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Chronic consumption of a flavanol- and procyanindin-rich diet is associated with reduced levels of 8-hydroxy-2′-deoxyguanosine in rat testes

Timothy J. Orozco[a], Janice F. Wang[a], Carl L. Keen[a,b,*]

[a]*Department of Nutrition, University of California, Davis, CA 95616, USA*
[b]*Department of Internal Medicine, University of California, Davis, CA 95616, USA*

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Abstract

Cocoa can contain a high concentration of flavanols and procyanidins which have been reported to have strong antioxidative activity. In the present study, male Sprague-Dawley rats were fed diets containing 0, 0.5, 1, or 2% cocoa rich in flavanols for two weeks. Blood, liver, heart and testes were collected and analyzed for markers of oxidative damage. Plasma epicatechin concentrations, 8-hydroxy-2′-deoxyguanosine (8OH2′dG), and oxidized and reduced glutathione were quantitated by HPLC with electrochemical detection. Plasma F_2-isoprostanes were measured using an enzyme immunoassay. Plasma epicatechin concentrations increased in a dose-dependant fashion according to the amount of cocoa in the diet (128 nM–790 nM). Cocoa supplementation was associated with lower than normal concentrations of 8OH2′dG in the testes (0.590 + 0.40 vs. 0.328 + 0.29; $p < 0.05$). Liver and heart 8OH2′dG levels were unaffected by dietary treatment. In erythrocytes, the glutathione pool was significantly less oxidized in the cocoa fed group compared to controls ($p < 0.05$). In liver and testes, no differences in superoxide dismutase activities were detected. Concentrations of plasma F_2-isoprostanes and thiobarbituric acid reactive substances were similar in all groups. These results support the concept that a diet rich in flavanols and procyanidins can improve oxidant defense and reduce tissue markers for oxidative stress, although these effects can be tissue specific.

Keywords: 8-hydroxy-2′-deoxyguanosine; Cocoa; Flavanols; Flavonoids; Oxidative damage; Epicatechin

1. Introduction

There is increasing interest in the identification of dietary factors that can reduce an individual's risk for chronic disease. In addition to the well-recognized essential nutrients, food can contain a multitude of other components that can promote health. In this regard, flavonoids represent a class of phytochemicals that has been postulated to have positive health effects, and the consumption of certain foods and beverages rich in these compounds has been reported to be associated with numerous positive health benefits including vessel relaxation, reductions in platelet reactivity, and increases in plasma antioxidant potential [1–6].

Given the chemical diversity of the flavonoid family, it is reasonable to speculate that the biological effects of these compounds in humans occur through a variety of mechanisms. With the above stated, a number of research groups have suggested that an important role for these nutrients can be to enhance the oxidant defense system. Using a variety of in vitro models, investigators have shown that the flavonoids found in wine, purple grape juice, tea, and cocoa can act as potent antioxidants by scavenging reactive radicals, thus reducing the rate of protein and lipid oxidation [7–13]. While a substantial body of literature has been developed on the antioxidant effects of flavonoids in vitro, studies documenting the potential of flavonoids to reduce oxidative damage in vivo are more limited. The paucity of in vivo data can be attributed, at least in part, to the difficulty in accurately measuring oxidative damage in vivo. For tissue oxidative damage, two markers that are reputed to be reliable indicators of oxidative damage are F_2-isoprostanes, [14] a marker of lipid oxidation, and 8-hydroxy-2′-deoxy-

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* Corresponding author. Tel.: +1-530-752-6331; fax: +1-530-752-8966.

E-mail address: clkeen@ucdavis.edu (C.L. Keen).

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guanosine (8OH2′dG) [15] a marker of DNA oxidation. In one of the few studies that have demonstrated an in vivo effect of flavonoids on tissue oxidative damage, the consumption of a flavonoid-rich red wine was associated with a reduction in plasma and urinary F_2-isoprostanes in smokers [16]. In addition, a few studies have shown that treating rats with flavonoids isolated from black tea and red wine significantly reduced the amount of chemically induced 8OH2′dG adducts in both the colonic mucosa and liver [17–19].

