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Research Article

Effect of the Dark Chocolate Consumption on Some Markers of Oxidative Stress, Endothelial Dysfunction and Inflammation of a Healthy Population

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Abstract

Several studies have indicated the benefits of dark chocolate consumption in human being, due to the polyphenols from the cocoa that give to its antioxidant capacity. This antioxidant capacity delay the oxidation of low density lipoprotein (LDL), and reduce the plasma concentration of F2-isoprostans, besides inducing the vasodilation dependent on nitric oxide, thereby reducing the cardiovascular risk. The objective of the study was to evaluate the effect of dark chocolate consumption on lipid markers, oxidative stress, endothelial dysfunction and inflammation in a metabolically healthy population. In order to perform the study 9 healthy metabolically adult individuals were selected by using a inclusion and exclusion criterium. The fasting individuals were evaluated in their nutritional level (anthropometrics and dietary indicators) and biochemical: glycemia, cholesterol, triglycerides, high density lipoprotein (HDL) and low density lipoprotein (LDL) before and after consuming 20 g/day of dark chocolate with 70% of cocoa solid during a 1 week. The determinations of the markers: 8-isoprostane (oxidative stress), nitric oxide (endothelial dysfunction) and interleukin 6 (inflammation) were also performed. It was observed a statistically significant diminution ($p < 0.05$) of the arterial pressure, in the oxidative stress marker 8-isoprostanes and Interleukin-6 and an increment of the nitric oxide (NO) in the consumer blood, possibly due to the effect of polyphenols present in dark chocolate. Lipid profile markers remained unchanged. It is concluded that consumption 20g per day of dark chocolate has a possible beneficial effect on cardiovascular health, due to the nitric oxide activation on blood, and its anti-inflammatory and antioxidant effect.

Keywords: *Dark Chocolate; Cardiovascular Markers; Oxidative Stress; Oxidative Stress; Inflammation*

Introduction

As chocolate was considered as candy, many physicians tended to warn patients about the potential health hazards of consuming large amounts of chocolate. Earlier, chocolate used to be criticized for its fat content and its consumption was a sin rather than a remedy, associated with acne, caries, obesity, high blood pressure, coronary artery disease and diabetes. However, the recent discovery of biologically active phenolic compounds in cocoa [1,2]. has changed this perception and stimulated research on its effects in ageing, oxidative stress, blood pressure regulation, and atherosclerosis [2-4]. Today, dark chocolate is praised for its antioxidant potential [2], and its benefits have been point out as a result of the polyphenols present in cocoa [1], which delay the oxidation of low-density lipoprotein and reduce the plasma concentration of F2-isoprostans, further induce the vasodilation dependent on nitric oxide, thereby reducing cardiovascular risk. Moreover, it has been considered that the dark chocolate may improve cardiac function and relief of angina, stimulation of the nervous system, which facilitates digestion and improves bowel function [4,5]. Beside of the proven of antioxidant capacity the chocolate has plenty mineral contents such as; iron, magnesium, copper, manganese potassium, phosphorus, zinc and selenium. However, in many studies,

contradictory results and concerns about methodological issues have made it hard for health professionals and the public to understand the available evidence on chocolate's effects on health [1]. Then the goal the research was to evaluate the effect of the dark chocolate consumption on cardiovascular risk markers; such as: the 8-isoprostane (for oxidative stress), nitric oxide (for endothelial dysfunction) and interleukin 6 (for inflammation).

Materials and Methods

Nine (9) metabolically healthy volunteers (78% female and 22% male) aged between 28 to 45 years were selected from a healthy population of consumers. In order to complete the selection, the subjects were evaluated at the Laboratory of Endocrinological and Metabolic Diseases of the Military Hospital "Dr. Carlos Arvelo", and by using a previously established criteria, those subjects with chronic diseases, diabetes, infections, diseases of immunological origin, obesity treated with surgery, as well as subjects with habitual consumption of alcoholic beverages or drugs, pregnant women, with antiplatelet or anti-oxidant treatment, and those allergic to chocolate were excluded from the study.

All volunteers included in the study have signed the informed consent, where they agreeing to participate. All participants, who were not excluded, previously were tested for their inclusion in order to be sure that they were metabolically healthy. It was assessed that they were normotensive, that they hematological, insulin, cortisol, TSH, and T4 indexes were normal, and that they body mass index were between 18 y 29,9.

