Special Article

Clinical benefit and preservation of flavonols in dark chocolate manufacturing

Andrew McShea, Emma Ramiro-Puig, Sandra B Munro, Gemma Casadesus, Margarida Castell, and Mark A Smith

The consumption of high-cacao-content chocolate has been associated with positive health benefits ascribed to flavonol antioxidants derived from the ground, fermented cocoa seeds of Theobroma cacao. *However, flavonols impart a bitter, astringent flavor to foodstuffs, frequently masked in chocolates and confections by aggressive processing and adulteration with other flavors. Recent reports have implied that not all varieties of dark chocolate are created equally, and significant caveats exist regarding its potential health benefits. It is perhaps not surprising that extensive processing, dilution, and the addition of flavor modifiers may improve the palatability of chocolate, but could have negative nutritional and clinical benefits. This article examines the chemical composition of chocolate and the clinical data associated with the consumption of flavonoid-rich cocoa. We review the steps in chocolate manufacturing that directly affect the antioxidant levels in chocolate products, and the caveats associated with claims of health benefits from the consumption of dark chocolate.*

INTRODUCTION

Chocolate is a three millennia old comestible that originated as a frothy, spicy, cocoa-like drink in Central America.[1] Between 1847 and 1879, fundamental improvements to the processing of cacao resulted in the more appetizing chocolate bars from Fry's (UK) and Nestle and Lindt (Switzerland). Interest in chocolate confections continued to grow to the point where cacao products have become almost ubiquitous in the Western diet. Consumption of chocolate products in northern climates is significant and estimated at 5–10 kg per person per year, with some geographical variation linked to cultural norms and local climate. Overall, the world demand for cacao remains very high (estimated at around 3.5 million tons for 2008) with chocolate occupying an unusual multifaceted niche as an indulgent luxury food with healthy connotations in the $17 billion US confectionary market.

In ancient times cacao was thought to confer magical properties when consumed, and it was associated with a variety of rituals. Despite the passage of time and advancement of technology, an air of mystery still surrounds chocolate. Indeed, the art and science of chocolate manufacturing has generally remained a covert operation performed "behind closed doors" and laden with trade secrets. Meanwhile, there is a significant body of evidence to suggest that chocolate's "magical" properties are associated with a spectrum of pharmacological effects on the human body, most of which are attributable to Flavan-3-ols.[2] There has now been over a decade of articles in scientific journals describing the connections of cocoa with antioxidant activity and a variety of significant clinical phenomena. The trend to highlight the health benefit of high-percentage-cacao-containing products has continued to increase based not only on scientific merit but also for business reasons. Ultimately, the morass of

Affiliations: *A McShea* and *SB Munro* are with Research and Development, Theo Chocolate, Seattle, Washington, USA. *E Ramiro-Puig* and *M Castell* are with Department of Physiology, Faculty of Pharmacy, Barcelona, Spain. *G Casadesus* is with Department of Neuroscience, Case Western Reserve University, Cleveland, Ohio, USA. *MA Smith* is with the Department of Pathology, Case Western Reserve University, Cleveland, Ohio, USA.

Correspondence: *MA Smith*, Department of Pathology, Case Western Reserve University, 2103 Cornell Road, Cleveland, Ohio 44106, USA. E-mail: mark.smith@case.edu, Phone: +1-216-368-3670, Fax: +1-216-368-8964.

Key words: antioxidant, chocolate, flavonoid, manufacturing

doi:10.1111/j.1753-4887.2008.00114.x

marketing and scientific detail has become challenging to untangle, and the vacillation in the lay press on whether chocolate is ultimately "good" or "bad" has done little to demystify a complex problem for the consumer.

This article details the complexity and inherent variation of the chocolate-making process to give a flavor of just how complex this material remains. In parallel, the article examines significant pharmacological data demonstrating the health benefits of cacao and how this may relate to process methodology in chocolate-making.

CACAO PROCESSING AND FLAVONOL CONTENT

Chocolate is derived from finely ground, fermented beans (seeds) of the *Theobroma cacao* tree, which is generally cultivated in small farms located in a climate belt approximately within 20 degrees of the equator. Cacao beans are harvested biannually using a labor-intensive process from squash-like pods that grow proximal to the trunk of the tree. Generally, three broad varietals of *Theobroma cacao* are employed in chocolate making: Criollo, Forastero, and Trinitario, with the last being the most common. Short terminal repeat analysis of *T. cacao* genomes suggests that the number of distinct varieties of the plant exceeds 14,000,[3] implying that the genetic diversity of cacao is significant. However, most cacao products currently consumed consist of a mixture of varietals, so genetic differences play a minimal role in the overall taste and character of mass-marketed chocolate products. Recently, smaller chocolate manufacturers have recognized this disparity and "single origin" and "single varietal" chocolate products have begun to appear in a manner similar to varietal and vintage trends in the wine-making industry.

Chocolate is a highly processed form of cacao, and although many forms of manufacturing process exist, they fundamentally all have the same basic steps: fermentation, roasting, and milling. These processing steps have been developed over centuries to render the cacao seed palatable by altering the chemical and physical properties of the bean to produce the chocolate flavors. Not surprisingly, a cacao seed is quite unpalatable in its native form, so a series of steps is required to break down the cotyledon, the shelled seed or nib, and neutralize the unpleasant flavor. The first step of seed fermentation occurs by the spontaneous inoculation of piles of harvested cacao seeds and lasts for up to 7 days. Yeasts (*S. cerevisiae* being the most abundant) dominate the fermentation for the first 24 hours, then lactic acid bacteria replace the yeasts, and the sugary pulp that surrounds the cacao seed is converted to ethanol. The piles are manually aerated during the fermentation, and as oxygen penetrates the fermentation, acetic acid bacteria take over, oxidizing ethanol to acetic acid, which persists in significant quantities after fermentation.[4] During this time, the temperature rises to about 50°C, with the combined heat and acid resulting in breakdown of the cacao seed. Organic acids (oxalic, phosphoric, succinic, and malic acids) are also generated and likely assist in the chemical breakdown of the bean cotyledons. These and other complex reactions contribute to precursors of the chocolate flavor. The acetic acid dominates the flavor of the fermented bean at this stage and is removed/reacted-off during downstream steps of chocolate manufacturing, as it imparts an unpleasant flavor at high concentrations.

It is perhaps obvious from this description that the fermentation is a relatively uncontrolled, non-sterile process, with a significant number of variables that can affect the final characteristics of the chocolate. For example, colonization of the beans by molds can frequently cause significant problems with the flavor profile and overall yield of usable material. In addition, fermentation is almost always unevenly distributed throughout the beans. Thus, a significant amount of knowledge specific to the cacao-growing region and fermentation practices is required to produce good results. Interestingly, controlled inoculations of large-scale fermentation efforts have proven to be challenging to industrialize. This is likely due to the low level of sterility in the process and difficulties in controlling the auto-thermal reactions driving the fermentation.

