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Chocolate Is a Powerful ex Vivo and in Vivo Antioxidant, an Antiatherosclerotic Agent in an Animal Model, and a Significant Contributor to Antioxidants in the European and American Diets

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Chocolate today is often viewed as a food or snack with little nutritional value. The high saturated fat content of chocolate has also contributed to the belief that its consumption increases the risk of heart disease. However, recent human studies have proven that chocolate has beneficial effects on some pathogenic mechanisms of heart disease such as endothelial function and blood pressure. Although the antioxidant properties of chocolate have been known for some time, there has been no examination of its place in the U.S. diet as a source of antioxidants. This paper demonstrates that chocolate makes a significant contribution to U.S. per capita dietary antioxidants and by inference the European Community's. In the U.S. diet chocolate is the third highest daily per capita antioxidant source. An ex vivo study shows that epicatechin, a major polyphenol in chocolate and chocolate extracts, is a powerful inhibitor of plasma lipid oxidation due to polyphenols' ability to bind to lower density lipoproteins. Conversely, the fat from chocolate alone is a pro-oxidant in this model. This is also demonstrated in an in vivo human study. After consumption of dark chocolate and cocoa powder, the lower density lipoproteins isolated from plasma were protected from oxidation compared to the lipoproteins isolated after cocoa butter consumption, which were put under oxidative stress. In an animal model of atherosclerosis, cocoa powder at a human dose equivalent of two dark chocolate bars per day significantly inhibited atherosclerosis, lowered cholesterol, low-density lipoprotein, and triglycerides, raised high-density lipoprotein, and protected the lower density lipoproteins from oxidation. Chocolate has thus been shown to have potential beneficial effects with respect to heart disease.

KEYWORDS: Chocolate; polyphenols; lipoprotein oxidation; atherosclerosis; heart disease

INTRODUCTION

Cardiovascular disease (CVD) is the primary chronic disease in industrialized societies and the number one cause of death in these populations (*1*). There have been a large number of epidemiological studies indicating that increased consumption of foods and beverages rich in flavonoids, or in more inclusive terms phenolic and polyphenolic antioxidants, reduces the risk of CVD death (*1*−*4*). Tea and red wine, both high in phenolic antioxidants, have been shown to be protective against CVD by epidemiology (*5*, *6*). However, wine's benefits may be partially due to a healthier lifestyle in wine drinkers.

Chocolate is normally not considered to be part of a heart-healthy lifestyle due to its high caloric and stearic acid content. In fact, chocolate is not a main contributor to stearic acid or total saturated fat intake in the U.S. diet (*7*). However, consumption of fat creates an oxidative stress, which results in an acute impairment of endothelial function and platelet aggregation, as well as enrichment of lipoprotein with triglycerides. The latter produces a low-density lipoprotein (LDL) that is more oxidizable with either cupric ion or macrophages (*8*). The oxidative stress hypothesis was strengthened by a study demonstrating that lipid hydroperoxides, potent oxidants and free radical generators, were increased 2−4 h after a high-fat meal and returned to baseline paralleling the hyperlipidemia (*9*). Further proof was given by a study showing that a low-fat meal produced no significant changes in triglycerides, flow-mediated endothelium-dependent dilation (FMD), or oxidative stress in the form of leukocyte superoxide production (*10*). However, a high-fat meal given to the same subjects caused an increase in all three parameters. Changes in triglycerides were negatively correlated with FMD but positively correlated with leukocyte superoxide. The superoxide production was negatively correlated with the detrimental changes in FMD. These pathological

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alterations are probably responsible for the high incidence of heart attacks following a high-fat meal.

Antioxidant assays have shown chocolate to be high in polyphenolics (*11*, *12*), in particular catechins and their oligomeric proanthocyanidins. A nutritional survey showed that chocolate contributes 20% of dietary catechin intake in the Dutch population (*13*). However, oligomeric procyanidins, the major phenols in chocolate, were not measured in the Dutch survey. Of importance is the finding that procyanidins are stable to human gastric conditions and thus available for human absorption (*14*). Thus, monomeric catechin and epicatechin analysis will underestimate the phenols available from chocolate.