In addition, a survey was conducted regarding their preference for dark chocolate, and whether they would rigorously will perform the study by consuming only the 20 g/day of dark chocolate at 70% supplied for one week, without the intake of another type of chocolate. The study was approved by the Ethics Committee of the Military Hospital Dr. Carlos Arvelo. Caracas Venezuela.

Subjects were carefully instructed to maintain their diet and refrain from consuming foods and drinks rich in flavonoids, including wine and other alcoholic beverages. All participants in the study were given a list of foods and beverages they should not consume.

The chocolate supplied was acquired from the local market and it had a composition of 70% cocoa and 30% sugars. The total polyphenols content of 39.20 ± 0.26 mg of gallic acid equivalents (GEA) /100 g chocolate [6,7], with an antioxidant capacity of 1.07 g/g ± 0.04 2,2-diphenyl-1-picrylhydrazyl (DPPH) [8]. Each individual was given 140g of dark chocolate for consumption throughout the week. The pack of 140g consisted of a presentation of 14 tablets, for a daily consumption of 2 tablets of 10g each.

Total Polyphenols in Chocolate

Total polyphenols were measured by the method adapted from Ainsworth and Gillespie [6] and Cicco., *et al* [7]. A sample of 100 mg chocolate was mixed with 1 mL 70% methanol using a vortex and extracted for 1 h with constant agitation. The mixture was centrifuged at 15000 rpm for 15 min and the liquid phase was removed for posterior analysis. An aliquot of the extraction solution (100 µL) was mixed with 200 µL of Folin-Ciocalteu (diluted 1:10 in distilled water) for 2 min. Then 800 µL of Na_2CO_3 700 mM was added and the reaction was allowed to proceed in the dark and at room temperature for 2 hours. 100 µl were analyzed after that the reaction spectrophotometrically at 710 nm and absorbance measure was collated with the calibration curve obtained by the reaction of Folin-Ciocalteu with different concentrations of gallic acid (15 - 150 ppm). Results were expressed in grams of gallic acid equivalents (GAE) per 100g of sample.

DPPH antioxidant capacity in chocolate

Antioxidant capacity was measured by measuring the radical scavenging properties of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) using the methodology reported by Rufino., *et al* [5]. About 50 mg of chocolate were subjected to extraction with 1 mL of a mixture methanol: water acidified (pH = 1) with constant stirring for one hour in vortex and after this time the sample was centrifuged at 15000 rpm for 15 min. The liquid phase was withdrawn and the solid phase was subjected to a second extraction with 1 mL of acetone: water with constant stirring for one hour in vortex thereupon the sample was centrifuged at 15000 rpm for 15 min. Both liquid phases were pooled and brought to a volume of 2 mL. The extraction was immediately stored at -18°C for the shortest time possible before spectrophotometric

analysis. Quantification was performed by mixed with 40 µL of sample, 1960 µL of methanol solution of DPPH (60 µM). The absorbance at 515 nm was recorded every 20 seconds while the reaction was conducted in the cell to achieve equilibrium. The absorbance at equilibrium was compared with the calibration curve of DPPH absorbance to calculate the number of free radicals in the reaction. The absorbance at equilibrium was transformed into trolox equivalents by using a standard trolox calibration curve prepared from concentrations between 0.5 and 1 mM). Trolox was used as a reference antioxidant.

Nutritional Assessment

The anthropometric evaluation that have included measurements of body weight, maximum height and waist circumferences, were performed following the methodology of the International Biology Program [9]. TANITA® UM-080 digital scale, fiberglass tape attached to the wall, and fiberglass, narrow, flexible and non-elastic anthropometric tape were used. With the measures obtained the body mass index (BMI) was determined using the classification of the World Health Organization [10]. The dietary assessment was performed before and after the intervention study through the application of the food recall reminder technique of a usual or typical day [11,12]. The calculation of Calories and nutrients were calculated following the data of the Venezuelan Food Composition Table [13].