Significant changes in the concentrations and composition of organic metabolites such as procyanidins and flavan-3-ols occur during the fermentation process.[5] The profiles of these antioxidants are complex in chocolate and the chemical terminology is somewhat redundant (see Figure 1).

Generally speaking, under-fermented cacao has a higher antioxidant content and a markedly more astringent flavor profile. Recent trends to seek chocolate products with high antioxidant content have even resulted in some demand for marginally fermented cacao. Note that the fermentation process is not homogeneous throughout a population of cacao beans, so it is possible to separate

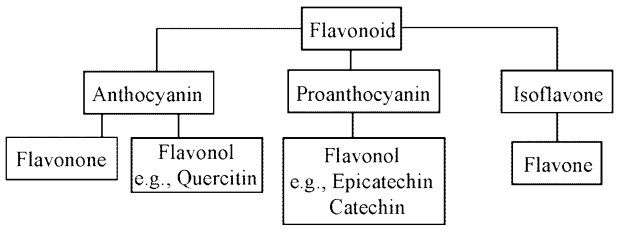

Figure 1 **Nomenclature of polyphenolic flavonoids.** Flavanols (Flavan-3-ols) and proanthocycanin polymers are primary components in cacao products.
Adapted from Murphy et al.[28]

flavor and antioxidant concentration to some degree. In addition, the type of microbial colonization and length of fermentation phase (i.e., alcohol, lactic, acetic) will also likely affect flavonol content and breakdown. Indeed significant variations in the flavonol content of cacao can be observed between batches and growing regions.

After the fermentation process is complete, a natural or assisted process to lower the moisture content of the beans dries the cacao. This completes the fermentation process and permits more straightforward storage and shipment of the cacao for downstream processing. However, spoilage of this microbiologically active material by molds and water damage is a common problem that requires significant diligence to avoid during transport and storage.

After a cleaning process, the fermented, dried cacao beans are then roasted. This serves as the first sterilization process for the beans, and is used to loosen the shell from the fermented cotyledon. Roasting also generates favorable flavor characteristics through Maillard and caramelization reactions of sugars, amino acids, and peptide oligomers. A wide variety of flavors can be produced, depending on the specific amino acids and sugars available, as well as the pH and temperature of the roasting process. More than 25 diketopiperizine roasting reaction products are readily detectable in cacao nibs, with proline-valine dimers predominating and producing bitter flavor qualities. In addition, characteristically bitter flavors of dark chocolate are imparted by theobromine, caffeine, l-leucine, and catechin flavonoids.[6]

After the roasting step, the woody husk of the cacao bean is removed in a violent vibratory process called winnowing. This separation step also breaks down the cacao bean into small 2–5 mm irregular-shaped fragments known as cacao nibs. Depending on the process being applied, cacao nibs can also be roasted a second time to further develop the flavor qualities of the cacao. At this stage, roasted cacao nibs represent the first truly palatable product generated in the chocolate-making process. The roasting process subjects the cacao to significant heat (120–150°C), so changes in the flavonoid biochemistry would be expected during this processing step. Catechin antioxidants are thermally labile to some degree, based on studies of thermal stability in tea;[7] however, the exact stability of epicatechin in chocolate will likely depend on stabilizing interactions with other components of the cacao nib. Indeed, studies suggest that the interaction between flavonoids and other small molecules in the cacao nib do determine the flavor as well as the final antioxidant activity. Thus, flavonoid degradation likely occurs but this depends on the starting material and the specific roasting process applied.[5]

Similar to coffee, cacao must be ground in order to develop the flavor further. However, unlike coffee, the cacao bean is milled to a much finer grain size and is consumed in its entirety, rather than having the flavors extracted. Furthermore, cacao contains between 50% and 56% cocoa butter, an unsaturated triglyceride, which results in the ground material becoming liquefied in the elevated temperature of the high-friction milling equipment. Environmental, genetic, and upstream processing differences in the cacao result in variations in the fat and water content of the cacao, so grinding conditions vary somewhat between processes and raw starting material.

A number of milling methods exist depending on whether it is desirable to separate the cocoa butter from the cocoa solids. Significant changes can be made to the flavor profile and chemical composition of the material depending on the process; however, in all methods, efforts are made to keep the milling temperature in check to avoid overheating the product and further altering the chemistry. Overall, the goal of milling is to reach a relatively homogeneous particle size of below 30 microns. Taste perception of grain sizes below 50 microns is indistinct, i.e., "grittiness" is not perceived; however, the final particle size and conformation has a significant impact on the flavor and "mouth-feel" of the final product. Thus, significant attention must be applied to the milling process to generate a suitable size distribution. Depending on the manufacturing process, the milled liquor may continue down the rest of the chocolate manufacturing process or be solidified into large blocks for transportation to secondary processing plants.

In the next stage, a mixing process combines the milled cocoa liquor with sugar. The quantities of ingredients vary very significantly, with dark chocolate monikers being given to products containing cocoa solids as low as 40% and as high as 100%. In the case of milk chocolate, a significant quantity of milk powder is also added. As chocolate viscosity increases rapidly with water content, the addition of powders or low moisture forms of milk (e.g., dried condensed milk) are required. Most industrialized processes also add an emulsifier such as soy lecithin to the mix to improve viscosity characteristics during manufacturing and to maintain a lower overall cost of production. Though dilution of cacao liquor per se does not directly result in chemical changes to the flavonols, it does greatly complicate the issue of potential beneficial pharmacological effects of cacao ingestion. A significant issue was whether the addition of milk to chocolate would impair the uptake of cacao-derived flavonols. However, recent studies indicate that the uptake of flavonols is not significantly inhibited by milk proteins[8,9] and, by inference, milk chocolate should retain the antioxidant activity proportional to its cacao flavonol content. However, the contribution of saturated fat to the product is likely

undesirable in terms of clinical benefit and there is no doubt that, in general, high-fat, high-sugar diets are undesirable. Indeed, the lax naming conventions for dark chocolate are frequently exploited to imply increased health benefits, which has been a point of significant controversy[10] and has been highlighted to the public by the media.

After the addition of sugar, milk powder, and other additives (note: at what point does chocolate cease to be chocolate?) the chocolate mass is subjected to further rounds of refining to ensure the homogeneity of particle sizes within the product, and enhance the characteristics and dispersion of the particles and fat. Although the material now has properties and an approximate taste profile similar to chocolate, a significant quantity of acetic acid remains from the upstream fermentation imparting an unpleasant tartness to the product.