Cocoa contains a relatively high concentration of flavonoids, specifically the flavanols epicatechin, catechin, and oligomers of epicatechin known as procyanidins [20]. Purified flavonoids isolated from cocoa have been shown to reduce the rate of LDL oxidation in vitro [8,11], and LDL isolated from the plasma of subjects who consumed flavonoid-rich cocoa has been reported to be more resistant to oxidation ex vivo [12,21]. The above work supports the concept that diets rich in certain flavonoids may be associated with improvements in the oxidant defense system. In addition, numerous other studies suggest that the consumption of flavonoids may have the potential to prevent oxidative damage in vivo.

The purpose of the current study, using a rodent model, was to determine if the addition of a flavonoid-rich cocoa to a typical control diet would result in a reduction in markers of oxidative damage in animals fed the diet for two weeks in the absence of an oxidative stress.

2. Methods and materials

2.1. Materials

All chemicals used were from Sigma (St. Louis, MO) unless otherwise noted. Alkaline phosphatase and nuclease P1 used for enzymatic hydrolysis of DNA were from Roche (Indianapolis, IN). HPLC solvents were from Fisher (Pittsburg, PA). The flavonoid rich cocoa was generously provided by Mars, Incorporated (Hackettstown, NJ).

2.2. Animals

The animal protocol was in accordance with NRC recommendations [22] and was approved by the University of California, Davis Animal Use and Care Committee. Forty-eight adult male Sprague-Dawley rats (200–240 g) were fed a purified egg white based diet formulated to meet the nutritional requirements of the rat [22]. Each animal received a diet containing by weight, 0, 0.5, 1.0, or 2% of cocoa (n = 12 per group). The cocoa used for this particular study was especially high in flavanols and procyanidins and contained 12.2 mg/g epicatechin, 2.8 mg/g catechin, and 53.3 mg/g procyanidins. The diets were fed *ad libitum* for 14 days. Food intake was recorded daily, and body weight was recorded every other day. At the end of the 14 day period, the food cups were removed 4 hr before sacrifice, after which the rats were anesthetized with carbon dioxide and blood was collected by cardiac puncture. Blood was collected in heparinized syringes and the plasma was separated by centrifugation at 1800 g at 4°C.

2.3. Sample processing

Butylated hydroxytoluene dissolved in ethanol, and indomethacin were added to plasma for plasma F_2-isoprostane analysis to give a final concentration of 0.8%, and 14 uM, respectively. For the plasma thiobarbituric acid reactive substances determination, 4% butylated hydroxytoluene was added to plasma samples (1:5 v/v) before storing at −80°C. Tissues used for 8OH2′dG analysis were removed and homogenized immediately using a glass and teflon homogenizer in 10 mM Tris-HCl pH 7.5, 320 mM sucrose, 5 mM $MgCl_2$ and 0.1 mM desferoxamine mesylate (1:5 w/v). For glutathione analysis, tissues were homogenized in 1 M trifluoroacetic acid. The homogenates were centrifuged at 12,000 g for 15 min at 4°C, and supernatant fractions were stored at −80°C until analysis. The pellets were dissolved in 0.1 N NaOH, 0.1% SDS, and 0.1% Triton X-100 and saved for protein determination. Protein was measured using the Bradford assay from Biorad (Hercules, CA). All tissues were stored at −80°C until analyzed.