There recently have been several reviews of the CVD and health effects of chocolate with emphasis on cardiovascular health (*15*−*17*). The phenolic antioxidants in chocolate are postulated to be the agents responsible for the health benefits. We wished to gather more information about chocolate's place as a source of antioxidants in our diet and determine its possible effects on mechanisms relating to heart disease. Thus, we measured the antioxidants in various chocolate products and estimated the contribution of chocolate to the phenolic dietary intake of the U.S. and European diets. In addition, we studied the effect of cocoa butter, the fat component of chocolate, and chocolate on lower density lipoprotein oxidation in an ex vivo spiking experiment. In an in vivo study, lipoprotein oxidizability was measured after human consumption of cocoa butter or chocolate. An animal model of atherosclerosis was used to determine the effect of chocolate on heart disease.

EXPERIMENTAL PROCEDURES

Antioxidant Assay and Dietary Study. Chocolate products (chocolate milk, hot chocolate mixes, and chocolate syrup) were obtained from local markets (three to six different brands). Homemade chocolate milk was prepared from chocolate syrup as per label instructions. Milk and dark chocolate were received as coded samples (four to six samples each) from the American Cocoa Research Institute. Hot and cold chocolate beverages were prepared as stated on the package and assayed immediately. We used our Folin−Ciocalteu assay with catechin as the standard for total phenol assay in the chocolate products after removing fat by multiple extractions with hexane (*11*). Consumption data were taken from European Union statistics for 2003 chocolate confectionery (*18*).

Ex Vivo Human Plasma Study. Human plasma from a normolipidemic male subject, age 58, was incubated for 2 h at 37 °C with the following: 50% methanol/water as a control, a saturated solution of cocoa butter in methanol, epicatechin, the major monomeric polyphenol in chocolate (8 mg/40 L) in 50% methanol, a 50% methanol/water extract of cocoa powder or dark chocolate (40 g/40 L), and a defatted methanol/water extract of cocoa powder or dark chocolate (40 g/40 L). The concentrations of chocolates are based on weights before defatting. Dark chocolate was 32% fat by weight and cocoa powder was 10%. The LDL plus very low-density lipoprotein (VLDL) was isolated from the plasma by affinity chromatography and subjected to oxidation with 25 μM cupric ion with the protein concentration adjusted to 70 μg/mL and a pH of 7.4 at 37 °C. The lag time of oxidation of LDL + VLDL was calculated from a graph of conjugated dienes' (lipid oxidation products) absorbance at 234 nm versus time. This ex vivo spiking method has been previously described in detail (*19*).

In Vivo Human Study. The study was performed with informed consent and approval by the Institutional Review Board at The Pennsylvania State University. Four normolipidemic subjects (two men and two women), 30−49 years of age, participated in a pilot study to determine bioavailability and short-term in vivo antioxidant effects of the consumption of chocolate. Two different formulations were given in a random fashion. Subjects consumed 22 g of commercial cocoa powder and 16 g of dark chocolate in the form of a chocolate muffin and chocolate bits in the morning after an overnight fast. The chocolate products provided 5.4 g of fat from the cocoa butter in chocolate, 111 mg of catechin and epicatechin, and 466 mg of procyanidins. Blood was sampled at 0, 2, 4, and 24 h following an overnight fast and converted to heparinized plasma. The subjects were allowed to eat their normal lunch after the 4-h draw, and they ate their normal dinner meal. In a second control study a muffin was consumed with the same amount of cocoa butter as the first study but no added chocolate. LDL + VLDL was isolated from the plasma and subjected to oxidation with cupric ion as described previously, and the lag time and rate of the fast oxidation or propagation phase were measured. Plasma triglycerides were measured using a Sigma Chemical Co. (St. Louis, MO) enzyme kit.

Animal Atherosclerosis Study. Male, weanling, Syrian Golden hamsters were received from Charles River Breeding Laboratories (Wilmington, MA) and given commercial nonpurified rodent chow (Ralston Purina, St. Louis, MO) for 4 weeks. They were then separated into groups of nine animals, each with comparable average weights. The animals were housed in plastic cages, three or our animals per cage with a bedding of wood chips, in a temperature-controlled room (20 °C) and a 12-h light/dark cycle. They were allowed free access to food and water. The animals were maintained following the guidelines of the University of Scranton Institutional Animal Care and Use Committee. The groups were given a high saturated fat and cholesterol diet as described by Nicolosi (*20*). To 1 kg of powdered chow were added 600 mL of water and 0.2% cholesterol and 10% coconut oil melted together and, while the mixture was hot, 1 g (low dose, 0.1%) or 10 g (high dose, 1.0%) of Hershey's cocoa powder (Hershey, PA) was added. The slurry was mixed, frozen, and cut in the form of a "brownie". The control group had no added cocoa powder. The animals were weighed after acclimatization and just before sacrifice. After 10 weeks of feeding and following 12 h of food deprivation, the animals were anesthetized with pentobarbital, and a cardiac puncture was performed. The aorta was then perfused with 10% formaldehyde in phosphate-buffered saline, and the aorta was isolated and prepared for histology as described (*21*). The blood was put in an EDTA microtainer, and the plasma ws isolated and stored at −90 °C until assay.