A multi-day heat treatment step, referred to as conching, is typically applied with gentle grinding to improve the flavor characteristics and reduce the concentration of free acids and other volatile by-products from the cacao bean. This is a delicate manufacturing step, as aggressive processing at this stage can result in significant loss of volatile small molecules that positively influence flavor. An alternative process to conching relies on neutralization of acetic acid with mild base in a process termed "Dutching". Antioxidant flavonols are significantly reduced during the Dutching process, making this process undesirable for maintaining the flavonol activity in chocolate products.[11]

At this point, the material resembles molten chocolate in look and taste. Prior to molding into bars or other shapes, the material is tempered to set a compact crystal structure within the triglycerides so that the final product has a glossy finish, hard consistency, and is resistant to blooming or cosmetic discoloration due to separation of fats within the final product.

CHEMICAL COMPONENTS OF CHOCOLATE

Chocolate is an unusual composite material (Table 1). It contains a significant concentration of astringent-tasting polyphenols embedded in a triglyceride matrix. Polyphenols (proanthocyanidins) are oligomeric and polymeric end products of the flavonoid biosynthetic pathway. They impart astringency to beverages such as wine, fruit juices, and teas, and are increasingly highlighted for their potential beneficial effects on human health. Astringent-tasting polyphenols can be isolated from the bark, leaves, fruit, and seeds of many plants, where they are thought to provide protection against predation.

Significant antioxidant activity has been associated with polyphenols, particularly the monomeric subunits catechin and epicatechin in the case of cacao products. It

Table 1 **Chemical components of fermented, dried, and roasted seed fragments (nibs) from *Theobroma cacao* that impart flavor associated with chocolate.***

Chemical component	µmol/kg
Bitter-tasting compounds	
Theobromine	63564.4
Caffeine	5218.3
Cyclo(L-Pro-L-Val)	8877.8
Cyclo(L-Pro-L-Ala)	1357.0
Cyclo(L-Val-L-Leu)	817.1
Cyclo(L-Ala-L-Leu)	734.0
Cyclo(L-Pro-L-Leu)	711.2
Cyclo(L-Ala-L-Ile)	639.5
Cyclo((L-Ala-L-Val)	633.5
Cyclo(L-Pro-L-Ile)	537
Cyclo(L-Val-L-Val)	237.6
L-leucine	6990.4
L-phenylalanine	4761.5
L-valine	4049.8
L-tyrosine	2719.6
L-isoleucine	2716.4
L-arginine	723.4
L-lysine	578.0
L-histidine	568.4
Sour-tasting compounds	
Citric acid	30974.9
Acetic acid	16717.7
Lactic acid	9260.7
Malic acid	3581.2
Oxalic acid	2810.5
Succinic acid	1725.0
Phosphoserine	744.7
Phosphoethanolamine	298.5
Umami-like tasting compounds	
Glutamic acid	1781.8
Aspartic acid	1357.9
Bitter and astringent tasting compounds	
Epicatechin	8613.1
Catechin	2363.9
Procyanidin B_2	2082.8
Procyanidin C_1	1628.0
[epicatchin-(4beta->8]3-epicatechin	1158.9
[epicatchin-(4beta->8]4-epicatechin	802.1
Procyanidin B_5	791.5
[epicatchin-(4beta->8]5-9-epicatechin	623.3
Gamma-aminobutyric acid	5011.4
N-[3′,4′-dihydroxy-(E)-cinnamoyl]-L-aspartic acid	1615.6
Beta-aminoisobutyric acid	1349.3
N-[3′,4′-dihydroxy-(E)-cinnamoyl]-3-hydroxy-L-tyrosine	851.5
N-[4′-hydroxy-(E)-cinnamoyl]-L-aspartic acid	551.6
Quercetin-3-O-alpha-L-arabinopyranoside	497.5
N-[4′-hydroxy-(E)-cinnamoyl]-L-tyrosine	132.6
Quercetin-3-O-alpha-D-glucopyranoside	101.4
Sweet-tasting compounds	
Sucrose	8827.3
L-alanine	6115.5
Fructose	4834.1
L-proline	2475.0
L-threonine	1899.0
L-serine	1823.4
Glucose	1669.1
Galactose	1110.1
Raffinose	1068.3
Glycine	873.0
Stachyose	533.6
Beta-alanine	339.0

* Composition continues to change during downstream processing (refining/conching) resulting in a reduction of acetic acids and some additional changes in flavor components.
Adapted from Stark et al.[6]

is likely no coincidence therefore that dark chocolate bars have a remarkably long shelf life when stored appropriately. This characteristic is likely attributable to the combination of a high level of antioxidants and a low oxygen permeation rate due to the high concentration of lipids.

Both the concentration and activity of antioxidant polyphenols can be detected at physiologically relevant concentrations in certain forms of chocolate containing high percentages of cacao. As a rough estimate, the dosage consumed during nominal consumption of antioxidant-rich dark chocolate corresponds to a dose of up to 2.8 grams of (−)-epicatechin per 40 gram serving (or 40 mg/kg for an average human). Of course truly "raw" chocolate that was under-fermented and under-roasted with very high cacao solid percentages (90–100%) may contain even higher levels of antioxidant polyphenols but would likely be exceedingly unpalatable and have a high microbiological burden from the fermentation process. More studies are required to correlate chocolate processing methodology with antioxidant activity.

Note that the antioxidant activity of chocolate is exceedingly high on a percentage dry weight basis even when compared with traditionally healthy berries, beans, and fruits. For instance, in comparison to the Acai berry (*Euterpe oleracea*), currently recognized as a highly antioxidant-rich natural foodstuff,[12] dark chocolate and cacao powder have approximately 20% and 75% of the antioxidant activity per gram based on measurements in oxygen radical absorbance capacity (ORAC) assays.[11]

In addition to polyphenols, chocolate also contains the structurally related methylxanthines theobromine and small amounts of caffeine. Theobromine concentrations are significant in chocolate, ranging from 1.5% to 3% for theobromine versus 0.2% to 0.4% for caffeine; however, theobromine has relatively mild vasodilative and diuretic effects. The methylxanthines do, however, contribute a characteristically astringent and bitter quality to the chocolate. Chocolate also contains a complex mixture of amino acids and dipeptide derivatives in various states depending on the amount of fermentation, roasting, and thermal treatment that has been applied to the cacao. As with many roasted foodstuffs there are complex mixtures of reaction products (e.g., diketopiperazines) produced by Maillard and associated reactions that impart a strong flavor to the chocolate.

