ORIGINAL ARTICLE

Effects of Chocolate Intake on Oxidative Stress/Oxidant-antioxidant Balance in Medical Students: A Controlled Clinical Trial

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ABSTRACT

Background and Aim: Cocoa polyphenols have been shown to exhibit antioxidant properties *in vivo* and *in vitro*. This study aimed to determine whether commercially available chocolate could improve oxidant/antioxidant balance in medical students.

Materials and Methods: Sixty students (30 males and 30 females) were given three different types of chocolate. Subjects were divided equally into three groups of 20 students (10 males and 10 females) as follows: (i) Dark chocolate group (DC), (ii) milk chocolate group (MC), and (iii) placebo group (PC). The placebo group was given white chocolate. Blood was drawn at baseline and after consumption of chocolate (40 g/day) for 2 weeks. Serum was analyzed for DNA/RNA oxidative damage, thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), and glutathione peroxidase (GPX) enzymes. Descriptive analyses were conducted to determine the frequency distributions of the study variables. Means were compared across the study groups by one-way Analysis of Variance and within the same group by paired *t*-test.

Results: Mean serum DNA/RNA damage, TBARS, SOD, and GPX enzymes compared between the groups revealed insignificant differences after 2 weeks of chocolate consumption ($P = 0.46$, 0.19, 0.11, and 0.06). Comparison within the same group also exhibited statistically insignificant differences in DNA/RNA damage in DC and MC groups (0.29 and 0.46, respectively); TBARS in DC and MC groups (0.11 and 0.19, respectively); SOD in DC and MC groups (0.06 and 0.11, respectively); and GPX in DC and MC groups (0.68 and 0.78, respectively).

Conclusion: Consumption of 40 g of DC or MC daily for a period of 2 weeks appears to be an ineffective way of improving oxidant/antioxidant balance in medical students.

Key words: Academic stress, chocolate, controlled clinical trial, medical students, oxidant/antioxidant balance

ملخص البحث:

هدفت هذه الدراسة إلى التحقق عما إذا كانت الشوكولاته تلعب دوراً في تحسين مستوى الأكسدة / ومضاداتها . شارك في البحث ستون طالباً (30 ذكور و 30 إناث)، قسموا لثلاث مجموعات حسب نوع الشوكولاته وهي : 1) الشوكولاته الداكنة ، 2) الشوكولاته بالحليب 3) الشوكولاته البيضاء. وقد أجاب المشاركون في الدراسة على استبانه معيار المستوى التصوري للإجهاد بعد تناول 40 غرام شوكولاته يوميا ولمدة أسبوعين. توضح نتائج المتوسطات الحسابية عند مقارنتها بين مجموعات الدراسة باستخدام تحليل التباين الأحادي (أنوفا) أن الاختلافات ضئيلة وغير

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مؤثرة إحصائيا بعد استهلاك الشوكولاته لمدة أسبوعين ($P=0.46$) وعند مقارنة المتوسطات الحسابية ضمن المجموعة نفسها أظهرت النتائج عدم وجود اختلافات ذات دلالة إحصائية في مستوى الضرر على DNA/RNA وخصوصا ضمن مجموعتي الشوكولاته الداكنة والشوكولاته بالحليب. يعتبر تناول 40 غراما من الشوكولاته الداكنة والشوكولاته بالحليب يوميا لمدة أسبوعين طريقة غير فعالة في تحسين مستوى التأكسد عند طلاب الطب.

INTRODUCTION

Oxidative stress (OS) is defined as the imbalance between reactive oxygen species and antioxidants in our body.[1] It may result from either an increased production of reactive oxygen species (ROS) or from an incapability of the antioxidant system to effectively remove the ROS.[2] OS is destructive for cell components, such as proteins, lipids, and DNA/RNA and can even cause disturbances in normal cell signaling mechanisms.[3] It is also believed to play a role in several human pathological states such as cardiovascular diseases and diabetes mellitus.[4,5]

Epidemiological studies suggest that regular consumption of foods and beverages rich in flavonoids, such as cocoa, is associated with emerging health benefits including a decreased risk of cardiovascular mortality owing to its significant natural antioxidant properties.[6,7] However, despite the well-known health properties of cocoa, its consumption in pure form is extremely rare owing to its bitter taste, mainly caused by the cocoa polyphenols and methylxanthines. The most widely consumed cocoa-derived confectionary product all over the world is chocolate. Surprisingly, few studies have explored the modulation of OS caused by chocolate. Most of the studies on beneficial effects of cocoa-derived products or chocolate have used pure extracts or special formulations (having high contents of flavonoids) instead of commercially available chocolate which people usually consume.[8-10] Thus, cocoa-induced benefits seen in those studies cannot be applied to commercially available chocolate. This has led to confusion among consumers. Even the published data regarding the effects of chocolate/cocoa on oxidant/antioxidant balance *in vivo* provide conflicting results. Few studies have shown an increase, in general, antioxidant defense with dark chocolate (DC) supplementation; others have shown no effect at all.[11-14]