2.4. 8-Hydroxy-2′-deoxyguanosine

8OH2′dG was quantitated using HPLC coupled with electrochemical detection. DNA extraction and enzymatic hydrolysis of DNA were conducted as previously described [23]. The HPLC system consisted of an Agilent 1100 series HPLC (Palo Alto, CA) with a quartenary pump and diode array detector connected to an ESA Coulochem II electochemical detector (Chelmsford, MA) with a 5011 analytical cell (E1: +150 mV; E2: +350 mV), and 5020 guard cell set at +550 mV. Separation was achieved with a 15 cm × 4.6 cm, 0.3 micron Supelco C-18 DB column (Bellefonte, PA) using a mobile phase consisting of 100 mM sodium acetate buffer, pH 5.2 with 7% methanol at a flow rate of 1 ml/min and column temperature of 25°C. After each analytical run the column was washed with 100 mM sodium acetate buffer pH 5.2, and 20% methanol for 8 min followed by a 15 min re-equilibration period with the mobile phase used for analysis. 8OH2′dG was detected electrochemically at 350 mV and 2′-deoxyguanosine was detected at 248 nm using UV detection. 8OH2′dG values are expressed relative to the amount of 2′-deoxyguanosine in the sample.

2.5. Plasma F_2-isoprostanes

Plasma samples were prepared using solid phase extraction as previously described [24]. An enzyme immunoassay kit from Cayman Chemical (Ann Arbor, MI) was used to measure F_2-isoprostanes.

2.6. Plasma thiobarbituric acid reactive substances

Plasma thiobarbituric acid reactive substances (TBARS) were measured using a modification [3] of the method described by Yagi [25]. Plasma lipids were isolated by precipitating them with a phosphotungstic acid/hydrochloric acid system. The lipid fraction was then reacted with thiobarbituric acid, and the resulting adducts were measured fluorometrically, using 1,1′,3,3′-tetraethoxypropane as the standard.

2.7. Tissue superoxide dismutase (SOD) activity

Activity was determined by the sample's ability to inhibit the auto-oxidation of pyrogallol using the method of Marklund and Marklund [26]. One unit of SOD activity is defined as the amount of sample needed to obtain 50% inhibition of pyrogallol oxidation, expressed per milligram protein.

2.8. Plasma epicatechin, catechin, and procyanidin dimers

Plasma samples were extracted as previously described [27]. The resulting solution was filtered with a 0.22 μm Millipore Ultrafree-MC low-binding Durapore centrifugal filter (Millipore, Bedford, MA) and centrifuged prior to injection.

Chromatography was carried out using an Agilent 1100 HPLC system, equipped with a quaternary pump, (Agilent, Palo Alto, CA) in series with an ESA CoulArray 5600 detector (Chelmsford, MA). Separation was achieved using gradient elution with an Alltima C18 column (5 μm, 150 mm $\times$ 4.6 mm; Alltech Associates, Deerfield, IL) and C18 5-μm guard column (Alltech Associates, Deerfield, IL). Solvent A consisted of 40% methanol, 60% 100 mM sodium acetate in water, pH 5.0 and solvent B consisted of 7% methanol, 93% 100 mM sodium acetate in water, pH 5.0. A gradient elution was conducted at a flow rate of 1 ml/min, and column temperature of 25°C with the initial concentration of A set at 20%, from 0-2 min. This was followed by a linear increase to 40% A from 2–10 min, immediately followed with another linear increase to 85% A from 10–13 min. The system continued to pump 85% A isocratically from 13-17 min followed by a linear increase to 100% A from 17–20 min. The system was then linearly decreased to 40% A by 23 min followed by another linear decrease to 20% A by 25 min.

Coulometric electrochemical array detection was carried out using the following cell settings: −50, +65, +150, +200, +250, +300, +700 and +800 mV. Epicatechin and catechin peak identification at + 150 mV was based on co-elution with authentic standards and quantified using external standards. Procyanidin dimer peak identification at +700 mV was based on co-elution with authentic standards and quantified using external standards extracted from cocoa (Cocoapro^{TM}, Mars Incorporated, Hackettstown, NJ) [13,20].