A variety of simple acids are present in cacao as a byproduct of the fermentation process; however, these are usually significantly reduced or neutralized in most cacao products. Naturally occurring sugars are also present in cacao, but frequently very significant quantities (25–90%) of sucrose are added to make the flavor palatable to a broad consumer base.

Naturally, the concentrations of all of these components can vary considerably depending on the origin and processing of the cacao, and the breadth of variance is currently not well understood.

BIOAVAILABILITY OF ANTIOXIDANTS IN CHOCOLATE

Having established that significant concentrations of antioxidants can persist in a variety of chocolate products,[11] the next issue to consider is their bioavailability. Given that thermal processing of cacao products can affect the final profile of polyphenols in chocolate and given the harsh environment of the digestive system, it is reasonable to question whether antioxidant polyphenols can transit to a site of uptake in the gut without being significantly degraded en route.

A study by Rios et al.[13] addressed this issue and focused attention on the stability of polyphenols and flavin-3-ols in gastric juices of the stomach. Surprisingly, cacao polyphenols introduced into human subjects through gastric feeder tubes for up to 40 minutes and then recovered showed no change in polymerization state by HPLC. This is significant, as in vitro treatment with acid and thermal treatment can cause changes in the polymerization state of polyphenols. Thus, it seems likely that the gastric environment has minimal effect on polyphenols and that they transit with minimal modification to the small intestine.

Both catechin and epicatechin are readily adsorbed by the gut into the bloodstream. However, there are differences in the uptake of the catechin enantiomers.[14] This is significant for the consumption of cacao, since the primary catechin enantiomer present in cacao is of the (−) form that is poorly adsorbed. Thus, it would seem that the most bioavailable antioxidant of significance in cacao is (−)-epicatechin.[15,16] Absorption of (−)-epicatechin in humans is efficient, and its metabolite (−)-epicatechin-glucuronide can be identified in plasma at a mean concentration of 625.7 ± 198.3 nmol/L 2 hours after consumption of a cocoa beverage containing 54.4 mg of (−)-epicatechin.[17] Epicatechin metabolites, (−)-epicatechin-glucuronide and (−)-epicatechin-sulfates can be detected in urine 6–12 hours after consumption.[15,17–19]

Interestingly, current data on uptake and bioavailability suggests that cocoa flavonols can be readily adsorbed regardless of the background of other foodstuffs. Mixing cacao products with common chocolate-making materials such as milk powder does not seem to prevent the uptake of epicatechin.[8,9] Further studies need to be carried out to understand the effects of dietary antioxidants as food additives, as well as on foodstuffs exhibiting other pharmacological effects, e.g., high dietary caffeine, etc. One intriguing study suggests

that certain antioxidants can compete with each other, suggesting that the interplay and intestinal transport systems are complex.[16]

Some studies have shown the access of flavonoids in brain tissue after their oral intake. Abd el Mohsen et al.[20] demonstrated for the first time that epicatechin metabolites, including glucuronide and its 3′-O-methylated form, were able to cross the blood brain barrier after a single oral administration of epicatechin (100 mg/kg) in rats. In addition, quercetin was detected in brain tissue from rats fed long-term with a diet containing 0.1% quercetin.[21] It is important to highlight that in both studies flavonoid concentrations were corrected, taking into account the presence of residual blood in the tissue.

The mechanisms by which flavonoids gain entry to the brain remain to be clarified. Flavonoids could enter the central nervous system (CNS) by diffusion, carrier, or receptor-mediated transport across the blood brain barrier.[22] Moreover, we cannot discard the possibility that epicatechin glucuronides could be hydrolyzed by tissue glucuronidases, resulting in free epicatechin penetration into the blood brain barrier and subsequent conjugation with glucuronic acid by CNS UDP-glucuronosyltransferase.

IN VIVO EFFECTS OF CHOCOLATE

Chocolate has been popularized as being able to satiate cravings. There is a wealth of both anecdotal and scientific evidence indicating that chocolate induces gender- and individual-specific responses when consumed. However, persistent evidence of a "smoking gun" for the psychoactive (even purportedly aphrodisiac-like) substances in chocolate remains controversial.[23] Indeed, in the absence of candidate drugs in chocolate that could provoke this type of physiological response, it has even been suggested that certain craving responses to chocolate may occur as a result of gut bacteria producing addictive secondary metabolites in certain hosts.[24] This remains an elusive problem.

The vasoactive effects of chocolate are somewhat better understood. In general, these effects can be linked to the consumption and bioavailability of (−)-epicatechin in chocolate.[25] Consumption of chocolate results in both acute modulations of blood pressure[26] as well as positive changes in cholesterol biochemistry.[27] Cocoa intake reduces cardiovascular disease risk due to a combination of different effects including the improvement of antioxidant status (shown by the decrease of oxidative stress biomarkers, including thiobarbituric acid reactive substances and low-density lipoprotein oxidation), vasodilation, and inhibition of platelet activation and aggregation.[28,29]

Epidemiological studies of long-term cocoa ingestion seem to tally with the shorter-term clinical studies, suggesting that the consumption of certain cacao products have significant clinical benefit.[30] Significantly, the doses used in the Taubert study[30] were exceedingly low (6.3 grams of chocolate per day). This demonstrated that the ingestion of flavonoids, through the medium of chocolate, had minimal impact on calorific intake (30 kcal per day) but produced statistically significant decreases in systolic and diastolic blood pressure and an increase in S-nitrosoglutathione. Furthermore, cacao flavonoids also seem to mediate very significant anti-inflammatory effects that can be measured as reductions in platelet and endothelial cell activation[31] and the expression of inflammatory mediators.[32] Of note, a negative effect on bone density in mature women who consume chocolate has recently been identified.[33]

In addition to the possible protection against coronary heart disease, cocoa intake improves cerebral blood flow in humans[34,35] and this may have a positive impact in aging and cerebrovascular diseases, such as stroke and dementia, in which endothelial function is impaired. Cocoa effects on brain perfusion seem to be attributed to NO-dependent vasodilatation mediated by flavonoids.[34] Although short-term cocoa intake increases blood oxygenation levels, it does not affect the reaction time in task-switching.[35] This lack of cognitive improvement is not surprising since this study was performed in young subjects with high cognitive abilities. Hence, using people with low cognitive performances (due to fatigue, aging, or cerebrovascular disease) is more likely to provide an effect in cognitive tasks. In this regard, long-term administration of cocoa extract (24 mg/kg p.o.) is capable of delaying the onset of age-related cognitive deficits in rats, as shown by the improved cognitive performances in light extinction and water maze paradigms.[36]

In addition, several in vivo studies demonstrate the benefits of cocoa flavonoids including catechin, epigallocatechin-3 gallate (EGCG) and quercetin in cerebral ischemia and hypoxia models.[37–43] For instance, flavonoids improve spatial memory[39] and decrease brain edema, neuronal death in the hippocampus,[39] and MDA[42] and NO[40] levels induced by ischemia. However, in almost all these studies, high doses of flavonoids were administered as intraperitoneal injections; therefore, the observed effects are not representative of oral consumption. Epidemiological studies suggest that the consumption of products with high flavonoid content delays the onset of dementia and Alzheimer's disease (AD), particularly among those who are at high risk.[44,45] Therefore, further studies using moderate doses administered orally should be performed to confirm the relevancy of dietary cocoa flavonoids in the improvement of cerebral ischemia.