Most studies have explored the effects of chocolate consumption in subjects under normal conditions when their natural antioxidant defenses are not compromised or challenged.[11-14] None of the studies have explored the beneficial effects of DC when the subjects are exposed to increased OS secondary to pathological or physiological stress. One of the physiological stress models is examination stress experienced by students. Students are expected to suffer from acute stress immediately before an examination, and chronic stress during an extended examination period.[15] The purpose of this study was to determine and compare OS and antioxidant activity of commercially available DC and milk chocolate (MC) with a placebo in university students, allegedly suffering from chronic stress, during an extended examination period.

MATERIALS AND METHODS

Students from the same academic year in the College of Medicine, University of Dammam were targeted. Inclusion criteria were (i) medical student, level 2; (ii) age range 18–20 years; (iii) body mass index = 18.5–24.9 m/kg$^{2;}$ (iv) nonsmoker; and (v) willing to consume 40 g of chocolate daily for 2 weeks. Exclusion criteria included (i) use of any medication; (ii) illness such as endocrine, metabolic, or psychiatric; (iii) pregnancy; (iv) females expected to menstruate during chocolate intake; and (iv) inaccessibility to follow-up.

Participants were drawn by nonprobability convenience sampling from the 2nd year medical students solely on a voluntary basis. Students who volunteered were reassured that all information obtained will be kept confidential. For sample size calculation, (based upon a pilot study on five volunteers, the results of whom were not included in the final data) the values of mean (± standard deviation [SD]) DNA/RNA oxidative damage, thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), and glutathione peroxidase (GPX) enzymes in the DC, MC, and PC group were assumed. At a power of 90% and at an alpha level (*P* value) of 0.05, the sample size was calculated to be 60 students (30 males and 30 females) to participate in the study. The subjects were advised to discontinue the intake of cocoa and chocolate other than the study chocolate 1-week prior to the start of study and maintain this restriction throughout the study period.

Based on the type of chocolate given, subjects were divided equally into three groups of 20 students (10 males and 10 females per group) as follows: (i) DC group, (ii) milk chocolate group (MC), and (iii) placebo group (PC).

The placebo group was given white chocolate. All the three different kinds of chocolate were bought from a local shop. These chocolates were similar in their chemical composition, nutrients content, and calories per bar except the amount of cocoa solids (DC = 35%, MC = 25%, PC = 0%); and milk solids (DC = absent, MC = present, PC = present). Special care was taken to ensure that study was scheduled during the period of the students' examinations; the time when students were under maximum academic stress. On the first visit, subjects were briefed about the study, and the study chocolate was given. Following this, 5 ml blood was extracted from each subject by a trained healthcare professional during the first visit (day 0) and on the second visit (day 14). Blood samples were allowed to clot and centrifuged within 30 min after venipuncture. The obtained serum was frozen at –80°C until further analysis by ELISA Kit.

Oxidized guanine; a product of DNA and RNA oxidative damage and TBARS; a product of lipid peroxidation were measured by Cayman Chemical EIA kits Item Numbers 589320 and 10009055, respectively, to reflect the amount of OS the subject was exposed to. SOD (Cayman Chemical EIA Kit 706002) and GPX (Cayman Chemical EIA kit 703102) were the antioxidant enzymes which were measured.

Permission and Ethical Approval to conduct this nonrandomized, parallel controlled clinical trial was sought and granted by the University Deanship of Scientific Research.

Statistical analysis

Statistical analysis of the data was performed using SPSS version 20 (IBM, Armonk, NY, United States of America), and the results were expressed as (means ± SEM). The difference between baseline and end-point values within the pooled groups were tested by paired *t*-test. Means were compared across the study groups by one-way analysis of variance (ANOVA). Differences with $P \leq 0.05$ were considered significant.