2.9. Oxidized and reduced glutathione

Supernatant fractions from tissue homogenates were diluted 1:1 in mobile phase, and filtered using 0.22 μm Millipore Ultrafree-MC low-binding Durapore centrifugal filters (Millipore, Bedford, MA) before injection onto the HPLC. The HPLC system consisted of an Agilent 1100 HPLC with quaternary pump and an ESA CouloArray 5600 detector. Separation was achieved using an Alltech (Deerfield, IL) Hypersil ODS C-18 column (250 mm $\times$ 4.6 mm, 5 um particle size), and guard column. The mobile phase consisted of solvent A: 50 mM sodium phosphate buffer with 0.0875% trifluoroacetic acid, 2% methanol (pH 2.5) isocratic at a flow rate of 1 ml/min and column temperature of 25°C from 0–15 min. At 15 min the mobile phase was ramped up to 50% solvent B: 50 mM sodium phosphate buffer, 0.0875% trifluoroacetic acid, 20% methanol, pH 2.5 and a flow rate of 1.5 ml/min for 2 min. At 17 min the mobile phase was shifted to 100% solvent A for 13 min. The voltage settings for the detector were: +100, +700, +750, +800, +850, +900, +950, and +950 mV. Glutathione was quantified at +750 mV and glutathione disulfide at +850 mV. Glutathione values are expressed relative to the amount of protein in the sample as determined by the Bradford assay. The redox potential (E_h) of the GSH/GSSG redox couple was calculated using the Nernst equation ($E_h = -264 + 0.03\log [(GSSG)/(GSH)^2]$ as previously described [28].

2.10. Statistical analysis

ANOVA was used to determine statistical significance and the Pearson product moment was used to ascertain any correlations using Sigma Stat Software from SPSS Science (Chicago, IL). Data are shown as mean ± standard error of the mean.

3. Results

Food intake and weight gains were similar among the groups (data not shown). As depicted in Fig. 1, plasma epicatechin concentrations increased in a dose-dependent manner according to the concentration of cocoa in the diet. Plasma catechin and dimer in the plasma were below the limit of detection in the plasma of rats in the low cocoa diet groups. Catechin was detected in plasma obtained from rats fed the 2% cocoa diet (40.2 nM ± 1.23) but at a much lower concentration than epicatechin (790.3 nM ± 113.0).

Oxidized and reduced glutathione were measured in liver, blood erythrocytes, and testes. Glutathione values are shown in Table 1 along with the calculated redox potential (E_h) of the GSH/GSSG redox couple. Consistent with plasma epicatechin concentrations, the glutathione pool was

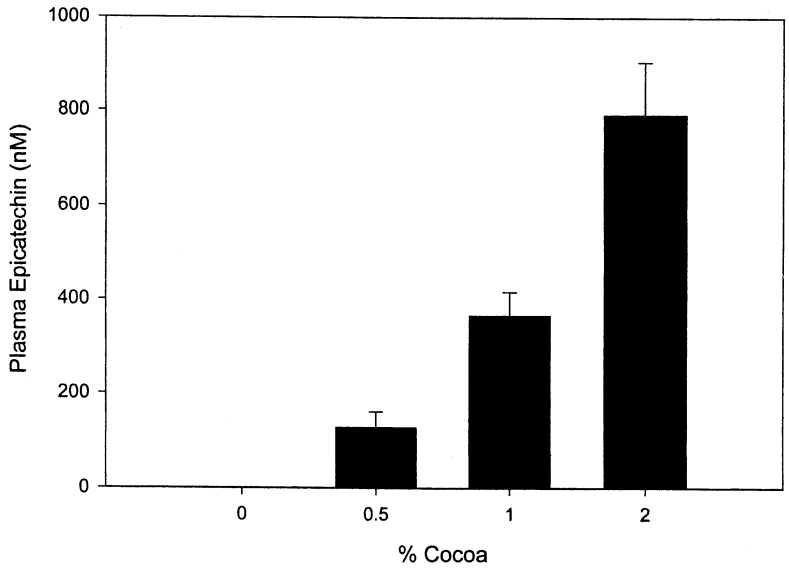

Fig. 1. Plasma epicatechin concentrations (nM) in rats fed diets supplemented with 0, 0.5, 1, or 2% cocoa as determined by HPLC with electrochemical detection. Epicatechin was not detected in the plasma of rats fed diets not supplemented with cocoa. All of the diet groups were statistically different from each other ($p < 0.05$).

significantly less oxidized in erythrocytes in the rats fed 2% cocoa relative to the other diet groups ($p < 0.05$). Liver and testes glutathione pools were similar in the groups.