Total cholesterol and triglycerides were measured with a Sigma enzyme assay. A pool was made of all the remaining plasma. Pooled plasma high-density lipoprotein (HDL) was measured as cholesterol after precipitation of LDL + VLDL by a Sigma phosphotungstate reagent. Pooled LDL was calculated from the Friedewald equation. LDL + VLDL was isolated from the pooled plasma and subjected to oxidation as described previously for human LDL + VLDL. The rate of oxidation, initial conjugated diene absorbance, and maximal conjugated diene absorbance were calculated from the oxidation curves. Aortal dissection and atherosclerosis assay were performed according to the method of Nicolosi (*20*). A 4−5 mm section of the aorta at 1 mm distance from the heart was removed, defatted, fixed, and stained with Oil Red O. The en face sample was observed under 16 × 40 magnification, and the number, size, and distribution of foam cells were determined. The area covered by foam cells (fatty streak lesion) was measured in duplicate using a computerized digitizer pad (SigmaScan, Jandel Scientific, Richmond, CA) after amplification with a microprojector. Atherosclerosis is calculated as percent of aortal surface covered with foam cells. Atherosclerosis and lipid results are expressed as means ± standard error (SE).

Statistical Analysis. Sigmastat (Jandel Scientific Software, San Rafael, CA) was used for all statistical analyses. Data were expressed as means ± SE. Comparisons were made by a paired *t* test, and differences were considered to be significant when $P < 0.05$. The statistical significance of differences between groups was assessed by a Student's *t* test for normal distribution or the Mann−Whitney test for non-normal distribution, after ANOVA testing of all the groups showed a significant difference existed. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Antioxidant Assay and Dietary Study. A comparison of different chocolate products according to phenols per serving

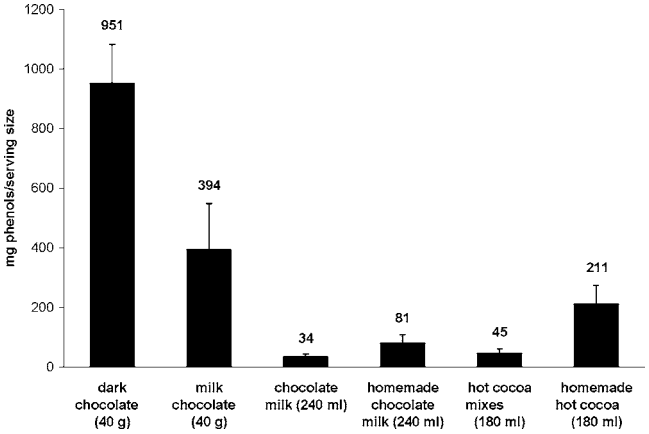

Figure 1. Phenol content of commercial chocolate products in the United States (means ± SD).

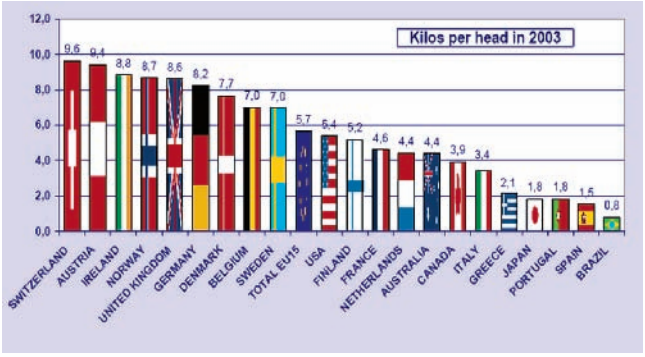

Figure 2. Per capita chocolate confectionery consumption in Europe, Asia, South America, and the United States (kilograms per year).

size is shown in **Figure 1**. Milk chocolate, dark chocolate, and hot cocoa values were calculated from previously published data (*11*). Milk chocolate and dark chocolate contained significantly more phenols than the other chocolate products ($P < 0.0001$). The homemade chocolate milk had significantly more phenols than commercial chocolate milk ($P < 0.001$). The hot cocoa made from chocolate syrup had over twice as many phenols as the cocoa mixes, and the difference was almost significant ($P < 0.1$). On a weight basis the average milk chocolate has 9.9 mg/g of phenolics, dark chocolate, 23.9 mg/g, and cocoa powder, 42.5 mg/g. These numbers agree quite well with published data for total catechins and procyanidins assay by liquid chromatography−mass spectrometry (LC-MS) (*21*).