RESULTS

Overall, compliance with the nutritional instructions and restriction was achieved, as none of the subjects reported consumption of the restricted foods (cocoa and chocolate products other than the study chocolate). Baseline DNA/RNA oxidative damage, TBARS, SOD, and GPX levels among all three groups compared by ANOVA revealed statistically insignificant differences (*P* value 0.46, 0.19, 0.11, and 0.06, respectively) [Tables 1-4], meaning that our groups were not different from one another in terms of oxidant-antioxidant balance at the beginning of the study. Likewise, there were statistically insignificant differences in DNA/RNA damage, TBARS, SOD, and GPX levels after 2 weeks of chocolate intake among all three groups (*P* values 0.61, 0.29, 0.48, and 0.47, respectively) [Tables 1-4]. Paired *t*-test was used for comparison before and after chocolate intake within the same group, and it exhibited statistically insignificant differences in all four parameters in the three groups [Tables 1-4]. When males and females were compared in each group for DNA/RNA oxidative damage, TBARS, SOD, and GPX enzyme, statistically insignificant differences were seen in all three groups [Tables 5 and 6].

DISCUSSION

The principal finding is a lack of favorable impact of chocolate intake on oxidant/antioxidant balance in young Saudi medical students. Our results are consistent with a study conducted by Wiswedel *et al*., who did not observe any significant changes in OS/antioxidant markers after flavonol-rich cocoa consumption.[16] In another report, these markers remained unchanged after dietary intervention with cocoa.[17] Likewise, our results are in accordance with Engler *et al*., who assessed OS by measuring oxygen radical absorbance capacity and reported no changes after DC intake.[14] Similarly, Heiss

Table 1: DNA/RNA oxidative damage in all three groups before and after chocolate supplementation

Groups	DNA/RNA oxidative damage (ng/ml) (mean±SEM)		*T*	*P* (paired *t*-test)
	Preintervention	Postintervention		
Dark chocolate	340.13±35.33	314.96±36.31	5.35	0.29
Milk chocolate	363.86±42.48	379.55±33.05	−0.288	0.77
Placebo chocolate	382.53±39.01	352.48±32.65	0.663	0.52
P (ANOVA)	0.46	0.61		

SEM – Standard error of mean; ANOVA – Analysis of variance

Table 2: TBARS in all three groups before and after chocolate supplementation

Groups	TBARS (µmole/liter) (mean±SEM)		*T*	*P* (paired *t*-test)
	Baseline	Postintervention		
Dark chocolate	2.50±0.08	2.29±0.13	1.69	0.11
Milk chocolate	2.24±0.10	2.28±0.13	−0.247	0.81
Placebo chocolate	2.49±0.15	2.54±0.14	−0.337	0.74
P (ANOVA)	0.19	0.29		

TBARS – Thiobarbituric acid reactive substances; SEM – Standard error of mean; ANOVA – Analysis of variance

et al. did not find any favorable change in OS/antioxidant markers after a daily cocoa drink intake over a 1-week period.[13]

Our findings are in contrast to Allgrove *et al*.,[18] and Davison *et al*.,[19] who reported a significant reduction in oxidative-stress markers following DC intake. The reason for the discrepancy between Allgrove, Davison, and our study might be because they both used DC containing 70% cocoa solids with high polyphenol contents in contrast to our study which used DC containing 35% cocoa solids with low polyphenol content. Moreover, Allgrove's subjects received a higher dose compared to ours; 40 g twice daily versus 40 g once daily. Likewise, Davison's subjects received 100 g of DC daily. Mellor *et al*. also showed a significant decrease in OS marker with the use of chocolate containing high polyphenol compared to chocolate with low polyphenol.[20] Wan *et al*.,[21] also showed favorable effects of 4 weeks of DC intake on reducing OS. Compared to our study, Wan's study subjects consumed 22 g cocoa powder, along with DC on a daily basis. In addition, their intake period was 4 weeks. Another report showed a decrease in TBARS *in vitro* and *in vivo* after 3 weeks of 50 g DC (rich in procyanidin 100 mg/100 g) supplementation on a daily basis.[11]

Furthermore, our results are in contrast to Fraga *et al*.,[12] who showed a reduction in OS markers following the consumption of 105g of chocolate (168 mg flavonols) over 14 days. There is convincing evidence for the *in vitro* antioxidant potential of pure cocoa. However, whether this *in vitro* antioxidant capacity decodes to *in vivo* antioxidant capacity postconsumption is dependent on many factors such as the absorption, bioavailability; and food matrix in which the cocoa polyphenols are