Liver 8OH2′dG concentrations were unaffected by diet group (Fig. 2). For heart 8OH2′dG, only the 0% and 2% cocoa diet groups were analyzed and no differences in 8OH2′dG concentrations (8OH2′dG per $10^5$2′dG) were observed between the two diet groups (0% cocoa: 2.32 ± 0.25; 2% cocoa: 2.23 ± 0.12). However, as depicted in Fig. 2, testes 8OH2′dG concentrations were lower in both the 1% (0.387 ± 0.054; $p < 0.05$), and 2% cocoa (0.328 + 0.029; $p < 0.01$) diet groups compared to the control group (0.590 ± 0.040). Testes 8OH2′dG concentrations in rats in the 2% cocoa diet group were significantly lower than those in the 0.5% cocoa diet group ($p < 0.05$). Across the groups, testes 8OH2′dG concentrations were inversely correlated with plasma epicatechin concentrations ($r = -0.406$, $p < 0.01$; Fig. 3).

Plasma F_2-isoprostane concentrations and plasma TBARS were similar across the diet groups. Plasma F_2-isoprostane concentrations were 434 pg/ml ± 42, 420 pg/ml ± 38, 443 pg/ml ± 43, and 381 pg/ml ± 38 pg/ml in the 0, 0.5, 1 and 2% cocoa diet groups, respectively. Plasma TBARS concentrations were 4.24 μM ± 0.25, 4.13 μM ± 0.11, 4.02 μM ± 0.26, 3.71 μM ± 0.27, for the 0, 0.5, 1 and 2% diet groups, respectively.

SOD activities were similar in all diet groups in liver and testes. Liver SOD activities were 8.62 ± 1.00, 9.09 ± 0.95, 8.78 ± .0.95, 9.33 ± 0.95 units/mg protein in the 0, 0.5, 1 and 2% cocoa groups, respectively. Testes SOD activities were 5.98 ± 0.51, 5.30 ± 0.51, 5.97 ± 0.51, and 5.96 ± 0.48 units/mg protein in the above groups.

4. Discussion

The results of this work support the concept that the chronic consumption of a diet rich in certain flavonoids can

Table 1
Redox potential of the GSH/GSSG redox couple (E_h), reduced glutathione (GSH) and oxidized glutathione (GSSG) concentrations in erythrocytes, liver, and testes obtained from rats fed 0–2% cocoa.

% Cocoa	Erythrocytes			Liver			Testes		
	Eh	GSH	GSSG	Eh	GSH	GSSG	Eh	GSH	GSSG
0.0	−225.7 ± 3.5	18.5 ± 3.1	0.56 ± 0.10	−249.5 ± 1.6	58.7 ± 4.8	1.02 ± 0.09	−249.2 ± 3.4	92.9 ± 13.2	2.40 ± 0.33
0.5	−225.3 ± 3.3	17.2 ± 2.3	0.54 ± 0.09	−248.2 ± 1.3	59.6 ± 10.2	1.33 ± 0.46	−249.9 ± 2.8	101.5 ± 14.6	2.82 ± 0.50
1.0	−222.8 ± 1.9	18.4 ± 3.5	0.76 ± 0.17	−248.3 ± 1.0	51.4 ± 2.2	0.90 ± 0.07	−249.0 ± 4.2	103.5 ± 20.8	2.51 ± 0.25
2.0	−239.3 ± 2.6*	26.1 ± 2.7	0.52 ± 0.09	−248.2 ± 1.8	66.4 ± 7.8	1.39 ± 0.14	−248.0 ± 3.5	105.2 ± 15.9	3.09 ± 0.23

E_h was calculated using the Nernst equation as described in the methods and is expressed in mV. More negative values indicate a less oxidized glutathione pool. Glutathione values are expressed in nmoles/mg protein.