Neurodegenerative diseases such as AD and Parkinson's are prompted by multiple factors including oxidative stress, inflammation, reduced expression of trophic factors, and accumulation of iron and protein aggregates, among others, which lead to neuronal loss.[46–53] One important feature of neurodegenerative diseases is the abnormal accumulation of iron in dying neurons, which enhances the production of reactive oxygen radicals and induces the aggregation of neurotoxic peptides including amyloid-β and α-synuclein (as reviewed by Mandel et al.[54]). Therefore, the combination of iron chelating and antioxidant therapies may be a useful strategy for neuroprotection.

Cocoa has recently attracted attention as a potentially neuroprotective natural product due to its antioxidant and potent iron-chelating activities.[54–57] Some studies suggest there are beneficial effects of cocoa on neurodegenerative disorders such as AD and Parkinson's disease. In this sense, cocoa extract, epicatechin, and catechin reduce the toxic effects of amyloid-β, a constituent of senile plaques in AD, on a neuronal cell line through membrane and mitochondrial protective mechanisms.[58] Moreover, EGCG decreases amyloid precursor protein (APP) levels and consequently amyloid-β formation in both *in vitro* and *in vivo* studies.[59,60] These effects seem to be mediated by the iron chelating capacity of EGCG since APP is post-transcriptionally regulated by iron regulatory proteins (IRPs).[61] In addition, oral administration of cocoa extract (100 mg/kg/day) has been recently shown to attenuate nigrostriatal dopaminergic cell loss in a murine model of Parkinson's disease induced by the infusion of the neurotoxin 6-hydroxidopamine.[62]

The mechanisms by which cocoa flavonoids exert neuroprotective and neurorescue activities seem to be beyond the simple radical scavenging activity. Accumulating evidence suggest that cocoa flavonoids interact selectively with mitogen-activated protein kinase signaling (MAPK) cascades that are involved in neuronal survival, regeneration, and cell death. Thus, epicatechin and its in vivo metabolite 3′O-methyl-epicatechin are potent intracellular inhibitors of oxidized LDL-induced neuronal cell death by counteracting JNK, c-Jun, and caspase-3 activation.[63] It is worth highlighting that although 3′O-methyl-epicatechin has a lower antioxidant activity than its precursor, it is as effective as epicatechin in protecting neurons against oxidized LDL; this provides evidence for the non-antioxidant mechanisms of cocoa flavonoids.

In serum-deprived neurons, EGCG (0.1–1 μM) modulates the expression of specific proteins associated with neurite outgrowth, cell survival, and iron chelation.[64] Thus, EGCG increases the level of cytoskeletal and structural proteins (e.g., the actin binding protein tropomyosin 3, beta-tubulin IV)[64] that are associated with the neurite outgrowth activity.[65] Moreover, EGCG enhances cell survival, in part, through the inhibition of caspase-3 pathway[66] and the upregulation of the binding protein 14-3-3 gamma, which blocks the apoptotic protein Bad.[64] EGCG also interferes in the iron-oxygen sensors of hypoxia-inducible factor (HIF)-1 alpha pathway. Recent findings have pointed out the importance of this pathway in iron regulation and its potential applicability in the treatment of neurodegenerative diseases.[67]

In addition to intracellular signaling modulation, we cannot discard the interaction of cocoa flavonoids with cell surface receptors, as described in cancer and immune cells.[68–70] In this regard, the specific binding of EGCG to the 67 kDa laminin receptor has been shown to be involved in anticancer and antiallergic effects.[68,70] This laminin receptor is also present in neuronal cells and its expression is associated with tumor malignancy and metastasis.[71]

A summary of in vivo work to date on CNS effects of cacao is shown in Table 2.

CONCLUSION

Dietary modification and exercise is a cheap, simple, and lower risk method of attempting to modulate or prevent the onset of disease. Clearly, the trend in the clinical and scientific data suggests that the consumption of dietary flavonols can produce mostly positive clinical benefits. The benefit to the vascular system seems clear and a number of studies suggest there may also be significant benefit to the cerebrovascular system and elements of brain function in neurodegenerative disease. However, the context in which flavonols are realistically consumed outside of controlled scenarios is unclear. More work needs to be done to determine the context in which dietary intervention would be most clinically efficient and result in the highest level of patient compliance. Given the broad popularity of chocolate, clearly significant potential exists here. However, the monikers of "chocolate" and even "dark chocolate" are applied to a very wide range of foodstuffs and processing techniques. This is due, in part, to the highly complex manufacturing processes that have developed but also to the wide and bewildering range of chocolate consumer products in a market in which the pressure to very aggressively differentiate and promote products is significant.

Indeed, the positive data regarding the consumption of cacao also has significant caveats, given that no one actually consumes raw, lightly fermented cacao beans anymore; note that this was, however, more like what was consumed by the Olmec/Aztec/Mayan populations millennia ago. So the health benefits of chocolate must be regarded in the context of the other ingredients in