To put the chocolate products in **Figure 1** in perspective, a 180 mL serving size of commonly consumed U.S. blended black tea leaf (1.25%) provides 630 mg of phenols (*22*), one glass (105 mL) of the average red wine, 190 mg (*23*), one glass or 240 mL of the average orange juice, 54 mg (*20*), and one 70 g serving of blueberries, 181 mg (*24*). Thus, a serving size of several chocolate products provides more phenolic antioxidants than beverages and fruits such as red wine, tea and blueberries, which are known to be high in antioxidants.

The data for yearly per capita consumption in Europe, the United States, Japan, and Brazil are shown in **Figure 2** for 2003 (*18*). The chocolate confectionery consumption (availability) is estimated to be 14.8 g/day for the United States, which includes solid chocolate candy and solid with inclusions, enrobed or molded (with fruit, nut, granola, bakery product center) candy, panned items, and assortments. Of this total, solid chocolate contributes 1.9 g/day. The remaining 12.8 g is estimated to average 70% solid chocolate mixed with something else, for a contribution of 8.9 g of solid chocolate. These two together give

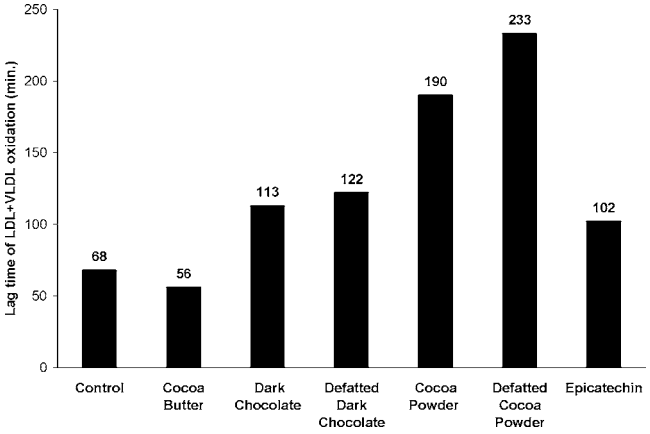

Figure 3. Ex vivo plasma spiking study with chocolate products.

a total of 10.8 g of solid chocolate/day (F. Seligson, personal communication). The catechin equivalents are conservatively estimated to be due to milk chocolate because milk chocolate has the lowest amount of phenols compared to dark chocolate and cocoa powder. In fact, the most popular of the chocolates consumed in the United States is milk chocolate. Also, these numbers do not reflect chocolate consumed in ice cream, beverages, and baked products. The calculated per capita contribution of chocolate in the U.S. diet based on chocolate confectionery consumption is 107 mg/day.

Another means of calculation utilized the U.S. Department of Agriculture Database (*25*). The consumption data are based on cocoa liquor equivalents or 5.2 g/day per capita. Using this value and assuming cocoa liquor is 55% cocoa butter and 45% cocoa phenols (cocoa solids or cocoa powder), we calculate 2.35 g of cocoa powder or 100 mg/day of phenolic antioxidants for the United States. The European Union (EU) has an average consumption of confectionery slightly higher than that of the United States (5.7 vs 5.4 kg/year per capita). Thus, the EU consumption of phenolic antioxidants should be slightly higher than that of the United States or 113 mg/day estimated for the EU using U.S. chocolate phenol assay data. On a per capita basis in the U.S. diet chocolate is third behind coffee and tea as a daily source of antioxidants.

Ex Vivo Human Plasma Study. We simulated fat and chocolate consumption by means of an ex vivo spiking experiment to determine their effect on lipoprotein oxidative susceptibility. An increase in lag time of the LDL + VLDL isolated after equilibration with antioxidants indicates a beneficial antioxidant effect due to binding of the antioxidant polyphenols to the lipoproteins. The concentration of chocolate products added to the plasma mimicked the complete absorption of 40 g of the chocolate (single serving size of a chocolate bar) after consumption and distribution into 40 L, the approximate water content of a typical 70 kg human subject.