Biochemical and paramedical evaluation

The nine (9) healthy adult subjects were evaluated biochemically before and after consuming 20 g/day of dark cocoa at 70% for 1 week. From each subject with 14-hour fast was removed 30 ml of peripheral blood and poured into 6 Vacutainer tubes with EDTA and 6 without EDTA. Tubes were and centrifuged at 1000 rpm for 20 minutes, and the serum and plasma were separated to determine: glycemia, total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol by Roche Diagnostic CA. The determination of Interleukin 6 (IL6) was completed by the use of a commercial Endogen Human kit with standard reference values plasma of 0 - 5 pg/ml. The 8-Isoprostane, was done using the commercial kit of Endogen Human with standard reference values of plasma 40 - 100 pg/ml. Determination of Nitric Oxide Synthase (NOs), by colorimetric method of Calbiochem Commercial Kit. Both of them were measured using a Microplate Reader Elisa Biotek Instruments, Inc. On each individual were also measurements, before and after consuming dark chocolate. their systolic (SBP) and diastolic blood pressure (DBP) with a mercury sphygmomanometer and with a dynamap with a 48 x 14 cm cuff, according to the technique described by Contrera, 2000 and Fragachan, 2001 [14,15].

Statistical Analysis

For statistical calculation, a database was made in the Office Excel 2007® program, the statistical treatment was performed using the database, which included the mean and standard deviation of the quantitative variables ($p < 0.05$). Student's T test was used with the Statistical Package for the Social Sciences (SPSS®) for Windows®, version 22. 2016.

Results and Discussion

Table 1 shows the mean and standard deviation of the anthropometric measurements and indicators, where there are not observed significant differences in weight, height, body mass index and waist circumference due to the effect of dark chocolate consumption, as well as That a study of 49 healthy women over 6 weeks showed no effect on weight gain after daily consumption of 41g of chocolate, 60g of almonds, or almonds and chocolate together [16].

Variables and indicators n = 9	Before	After
Weight (Kg)	61.64 ± 7.31[a]	61.23 ± 7.28 [a]
Size (m)	1.62 ± 0.08[a]	1.62 ± 0.08[a]
BMI (Kg/m^2)	23.23 ± 2.70[a]	23.10 ± 2.70[a]
Wait Circumference (cm)	84.21 ± 6.53[a]	83.99 ± 7.19[a]

Table 1: *Effect of the dark chocolate consumption on the anthropometric measures.*

The results are expressed as the mean ± standard deviation. Average followed by different letters in the same row are significantly different ($p < 0.05$).

There were no differences due to the effect of dark chocolate consumption on the dietary indicators. On average, the study participants maintained their constant intake: of energy, animal and vegetable protein, animal and vegetable fat, carbohydrates and fiber before and after the study.

Table 2, shows the effect of dark chocolate consumption on the paramedical evaluation. It is observed significant differences for systolic and diastolic pressure. A 2003 study [17] indicated a reduction in systolic and diastolic pressure in elderly hypertensive patients consume chocolate. Other studies [18,19] are showing a decreasing of 12 mmHg in blood pressure during day and night, according to an ambulatory evaluation completed for 24 hours, after the intake of 100g dark chocolate rich in flavonoids, daily for 2 weeks.

Parameters n = 9	**Before**	**After**
Systolic blood pressure (mmHg)	115.50 ± 7.53[a]	112.75 ± 8.52[b]
Diastolic blood pressure (mmHg)	81.50 ± 6.32 [a]	79.67 ± 4.29[b]
Glycemia (mg/dl)	86.00 ± 4.58 [a]	86.22 ± 6.92 [a]
Total Cholesterol (mg/dl)	171.78 ± 25.98 [a]	167.00 ± 19.96[a]
Triglycerides (mg/dl)	83.22 ± 20.41 [a]	88.44 ± 24.69 [a]
HDL-Cholesterol (mg/dl)	52.11 ± 9.57 [a]	50.56 ± 7.91 [a]
LDL-Cholesterol (mg/dl)	108.00 ± 25.02 [a]	102.33 ± 19.62 [a]
8-Isoprostane (pg/ml)	57.08 ± 11.44 [a]	51.31 ± 10.45[b]
Nitric oxide (μM)	19.37 ± 6.45 [a]	24.87 ± 3.67 [b]
IL6 (pg/ml)	4.02 ± 0.79 [a]	3.55 ± 0.89 [b]

Table 2: *Effect of consumption of dark chocolate in the quantification of blood pressure, blood glycemia, lipid markers of oxidative stress, inflammation and endothelial dysfunction in a metabolically healthy population.*

The Dutch study Zutphen corroborated that the cocoa intake was inversely proportional to blood pressure, and it was associated with a reduction in cardiovascular disease and mortality [20]. Other studies have shown no effect on the arterial pressure for effect the dark chocolate consumption [21-24].