Table 2 **Effects of cocoa and related flavonoids on central nervous system.**

Subjects	Intervention	Flavonoid intake/day	Main outcome
Brain perfusion and cognition			
Healthy patients (>50 years old)[34]	920 mL cocoa beverage/5 days	821 mg	Peripheral vasodilatation dependent on NO synthase
	1000 mL cocoa beverage/7 days	900 mg	↑ Cerebral blood flow
Female patients ($n = 16$; 18–30 years)[35]	High-flavonol cocoa beverage/5 days	172 mg	↑ Blood oxygenation
			No effect on cognition
Wistar rats (15 mo)[36]	Cocoa flavonoid extract/1 year	24 mg/kg	↑ Cognition
			↑ Lifespan
			Preservation of high urinary free dopamine levels
Cerebral ischemia models			
Model	Induction of damage	Flavonoid treatment	Main outcome
Repeated cerebral ischemia (Wistar rats)[39]	10 m artery occlusion × 2	Quercetin (50 mg/kg) 30 min before occlusion	↑ Spatial memory
			↓ Neuronal cell death in the hippocampal CA1
Excitoxic neuronal damage (rat primary hippocampal neurons)[42]	10 μM NMDA; 10 μM AMPA; 20 μM Kainate	EGCG (10 μM); at time of occlusion	↑ Cell survival
			↓ MDA production
Unilateral cerebral ischemia (gerbils)[42]	30, 60, or 90 min carotid artery occlusion	EGCG (50 mg/kg) 30 min before occlusion and after ischemia	↓Brain edema
			↓Infarct volume
			↓MDA production
Transient forebrain ischemia (gerbils)[41]	3 min carotid arteries occlusion	EGCG (50 mg/kg) 1–3 h after occlusion	↓ Hippocampal damage
			↓ Polyamine (putrescine) levels in brain
Bilateral ischemia (Wistar rats)[40]	10 min carotid/vertebral arteries occlusion	EGCG (50 mg/kg i.p.) before occlusion	↓ NO currents in the hippocampus
Transient ischemia (gerbils)[37]	Ischemia/reperfusion	Catechin for 2 weeks before ischemia	↓ Neuronal cell death in hippocampal CA1
			↑ Superoxide scavenging activity
Hypoxia (Wistar rats)[43]	Altitude chamber 10,000 m for 4 h	EGCG (25–50 mg/kg i.p.)	↑ Cell survival
			↓ NADPH-d/nNOS
Neurodegenerative disease			
Alzheimer's disease			
Type of model	Induction of damage	Flavonoid treatment	Main outcome
Rat PC12 cells[58]	$A\beta_{25-35}$ (25 μM, 24 h)	Cocoa extract (one or both: 7–45 μg/mL or 10–100 μM epicatechin and catechin) 10 min before cell damage	↓ Aβ cytoxicity preservation of membrane integrity and mitochondrial function
Human neuroblastoma SHSY5Y[59]		EGCG (1–10 μM) for 2 h	↑ sAAPα mediated via an increase of α-secretase activity

Table 2 **Continued**

Subjects	Intervention	Flavonoid intake/day	Main outcome
Rat PC12 cells[59]	$A\beta_{25-38}$, $A\beta_{1-42}$, $A\beta_{1-40}$ (10 μM 48 h)	EGCG (1 μM) 30 min before cell damage	↓ Aβ cytoxicity ↑mitochondrial function ↓ Apoptosis
Rat PC12 cells[59]	$A\beta_{25-35}$ (10 μM 48 h)	EGCG (0.1–1 μM) 2 h after	Neurorescue
C57/Bl mice[59]	NO	EGCG (2 mg/kg/day p.o.) for 7 and 14 days	↓ Holo-APP levels in the hippocampus ↑ sAPP; PKC activation
Human neuroblastoma SHSY5Y[60]	Serum withdrawal	EGCG (1–10 μM) for 48 h	↑ Transferring receptor ↓Holo-APP by suppressing APP translation ↑ sAPPα /holo-APP
CHO/ΔNL (sweAPP)[60]	NO	EGCG (1–10 μM) for 48 h	↓ Aβ secretion ↓ Holo-APP
Parkinson's disease			
Sprague-Dawley rats[62]	Neurotoxin 6-OHDA 12 μg	Cocoa rich in procyanidins (100 mg/kg/d) 4 days before	↑ Dopaminergic neuron survival
Neurodegenerative disease and aging			
Rat PC12 cells[65]	Serum withdrawal	EGCG (0.1–10 μM) for up to 3 days after serum withdrawal	↑ Cell survival ↑ Neurite outgrowth ↑ PKC activation
Human neuroblastoma SHSY5Y[64]	Serum withdrawal (5 days)	EGCG (0.1–1 μM) for 48 h 3 days after serum withdrawal	↑ Cytoskeletal and structural proteins ↓ Metabolic enzymes ↓ Heat shock proteins
Rat PC12 cells[72]	Sodium nitroprusside (NO donor)	EGCG (100–300 μM) 30 min before cell damage	↓ Cytotoxicity ↓ Released cytochrome C ↓ ROS ↓ Apoptotic changes ↓Bax/Bcl2, caspase 9, –8, and –3
Mouse primary striatal neurons[63]	Oxidized LDL (12.5 μg of protein/mL) for up to 24 h	Epicatechin and 3′-O-methylepicatechin (30 μM) up to 18 h before	↓Cytotoxicity ↓ JNK and c-Jun activation ↓Caspase-3 activation

chocolate bars, an area that is still unclear. Certainly chocolate can contain very high concentrations of refined sugar and have a very high saturated fat content derived from the cacao bean itself, added milk powder, or the addition of vegetable oils to chocolate products. Thus, the net benefit of cacao must be weighed against the other components in chocolate and reformulated if necessary. In the meantime, strict adherence to labeling standards, additional consumer education and more openness in the chocolate industry can also help improve consumer understanding of the issues.

Acknowledgment

Declaration of interest. AM and SBM are employees of Theo Chocolate (www.theochocolate.com).