As can be seen in **Figure 3**, the cocoa butter fat was a mild pro-oxidant, that is, it decreased the lag time compared to the baseline, thus indicating an increase in LDL + VLDL oxidizability. From an examination of the difference between the lag times for chocolate with and without fat, it is seen that the fat in both dark chocolate and cocoa powder was a pro-oxidant. Cocoa butter contains ~60% of total fatty acids as saturated fat (*26*), the remainder being unsaturated and oxidizable. The addition of unsaturated fat provided more oxidizable lipids, thus decreasing the lag time of lipoprotein oxidation. Conversely, from the data it is evident that pure epicatechin and the polyphenols in the three forms of chocolate were antioxidants, the latter in spite of the presence of the pro-oxidants cocoa butter

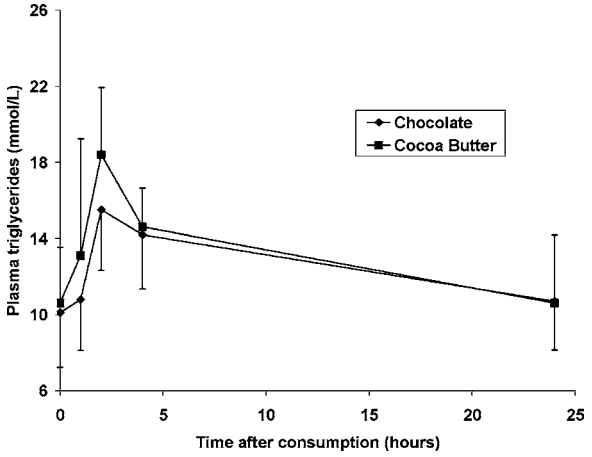

Figure 4. Changes in plasma triglycerides after consumption of cocoa butter alone or in chocolate. Data are expressed as means ± SE, $n = 4$.

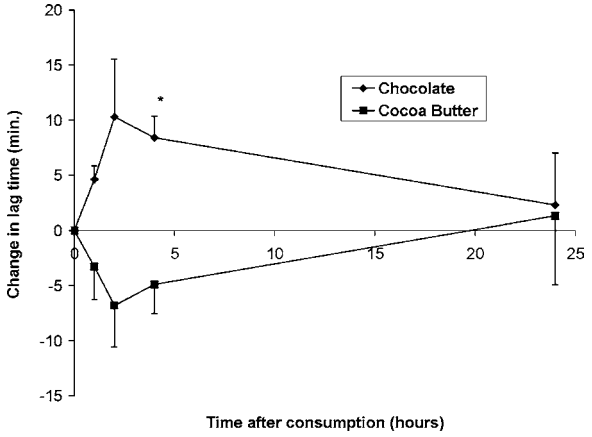

Figure 5. Effect of human consumption of cocoa butter alone or in chocolate on the lag time of LDL + VLDL oxidation. Data are expressed as means ± SE, $n = 4$; *, P value < 0.05.

fat and sugar. The pure epicatechin concentration in the spiked plasma was 0.7 μM, which was similar to the maximum plasma concentration found after human consumption of dark chocolate, 0.4 μM (*26*), or cocoa, 3 μM (*27*). Defatted cocoa, containing the most polyphenols and no pro-oxidants, was the best antioxidant among those tested.

In Vivo Human Study. As seen in **Figure 4**, the triglyceride pharmacokinetics of cocoa butter (control) and chocolate were identical. After an overnight fast, as expected, the triglycerides returned to baseline. The postprandial plasma triglyceride concentrations were lower with the chocolate at 2 and 4 h, but the results were not significantly different due to the large variation within the small group ($n = 4$). Fiber or polyphenols in the cocoa powder and dark chocolate might have inhibited the absorption of the fat but apparently did not affect the kinetics.