Table 3: SOD in all three groups before and after chocolate supplementation

Groups	SOD (IU/ml) (mean±SEM)		*T*	*P* (paired *t*-test)
	Baseline	Postintervention		
Dark chocolate	34.22±3.48	26.01±1.26	2.381	0.06
Milk chocolate	28.40±1.67	28.29±2.13	0.034	0.97
Placebo chocolate	27.05±1.96	25.86±1.16	0.458	0.65
P (ANOVA)	0.11	0.48		

SEM – Standard error of mean; ANOVA – Analysis of variance, SOD – Superoxide dismutase

Table 4: GPX in all three groups before and after chocolate supplementation

Groups	GPX (μM) (mean±SEM)		*T*	*P* (paired *t*-test)
	Preintervention	Postintervention		
Dark chocolate	11.07±0.75	11.45±0.73	−0.426	0.68
Milk chocolate	14.11±2.43	13.28±1.04	0.281	0.78
Placebo chocolate	20.15±3.75	12.60±1.33	1.84	0.08
P (ANOVA)	0.06	0.47		

SEM – Standard error of mean; ANOVA – Analysis of variance; GPX – Glutathione peroxidase

Table 5: SOD and TBARS in males and females of all three groups (pre- and post-intervention)

Gender	Groups	SOD in IU/ml (Mean±S.E.M)			TBARS in μmol/liter (Mean±S.E.M)		
		Pre-intervention	Post-intervention	*P* value	Pre-intervention	Post-intervention	*P* value
Females	Dark Chocolate	35.00±4.82	26.01±1.85	0.09	2.44±0.13	2.20±0.17	0.13
	Milk Chocolate	26.95±2.09	25.86±3.38	0.83	2.01±0.09	2.36±0.19	0.11
	Placebo Chocolate	29.25±3.25	23.27±1.14	0.13	2.22±0.13	2.42±0.25	0.36
Males	Dark Chocolate	33.43±5.28	26.01±1.81	0.19	2.56±0.10	2.38±0.19	0.41
	Milk Chocolate	29.85±2.62	30.71±2.53	0.85	2.47±0.16	2.20±0.18	0.3
	Placebo Chocolate	24.85±2.15	28.46±1.71	0.29	2.77±0.25	2.66±0.15	0.61

SOD – Superoxide dismutase; TBARS – Thiobarbituric acid reactive substances; SEM – Standard error of mean

Table 6: DNA/RNA oxidative damage and GPX in males and females of all three groups (pre- and post-intervention)

Gender	Groups	DNA/RNA oxidative damage in ng/ml (Mean±S.E.M)			GPX in μM/liter (Mean±S.E.M)		
		Pre-intervention	Post-intervention	*P* value	Pre-intervention	Post-intervention	*P* value
Females	Dark Chocolate	398.75±41.90	288.95±14.81	0.72	10.30±0.95	9.92±1.15	0.78
	Milk Chocolate	364.75±59.54	241.69±24.23	0.13	11.04±0.92	13.62±1.52	0.14
	Placebo Chocolate	292.29±50.53	313.46±30.54	0.36	25.4±1.76	13.84±2.18	0.17
Males	Dark Chocolate	507.51±33.07	440.98±42.63	0.28	11.84±1.17	12.98±0.65	0.38
	Milk Chocolate	486.03±60.97	394.95±31.91	0.20	17.17±3.25	12.95±1.49	0.47
	Placebo Chocolate	391.50±56.84	472.77±45.40	0.55	14.89±2.31	11.35±1.53	0.23

SEM – Standard error of mean; GPX – Glutathione Peroxidase

delivered.[22] Factors such as their metabolic conversion in intestinal cell, liver, and other tissues, binding to proteins and accumulation in cells and urinary elimination rate will all affect their efficacy *in vivo*[23], and consequently, vigilant distinction between the *in vitro* and *in vivo* effect of cocoa flavonols is needed.

Processing of cocoa beans into commercial chocolate may result in loss of flavonoids as flavonoids are deliberately removed during processing because of their bitter taste. The inability of the DC to reduce the OS in our study may explain that by constantly "improving" or "adjusting" taste, the general health potential of cocoa is simply lost in commercially prepared chocolate. There may be limited chances of retaining significant health components in chocolates. Hence, there seems to be a need to re-visit the "recipe" of commercial chocolate to preserve the healthy components and gain the full benefit from cocoa. The absence of any gender-specific difference in oxidant-antioxidant balance following chocolate intake excludes the role of sex hormones.

CONCLUSION

The results of this study revealed that DC or MC intake (40 g/day for 2 weeks) appears to be ineffective in modulating oxidant/antioxidant balance in young Saudi male and female medical students during their examination periods.

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Conflicts of interest

There are no conflicts of interest.

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