REFERENCES

1. Henderson JS, Joyce RA, Hall GR, Hurst WJ, McGovern PE. Chemical and archaeological evidence for the earliest cacao beverages. Proc Natl Acad Sci USA. 2007;104:18937–18940.
2. Aron PM, Kennedy JA. Flavan-3-ols: nature, occurrence and biological activity. Mol Nutr Food Res. 2008;52:79–104.
3. Turnbull CJ, Wadsworth RM, Hadley P. *International Cocoa Germplasm Database. ICGD 2003, Version 5.2*. Reading, UK: University of Reading; 2003.
4. Schwan RF. Cocoa fermentations conducted with a defined microbial cocktail inoculum. Appl Environ Microbiol. 1998; 64:1477–1483.
5. Counet C, Ouwerx C, Rosoux D, Collin S. Relationship between procyanidin and flavor contents of cocoa liquors from different origins. J Agric Food Chem. 2004;52:6243–6249.
6. Stark T, Bareuther S, Hofmann T. Molecular definition of the taste of roasted cocoa nibs (*Theobroma cacao*) by means of quantitative studies and sensory experiments. J Agric Food Chem. 2006;54:5530–5539.
7. Wang R, Zhou W, Wen RA. Kinetic study of the thermal stability of tea catechins in aqueous systems using a microwave reactor. J Agric Food Chem. 2006;54:5924–5932.
8. Keogh JB, McInerney J, Clifton PM. The effect of milk protein on the bioavailability of cocoa polyphenols. J Food Sci. 2007;72(Suppl):S230–S233.
9. Roura E, Andres-Lacueva C, Estruch R, et al. Milk does not affect the bioavailability of cocoa powder flavonoid in healthy human. Ann Nutr Metab. 2007;51:493–498.
10. The devil in the dark chocolate. Lancet. 2007;370:2070.
11. Miller KB, Stuart DA, Smith NL, et al. Antioxidant activity and polyphenol and procyanidin contents of selected commercially available cocoa-containing and chocolate products in the United States. J Agric Food Chem. 2006;54:4062–4068.
12. Schauss AG, Wu X, Prior RL, et al. Antioxidant capacity and other bioactivities of the freeze-dried Amazonian palm berry, *Euterpe oleraceae* mart (acai). J Agric Food Chem. 2006; 54:8604–8610.
13. Rios LY, Gonthier MP, Remesy C, et al. Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects. Am J Clin Nutr. 2003; 77:912–918.
14. Donovan JL, Crespy V, Oliveira M, Cooper KA, Gibson BB, Williamson G. (+)-Catechin is more bioavailable than (–)-catechin: relevance to the bioavailability of catechin from cocoa. Free Radic Res. 2006;40:1029–1034.
15. Baba S, Osakabe N, Yasuda A, et al. Bioavailability of (–)-epicatechin upon intake of chocolate and cocoa in human volunteers. Free Radic Res. 2000;33:635–641.
16. Baba S, Osakabe N, Natsume M, Muto Y, Takizawa T, Terao J. In vivo comparison of the bioavailability of (+)-catechin, (–)-epicatechin and their mixture in orally administered rats. J Nutr. 2001;131:2885–2891.
17. Roura E, Andres-Lacueva C, Jauregui O, et al. Rapid liquid chromatography tandem mass spectrometry assay to quantify plasma (–)-epicatechin metabolites after ingestion of a standard portion of cocoa beverage in humans. J Agric Food Chem. 2005;53:6190–6194.
18. Ito H, Gonthier MP, Manach C, et al. Polyphenol levels in human urine after intake of six different polyphenol-rich beverages. Br J Nutr. 2005;94:500–509.
19. Baba S, Osakabe N, Natsume M, Muto Y, Takizawa T, Terao J. Absorption and urinary excretion of (–)-epicatechin after administration of different levels of cocoa powder or (–)-epicatechin in rats. J Agric Food Chem. 2001;49:6050–6056.
20. Abd El Mohsen A, Kuhnle MM, Rechner G, Schroeter H, et al. Uptake and metabolism of epicatechin and its access to the brain after oral ingestion. Free Radic Biol Med. 2002;33:1693–1702.
21. de Boer VC, Dihal AA, van der Woude H, et al. Tissue distribution of quercetin in rats and pigs. J Nutr. 2005;135:1718–1725.
22. Youdim KA, Qaiser MZ, Begley DJ, Rice-Evans CA, Abbott NJ. Flavonoid permeability across an in situ model of the blood-brain barrier. Free Radic Biol Med. 2004;36:592–604.
23. Di Marzo V, Sepe N, De Petrocellis L, et al. Trick or treat from food endocannabinoids? Nature. 1998;396:636–637.
24. Rezzi S, Ramadan Z, Martin FP, et al. Human metabolic phenotypes link directly to specific dietary preferences in healthy individuals. J Proteome Res. 2007;6:4469–4477.
25. Schroeter H, Heiss C, Balzer J, et al. Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. Proc Natl Acad Sci USA. 2006;103:1024–1029.
26. Heiss C, Kleinbongard P, Dejam A, et al. Acute consumption of flavanol-rich cocoa and the reversal of endothelial dysfunction in smokers. J Am Coll Cardiol. 2005;46:1276–1283.
27. Baba S, Natsume M, Yasuda A, et al. Plasma LDL and HDL cholesterol and oxidized LDL concentrations are altered in normo- and hypercholesterolemic humans after intake of different levels of cocoa powder. J Nutr. 2007;137:1436–1441.
28. Murphy KJ, Chronopoulos AK, Singh I, et al. Dietary flavanols and procyanidin oligomers from cocoa (*Theobroma cacao*) inhibit platelet function. Am J Clin Nutr. 2003;77:1466–1473.
29. Cooper KA, Donovan JL, Waterhouse AL, Williamson G. Cocoa and health: a decade of research. Br J Nutr. 2008;99:1–11.
30. Taubert D, Roesen R, Lehmann C, Jung N, Schomig E. Effects of low habitual cocoa intake on blood pressure and bioactive nitric oxide: a randomized controlled trial. JAMA. 2007; 298:49–60.
31. Hermann F, Spieker LE, Ruschitzka F, et al. Dark chocolate improves endothelial and platelet function. Heart. 2006;92: 119–120.
32. Ramiro E, Franch A, Castellote C, et al. Flavonoids from *Theobroma cacao* down-regulate inflammatory mediators. J Agric Food Chem. 2005;53:8506–8511.