Results for oxidation curves of LDL + VLDL are shown in **Figure 5**. Cocoa butter alone decreases the resistance to oxidation, that is, it is a pro-oxidant for the LDL even after 4 h, and then the oxidizability of the LDL + VLDL returns to baseline after an overnight fast. The change in lag time was highly negatively correlated with the change in triglycerides, $r = -0.9762$ Pearson correlation coefficient, $P < 0.02$, indicating that triglycerides were the pro-oxidant. This negative correlation was also seen previously with triglycerides and leukocyte superoxide production after human fat consumption (*10*). With chocolate there was a positive correlation, with triglycerides and lag time changes, $r = 0.986$, $P < 0.02$, due to the presence of polyphenol antioxidants. The rate of oxidation for the LDL + VLDL conjugated diene formation versus time curves averaged from 1 to 24 h for all subjects and was significantly higher after the cocoa butter consumption than after the chocolate, 5.30 versus 4.62 $\times 10^{-3}$ abs/min, $P < 0.001$. This indicates a lower rate of susceptibility to oxidation following chocolate consumption. We showed that the plasma concentration of epicatechin, a major polyphenol antioxidant in chocolate, was elevated 2 and 4 h after the ingestion of the chocolate used in this study (*28*). However, there was no correlation of the plasma epicatechin concentration (data not shown but found in ref *28*) and LDL + VLDL oxidation lag time after chocolate consumption. This is presumably due to the fact that the binding of polyphenols to the lipoprotein takes some time to occur. In stark comparison to cocoa butter, chocolate was an in vivo antioxidant at least throughout the 4-h period after consumption and made the lipoproteins more resistant to oxidation.

Our results indicate that the chocolates' polyphenolic antioxidants enriched the lipoproteins and protected them from oxidation. We have also demonstrated this in our plasma spiking study reported herein and previously with pure polyphenols (*29*) and polyphenols in beverages (*30*). Kondo demonstrated a similar increase in LDL lag time 2 h after human consumption of 35 g of delipidated cocoa (*31*). In our study cocoa butter fat is a significant in vivo pro-oxidant. The postprandial oxidative stress of cocoa butter is completely blocked and overcome by the antioxidants from the chocolate.

Animal Atherosclerosis Study. The initial average weights of the hamsters were 110 ± 5, 114 ± 6, and 113 ± 8 g for control, low cocoa, and high cocoa, respectively. The final weights were 189 ± 17, 200 ± 25, and 193 ± 23 g, respectively. There was no significant weight difference among the groups, nor were there any significant differences in weight gain. The lipid results and oxidation parameters are shown in **Table 1**. Remarkably, both doses of cocoa significantly decreased the elevated cholesterol levels, 36% for both doses. This result is in contrast to the study of cocoa liquor extract given at the 1% level to Kusanagi spontaneously cholesterolemic rabbits (*32*). The Japanese group found that cocoa liquor elevated plasma cholesterol after 6 months of supplementation. In addition, the low dose of cocoa given to the hamsters produced a significant reduction in plasma triglycerides, 34%. Pooled LDL was lowered 62 and 66% by the low and high doses of cocoa, respectively. Pooled plasma HDL was increased in a dose-dependent manner by cocoa. The low dose increased HDL 12% and the high dose by 23%. In several human supplementation studies chocolate consumption (dark chocolate and cocoa powder, milk chocolate, and dark chocolate) produced significant increases of HDL of 4, 10, and 11%, respectively (*28*, *33*, *34*). On a body weight basis/day basis these human studies ranged from 0.5 to 1 g of chocolate/kg of body weight. This is similar to our hamster study of about 0.5−1 g of cocoa powder/kg of body weight (at the beginning and end of the study, respectively).

The oxidation data are found in **Table 1**. The conjugated diene oxidation parameters gave meaningful results. The initial conjugated dienes (before oxidation) were much lower for the cocoa groups than the control. This is indicative of less oxidation in the plasma. This result confirms the rabbit study that showed lower plasma lipid peroxides in the cocoa group versus the control (*33*). The final conjugated dienes are also higher in the control group than the cocoa groups showing less oxidation with cocoa. In fact, the initial and final conjugated dienes are highly correlated (Pearson correlation coefficient = 1.000, $P = 0.01$),

Table 1. Plasma Lipid and Oxidation Parameters in Hamsters Given Cocoa[a]

group	cholesterol (mM)	pooled HDL (mM)	pooled LDL (mM)	triglycerides (mM)	pooled initial LDL + VLDL conjugated diene abs	pooled final LDL + VLDL conjugated diene abs
control	5.56 ± 1.63	1.34	2.35	39.1 ± 13.5	0.868	1.631
low cocoa	3.54 ± 0.67*	1.50	0.90	9.9 ± 12.5**	0.307	0.523
high cocoa	3.57 ± 0.59*	1.65	0.80	28.8 ± 8.8	0.340	0.614

[a] Data are expressed as means ± SE, $n = 9$; *, $P < 0.01$ vs control; **. $P < 0.05$ vs control.