* Significantly different from 0.0, 0.5, 1.0% cocoa diet groups ($p < 0.05$).

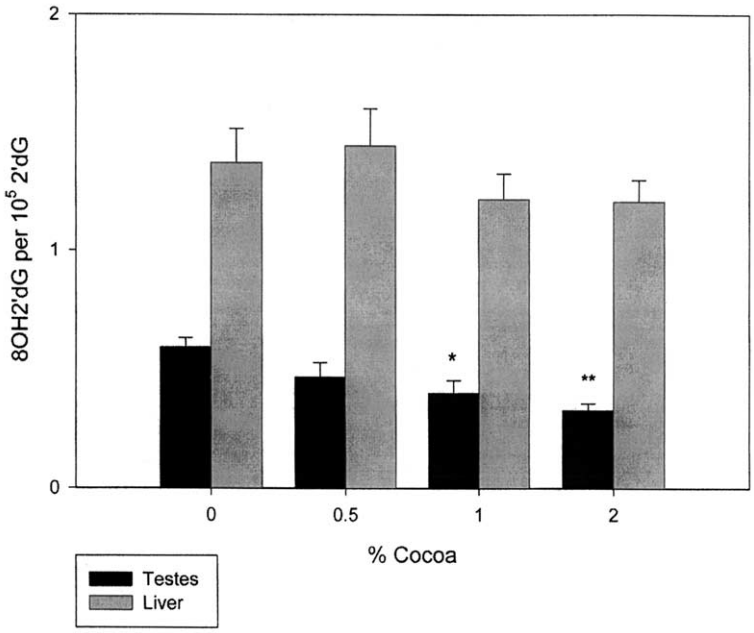

Fig. 2. 8-Hydroxy-2′-deoxyguanosine concentrations in testes and liver obtained from rats fed diets containing 0-2% cocoa. In testes, 8OH2′dG concentrations were lower in the 1% and 2% cocoa diet groups relative to rats receiving control diet. No differences were observed in liver. *Statistically different from the 0% cocoa diet group ($p < 0.05$). **Statistically different from the 0% cocoa diet group ($p < 0.01$).

be associated with improvements in the oxidant defense system. Significantly, there was a strong inverse correlation between plasma epicatechin concentrations and testes 8OH2′dG levels. Previous experiments with flavonoids have shown an inverse association between dietary flavonoid intake and tissue oxidative damage when the study subjects were challenged with an oxidative stress [16–19]. In the present study, animals were not challenged with an oxidative stress and we did not observe any diet related changes in markers of lipid oxidation in plasma. The lack of changes in the concentration of plasma TBARS is not surprising given that the American Institute of Nutrition dietary recommendations that were followed for this study provide for a rich supply of antioxidant vitamins and minerals. Similarly, while F_2-isoprostanes levels have been reported to be elevated under conditions of oxidative stress [16] they have not been shown to be influenced by diet in non-stressed individuals [21]. Other researchers have observed diet induced differences in F_2-isoprostanes, when using GC/MS [16].

