubert et al. (Taubert et al., 2007), long-term consumption of DC resulted in neutral effects in the oxidative biomarker (8-isoprostane) of participants.

On the one hand, many interventions have been conducted on healthy individuals, as well as overweight, hyperlipidemic, hypertensive, and prediabetic patients (Grassi et al., 2005, Taubert et al., 2003) which mostly have shown favorable effects of cocoa and DC consumption on oxidative stress biomarkers and BP. Some of these studies mentioned oxidative stress reduction as the reason for lowering BP effect of rich cocoa products (Fraga et al., 2011, Spadafranca et al., 2010). Some other studies indicated that increased plasma or urinary nitric oxide (NO) derived species and decreased oxidative stress are associated with BP lowering effect of cocoa (Fraga et al., 2011). Yet another research, associated adequate NO level with sufficient vessel relaxation (Davison et al., 2008). NO's decreased availability was also associated with endothelial dysfunction, atherosclerosis, and MetS in a different research (Corti et al., 2009) and finally, it was indicated that NO level maintenance have different stages that can be regulated with flavonoids (Wasir et al., 2008).

On the other hand, in accordance with the present study, Vlachopoulos et al. (Vlachopoulos et al., 2005), Fisher et al. (Fisher et al., 2003), and Engler et al. (Engler et al., 2004) had not observed positive effects of DC on BP and oxidative stress status. These studies were performed on a smaller number of healthy people with smaller amounts of chocolate or were conducted in a shorter time span.

Cocoa fatty acids contain 33% oleic acid (cis-18:1 monounsaturated), 25% palmitic acid (16:0 saturated), and 33% of stearic acid in average. Monounsaturated oleic acid, occurring in high amounts in chocolates, could have been responsible for neutral effects observed In order to investigate the effects of chocolate fatty acids on different variables, we designed our study with different amounts of DC fatty acids but in similar

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proportions of the total fat content, as it was recommended by Mursu et al. (Mursu et al., 2004). However, it should be considered that as chocolate consumption may increases the proportion of fat in the diet, it consequently decreases the proportion of carbohydrates, the amount of several nutrients, and thus, may affect detrimentally the quality of the diet.

The good compliance of patients in chocolate consumption and existence of a group as the control group are the strengths of present intervention while its short-term intervention is considered as a limitation. Further studies with high polyphenolic content and different protocols can be conducted. The effects of chocolate consumption on other CVD risk factors, such as endothelial function and thrombogenic factors, can also be studied.

Conclusions

Even daily intake of 40 g of DC with 76% purity for an 8-week period had no effect on body weight, BMI, BP, and oxidative stress in patients with MetS.

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Author contributions

Mozaffari-Khosravi H participated to conception and design of study, managing the project and drafting the manuscript. Naghdipour-Biregani A and Poursoleiman F participated to acquisition of data, data analysis and drafting the manuscript. Zavar-Reza J participated to laboratory evaluation and drafting the manuscript. All authors read manuscript and they finally verified it.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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