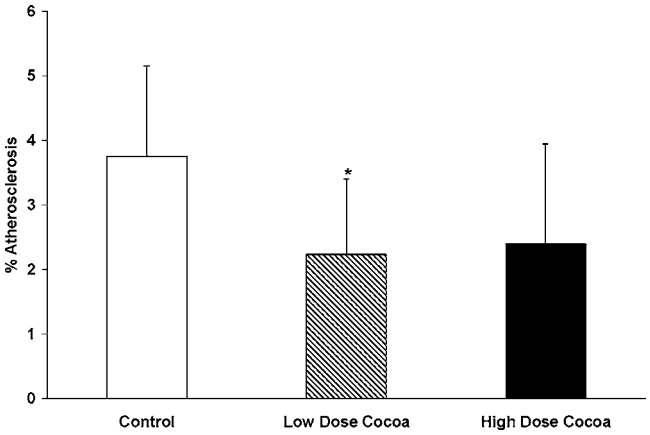

Figure 6. Effect of cocoa consumption on percent atherosclerosis in hamsters. Data are expressed as means ± SD, $n = 9$; *, P value < 0.05.

indicating that initial oxidation of LDL + VLDL produces more final oxidation products with cupric ion oxidant.

Hamsters are a good model for human atherosclerosis because with the high-fat and -cholesterol diet their lipid profile resembles that of humans. The clinical endpoint atherosclerosis provided the most important data for this animal study and are shown in **Figure 6**. The low dose of cocoa produced a significant 40% decrease in atherosclerosis ($P < 0.05$). The high dose decreased the atherosclerosis almost as much 36%, and the effect was almost significant, $P = 0.08$. This same high dose of 1% cocoa liquor decreased the atherosclerosis a similar 32% in the Kusagni rabbit model (*32*). This is a much more severe model of atherosclerosis as the control had 47% of the aorta covered with lesions. Our model is the initial stage of atherosclerosis with the control aortas <4% covered. There seems to be a saturation effect of the polyphenols on cholesterol, triglycerides, oxidation parameters, and atherosclerosis as there was no difference between the low and high doses of cocoa. It is difficult to extrapolate animal results to humans. The calculated human dose for the low-dose cocoa is equivalent to 2−40 g of dark chocolate bars/day. This is a reasonable consumption if the calories in the chocolate are substituted for other caloric food components of the diet.

Conclusion. It is apparent from the results that chocolate products contain a great deal of polyphenol antioxidants. Although mostly consumed as a snack rather than as a food in the United States (*35*), chocolate provides an estimated 100−107 mg/day of antioxidants. The significance of chocolate antioxidants in the U.S. diet is seen by comparing chocolate to the contribution from fruits (255 mg) and vegetables (233 mg) (*24*, *36*). Thus, chocolate contributes over 20% of the antioxidants provided by fruits and vegetables (total 588 mg) but is certainly no substitute. Chocolate provides even more antioxidants for residents of the European Community because they consume more chocolate per capita than the U.S. population. Chocolate polyphenols can enrich the LDL + VLDL with antioxidants after a single dose in spite of the presence of prooxidants in the cocoa butter. This was shown in both ex vivo and in vivo human experiments in this work. It remains to be shown whether chocolate can provide antioxidants when given with a high-fat meal similar to that found with tea and wine. Chocolate supplementation studies with humans have shown that LDL oxidizability significantly decreases after chocolate consumption, an effect hypothesized to decrease the risk of atherosclerosis and stroke (*17*). The first human epidemiology report regarding chocolate and cardiovascular disease has recently shown in the Zutphen elderly men study; the highest tertile of cocoa intake had a 50% decreased risk for cardiovascular mortality versus the lower tertile (*37*). Our animal atherosclerosis study shows the benefit of chocolate consumption on slowing the initial atherosclerosis process, that is, a preventive effect, thus complementing the epidemiology study. Cocoa intake in the Dutch study was also significantly associated with lower systolic and diastolic blood pressure. Thus, chocolate consumption may have cardiovascular benefit via a number of mechanisms. This must be tempered with the proviso that the added sugar and fat from chocolate consumption should be accounted for by adjustments in the rest of the diet. Large long-term randomized trials need to be performed to definitely prove that chocolate consumption can improve human heart health.

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