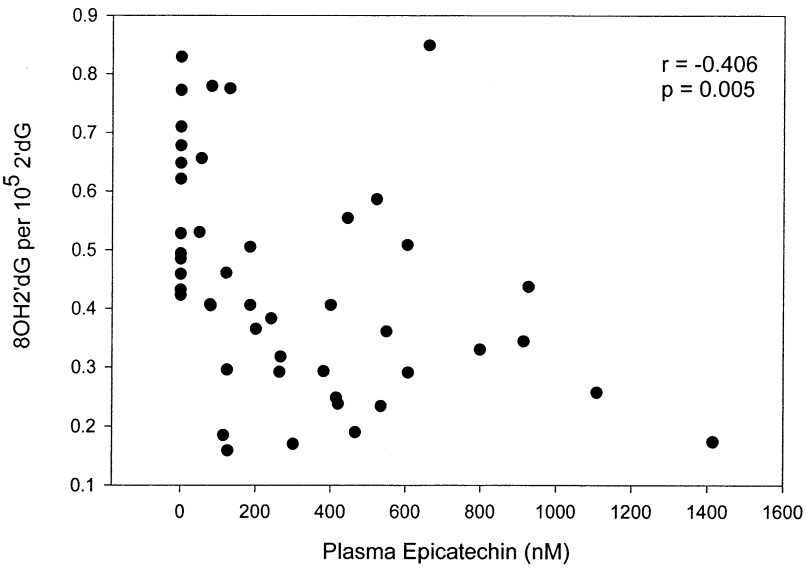

Fig. 3. Testes 8OH2′dG concentrations were inversely correlated to plasma epicatechin concentrations (r = −0.406).

While we observed no diet-induced change in 8OH2′dG concentrations in liver or heart, we observed significant reductions in 8OH2′dG levels in the testes of rats fed the high cocoa diets. This observation supports the concept that diets rich in certain flavonoids can be associated with a reduced risk of DNA damage in select tissues. The above observations also support the idea that the testes represent a sensitive tissue for the detection of oxidative damage to DNA [29], and underscore the fact that individual tissues can show a differential response to flavonoids.

The mechanisms by which flavonoids reduce oxidative damage to DNA are unclear. Flavonoids have been shown to be effective scavengers of both reactive oxygen species [30], and reactive nitrogen species [31,32]. Furthermore, they are capable of chelating transition metals such as iron and copper [33] which are capable of generating free radicals through initiation of Fenton type reactions. Flavonoids have also been reported to be capable of repairing 8OH2′dG by donating a hydrogen atom to oxidized guanine in vitro [34].

8-Hydroxy-2′-deoxyguanosine is known to cause G→T transversions which can be mutagenic [35,36]. However, oxidation of DNA can result in either the mutagenic 8OH2′dG lesion, or the nonmutagenic ring opened Fapy lesion [37]. Malins et al demonstrated that the log ratio of 8OH2′dG to Fapy increased with age and in prostate cancer tissues [38]. It was suggested by Malins et al that the lesion that is formed is dependant on the redox environment of the cell [38]. More oxidatively stressed environments give rise to more 8OH2′dG lesions, whereas a more reductive environment will give rise to the ring opened Fapy lesion. Therefore, it is possible that the flavonoids in cocoa are creating a more reductive environment and shifting the formation of lesions toward Fapy lesions. We did not measure Fapy lesions in the present study. This is an area that clearly merits additional study.

Although the biological significance of 8OH2′dG in testes is a subject of debate, several groups have reported higher levels of 8OH2′dG in the sperm of infertile males relative to fertile males [39,40], and increased levels in populations, such as smokers, who have a high oxidant load [41,42]. Furthermore, the levels of 8OH2′dG in sperm have been shown to be inversely correlated to concentrations of antioxidants in seminal plasma [43], and the provision of antioxidant supplements has been shown to reduce the high levels of 8OH2′dG observed in the sperm of infertile males [40]. 8OH2′dG adducts have also been shown to increase with age, an observation that might be attributed to an age-related increase in oxidative cellular stress [44,45].

In summary, the results obtained in the current study support the idea that certain dietary flavonoids can impact oxidative defense mechanisms, even in animals fed high quality diets. It is interesting to speculate that the above observation suggests that the consumption of foods rich in certain flavonoids may provide one mechanism by which dietary supplementation with antioxidant rich foods such as cocoa can reduce the accumulation of oxidative damage seen with aging. The results obtained in the current study also complement previous reports that the type of flavonoids present in cocoa have a positive effect on tissue oxidative defense mechanisms [4]. Finally, the present results show that the inclusion of certain “nonessential” nutrients in the diet, such as flavanols and procyanidins, may influence the risk for age-related chronic disease. The observation that this can be demonstrated even in animals fed “nutritionally adequate” diets provides support for the concept that inclusion of certain “nonessential” nutrients in the diet may reduce the risk for certain age-related chronic diseases.

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