Several studies point out to the beneficial effect of dark chocolate consumption on the lipid profile. Kondo., *et al*. [25] showed that cocoa inhibits the oxidation of LDL. In healthy individuals, daily consumption of 75g of dark chocolate with polyphenols, for more than 3 weeks have increased the HDL cholesterol by up to 14%, and have inhibits lipid peroxidation [26]. On the other hand, a study in hypertensive patients, where daily consumption was 100g of dark chocolate rich in flavonoids for more than 2 weeks, have produced a significant reduction of 12% levels of total cholesterol and LDL in blood serum [18]. Another study showed that in patients with hypercholesterolemia, the consumption of dark chocolate rich in flavanols have decreased the plasma levels of LDL, and have oxidized the LDL cholesterol with increment in the blood serum of the HDL concentrations [27].

The results are expressed as the mean ± standard deviation. Average followed by different letters in the same row are significantly different ($p < 0.05$).

The isoprostanes are eicosanoids of non-enzymatic origin formed *in vivo* from the random free radical-catalyzed peroxidation of the tissue phospholipid with oxygen. They are considered as a marker of oxidative stress determining the lipid peroxidation degree [28]. The determination of F2-isoprostane is currently the most reliable method for determining oxidative stress *in vivo*. In particular, 8-isoprostane is considered a marker of antioxidant deficit and oxidative stress in tissue in order to evaluate the vascular pathologies.

Table 2, also shows the effect of dark chocolate consumption on the formation of 8-isoprostane. It is observed a statistically significant decrease of 4.66 pg/ml in the 8-Isoprostane values when comparing the dark chocolate consumption before and after. This behavior is similar to the reported in literature [29] in a study where flavanol rich dark chocolate was supplied to the subjects. In this studied case, the consumption of dark chocolate rich in flavanols counteracts the lipid peroxidation and therefore reduces the plasma concentration of F2-isoprostanes, the marker of lipid peroxidation.

Some studies are indicative that cocoa polyphenols delay the oxidation of LDL [30]. Other studies showed a reduction in the production of reactive oxygen species in activated leukocytes [31] and also the inhibition of the DNA-oxidation induced by ultraviolet light [32].

This fact suggests that phytochemicals present in dark chocolate is promoting the induction of the endothelial NOs, which in turn elevates nitric oxide levels and reduces the production of reactive oxidant species, improving the endothelial function [33]. Certainly, the antioxidants can prevent the transformation NO into peroxynitrite, and protect against vascular damage and vasoconstriction [34].

Oxidative stress and the antioxidant defense play a crucial role in the pathogenesis of atherosclerosis, particularly in Transplant Vasculopathy (TV). In a double-blind, randomized study the effect of flavonoid-rich black chocolate was evaluated and compared to a control (cocoa-free) in patients with heart transplantation. Interestingly, the consumption of 40g of dark (70% cocoa) chocolate induced coronary vasodilation, improved coronary vascular function, and decreased platelet adhesion. These beneficial effects were also accompanied by a reduction in the serum oxidative stress, according to the data of the assessment of plasma isoprostanes that were positively related to concentrations of epicatechin in the serum [35].

The cocoa and its derivates are increasingly being recognized as a source rich in flavonoids (mainly flavanols). These flavonoids are potent antioxidants and anti-inflammatory agents with consequential benefits in cardiovascular health [36]. The cocoa flavonoids have the potential to modify a number of processes involved in the initiation and progression of the cardiovascular disease: (1) inhibiting the enzyme oxidase-nicotinamida-adenina-dinucleótido-fosfato (NADPH) dependent on superoxide production, (2) activating the enzyme nitric oxide synthases (NOs), (3) collecting superoxide, hydrogen peroxide (H_2O_2) and other oxidants that facilitate cell damage, and (4) modifying membrane-related events, leading to changes in NO and superoxide production, including effects of antioxidant, anti-inflammatory and antiplatelet, as well as improving health and functioning of the endothelial lining of the vascular system [37].

The circulating basal NO is from endothelial origin, and it exerts a regulatory effect on vascular tone; Failures in endothelial NO production are considered as endothelial dysfunction. The endothelial dysfunction is related in turn to diseases such as hypertension, aging, atherosclerosis and in the acute phase of myocardial infarction [38].

At the cardiovascular system, the level of NO produced by the endothelium is the responsible for the vasodilatory response. And this vasodilatory response is essential for the regulation of blood pressure, since, it inhibits platelet aggregation, decreases the harmful effects of atherosclerosis, protects against pulmonary hypoxia and controls the collateral circulation [39-41]. Due to the participation of NO in these functions, several investigations have been carried out, in order to verify its participation in different pathologies, such as; hypertension, cardiac insufficiency, atherosclerosis and in the acute phase of myocardial infarction. These studies have revealed faults of NO endothelial production connected to an endothelial dysfunction [38]. Alteration in endothelial function precedes the development of morphological atherosclerotic changes and can also contribute to lesion development and later clinical complications [42].

A significant increase in NO levels ranging from 19.37 ± 6.45 before chocolate intake to 24.43 ± 4.06 μM after consumption was observed in this study (see Table 2). These results agree with the findings of several authors; for example, in study with rats [43], since cocoa induces nitric oxide-dependent vasodilation in the rat aorta; as well as, in different studies performed in healthy humans [21,22,44].

In healthy smokers, the consumption of green tea has similar effects [45], and it is reinforced by studies, that pointed out an fast increment of over one third of the circulating bioactive NO and the flow-mediated vasodilation, in patients with cardiovascular risk factors, that had consuming a high-flavanol (176 to 185 mg) cocoa beverage [46,47].

Cocoa and dark chocolates are rich source of polyphenols that have potential to be used in treatments for various types of inflammation-related diseases. Polyphenols from cocoa have been revealed to have anti-inflammatory effects by assessments *in vivo* and *in vitro* [24]. The inflammation is a key mechanism in the atherogenesis, and rapid progression of coronary artery disease [48].

Di Giuseppe., *et al*. [49] have indicated that the dark chocolate contains high concentrations of flavonoids and may have anti-inflammatory properties. The authors have assessed the relationship of the black chocolate intake with serum C-reactive protein (CRP) levels, which is an inflation marker. In a study conducted in men and women over 35 years randomly recruited from a population seemingly healthy, the authors found that the consumers of up to 1 serving (20g) of dark chocolate every 3 days shown serum CRP levels significantly lower than consumers, who did not consume it. These findings suggest that a regular consumption of small doses of dark chocolate may reduce inflammation.

In order to establish the presumption, of what since cocoa flavanols have strong anti-inflammatory properties *in vitro*, its consumption could contribute to the prevention or treatment of diseases mediated by chronic inflammation. A critical review [50] judged the evidence for such effects occurring after cocoa consumption. As conclusions, the authors have pointed out that little evidence exists that consumption of cocoa-rich food may reduce inflammation, probably by lowering the activation of monocytes and neutrophils. The efficacy seems to depend on the extent of the basal inflammatory burden. Further well-designed RCTs with inflammation as the primary outcome are needed, focusing on specific markers of leukocyte activation and considering endothelial microparticles as marker of vascular inflammation.

IL-6 is a cytokine that has pro-inflammatory (as cytokine), and anti-inflammatory (as myokine) effects. For example, during infection or damage of tissues the cytokine leading to inflammation, while myokine (several hundreds of cytokines) have an anti-inflammatory response to muscular contractions [51].

IL-6 is currently considered as a marker of cardiovascular risk, because it is raise in pathologies, such as; diabetes, metabolic syndrome, obesity, atherosclerosis and ischemic heart disease.

In this study, the effect of dark chocolate intake on the inflammatory mechanism, measured by the IL-6, shown statistically significant differences, as is seen in Table 2. This tendency may be due to the circumstance that the studied population is metabolically healthy, and the effect on this marker of inflammation was evident, although other authors point out that there are controversies about the effect of chocolate consumption on IL-6 and other markers such as CRP [50].

Different authors pointed out a number of possible mechanisms through which cocoa could exert its benefits on cardiovascular health, including activation of nitric oxide, antioxidant effects, anti-inflammatory drugs and avoidance of endothelial dysfunction [52].

Conclusion

Glycemia and lipids values remained unchanged in the studied population. However, a statistically significant decrease in the 8-Isoprostane and inflammation values and an increasing in the nitric oxide values were observed. It can be concluded that the consumption of 20g per day of dark chocolate are efficient to improve the endothelial function and decreasing arterial pressure, due to it antioxidant effect can activated the production of NO with decreasing the 8-isoprostane and Interleukin-6 concentration, consequently resulting into the inherent beneficial effect on the cardiovascular health.

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Conflict of Interest

The authors of the paper certify that they have NO affiliations with or involvement in any organization or entity with any financial

interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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