33. Hodgson JM, Devine A, Burke V, Dick IM, Prince RL. Chocolate consumption and bone density in older women. Am J Clin Nutr. 2008;87:175–180.
34. Fisher ND, Sorond FA, Hollenberg NK. Cocoa flavanols and brain perfusion. J Cardiovasc Pharmacol. 2006;47(Suppl 2):S210–S214.
35. Francis ST, Head K, Morris PG, Macdonald IA. The effect of flavanol-rich cocoa on the fMRI response to a cognitive task in healthy young people. J Cardiovasc Pharmacol. 2006;47 (Suppl 2):S215–S220.
36. Bisson JF, Nejdi A, Rozan P, Hidalgo S, Lalonde R, Messaoudi M. Effects of long-term administration of a cocoa polyphenolic extract (Acticoa powder) on cognitive performances in aged rats. Br J Nutr. 2008;100:94–101.
37. Inanami O, Watanabe Y, Syuto B, Nakano M, Tsuji M, Kuwabara M. Oral administration of (–)catechin protects against ischemia-reperfusion-induced neuronal death in the gerbil. Free Radic Res. 1998;29:359–365.
38. Lee S, Suh S, Kim S. Protective effects of the green tea polyphenol (–)-epigallocatechin gallate against hippocampal neuronal damage after transient global ischemia in gerbils. Neurosci Lett. 2000;287:191–194.
39. Pu F, Mishima K, Irie K, et al. Neuroprotective effects of quercetin and rutin on spatial memory impairment in an 8-arm radial maze task and neuronal death induced by repeated cerebral ischemia in rats. J Pharmacol Sci. 2007;104:329–334.
40. Nagai K, Jiang MH, Hada J, et al. Epigallocatechin gallate protects against NO stress-induced neuronal damage after ischemia by acting as an anti-oxidant. Brain Res. 2002; 956:319–322.
41. Lee SY, Kim CY, Lee JJ, Jung JG, Lee SR. Effects of delayed administration of (–)-epigallocatechin gallate, a green tea polyphenol on the changes in polyamine levels and neuronal damage after transient forebrain ischemia in gerbils. Brain Res Bull. 2003;61:399–406.
42. Lee H, Bae JH, Lee SR. Protective effect of green tea polyphenol EGCG against neuronal damage and brain edema after unilateral cerebral ischemia in gerbils. J Neurosci Res. 2004;77:892–900.
43. Wei IH, Wu YC, Wen CY, Shieh JY. Green tea polyphenol (–)-epigallocatechin gallate attenuates the neuronal NADPH-d/nNOS expression in the nodose ganglion of acute hypoxic rats. Brain Res. 2004;999:73–80.
44. Commenges D, Scotet V, Renaud S, Jacqmin-Gadda H, Barberger-Gateau P, Dartigues JF. Intake of flavonoids and risk of dementia. Eur J Epidemiol. 2000;16:357–363.
45. Dai Q, Borenstein AR, Wu Y, Jackson JC, Larson EB. Fruit and vegetable juices and Alzheimer's disease: the Kame Project. Am J Med. 2006;119:751–759.
46. Zecca L, Youdim MB, Riederer P, Connor JR, Crichton RR. Iron, brain ageing and neurodegenerative disorders. Nat Rev Neurosci. 2004;5:863–873.
47. Smith MA, Harris PL, Sayre LM, Perry G. Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. Proc Natl Acad Sci USA. 1997;94:9866–9868.
48. Castellani RJ, Siedlak SL, Perry G, Smith MA. Sequestration of iron by Lewy bodies in Parkinson's disease. Acta Neuropathol (Berl). 2000;100:111–114.
49. Smith MA. Alzheimer disease. Int Rev Neurobiol. 1998;42:1–54.
50. Perry G, Castellani RJ, Hirai K, Smith MA. Reactive oxygen species mediate cellular damage in Alzheimer disease. J Alzheimers Dis. 1998;1:45–55.
51. Smith MA, Richey Harris PL, Sayre LM, Beckman JS, Perry G. Widespread peroxynitrite-mediated damage in Alzheimer's disease. J Neurosci. 1997;17:2653–2657.
52. Smith MA, Taneda S, Richey PL, et al. Advanced Maillard reaction end products are associated with Alzheimer disease pathology. Proc Natl Acad Sci USA. 1994;91:5710–5714.
53. Smith MA, Perry G, Richey PL, et al. Oxidative damage in Alzheimer's. Nature. 1996;382:120–121.
54. Mandel S, Amit T, Reznichenko L, Weinreb O, Youdim MB. Green tea catechins as brain-permeable, natural iron chelators-antioxidants for the treatment of neurodegenerative disorders. Mol Nutr Food Res. 2006;50:229–234.
55. Morel I, Lescoat G, Cogrel P, et al. Antioxidant and iron-chelating activities of the flavonoids catechin, quercetin and diosmetin on iron-loaded rat hepatocyte cultures. Biochem Pharmacol. 1993;45:13–19.
56. Arteel GE, Sies H. Protection against peroxynitrite by cocoa polyphenol oligomers. FEBS Lett. 1999;462:167–170.
57. Hatano T, Miyatake H, Natsume M, et al. Proanthocyanidin glycosides and related polyphenols from cacao liquor and their antioxidant effects. Phytochemistry. 2002;59:749–758.
58. Heo HJ, Lee CY. Epicatechin and catechin in cocoa inhibit amyloid beta protein induced apoptosis. J Agric Food Chem. 2005;53:1445–1448.
59. Levites Y, Amit T, Mandel S, Youdim MB. Neuroprotection and neurorescue against Abeta toxicity and PKC-dependent release of nonamyloidogenic soluble precursor protein by green tea polyphenol (–)-epigallocatechin-3-gallate. FASEB J. 2003;17:952–954.
60. Reznichenko L, Amit T, Zheng H, et al. Reduction of iron-regulated amyloid precursor protein and beta-amyloid peptide by (–)-epigallocatechin-3-gallate in cell cultures: implications for iron chelation in Alzheimer's disease. J Neurochem. 2006;97:527–536.
61. Rogers JT, Randall JD, Cahill CM, et al. An iron-responsive element type II in the 5'-untranslated region of the Alzheimer's amyloid precursor protein transcript. J Biol Chem. 2002;277:45518–45528.
62. Datla KP, Zbarsky V, Rai D, et al. Short-term supplementation with plant extracts rich in flavonoids protect nigrostriatal dopaminergic neurons in a rat model of Parkinson's disease. J Am Coll Nutr. 2007;26:341–349.
63. Schroeter H, Spencer JP, Rice-Evans C, Williams RJ. Flavonoids protect neurons from oxidized low-density-lipoprotein-induced apoptosis involving c-Jun N-terminal kinase (JNK), c-Jun and caspase-3. Biochem J. 2001;358:547–557.
64. Weinreb O, Amit T, Youdim MB. A novel approach of proteomics and transcriptomics to study the mechanism of action of the antioxidant-iron chelator green tea polyphenol (–)-epigallocatechin-3-gallate. Free Radic Biol Med. 2007;43: 546–556.
65. Reznichenko L, Amit T, Youdim MB, Mandel S. Green tea polyphenol (–)-epigallocatechin-3-gallate induces neurorescue of long-term serum-deprived PC12 cells and promotes neurite outgrowth. J Neurochem. 2005;93:1157–1167.
66. Weinreb O, Mandel S, Youdim MB. cDNA gene expression profile homology of antioxidants and their antiapoptotic and proapoptotic activities in human neuroblastoma cells. FASEB J. 2003;17:935–937.
67. Lee DW, Andersen JK. Role of HIF-1 in iron regulation: potential therapeutic strategy for neurodegenerative disorders. Curr Mol Med. 2006;6:883–893.

68. Tachibana H, Koga K, Fujimura Y, Yamada K. A receptor for green tea polyphenol EGCG. Nat Struct Mol Biol. 2004; 11:380–381.
69. Williamson MP, McCormick TG, Nance CL, Shearer WT. Epigallocatechin gallate, the main polyphenol in green tea, binds to the T-cell receptor, CD4: potential for HIV-1 therapy. J Allergy Clin Immunol. 2006;118:1369–1374.
70. Fujimura Y, Umeda D, Yano S, Maeda-Yamamoto M, Yamada K, Tachibana H. The 67kDa laminin receptor as a primary determinant of anti-allergic effects of O-methylated EGCG. Biochem Biophys Res Commun. 2007;364:79–85.
71. Castronovo V. Laminin receptors and laminin-binding proteins during tumor invasion and metastasis. Invasion Metastasis. 1993;13:1–30.
72. Jung JY, Han CR, Jeong YJ, et al. Epigallocatechin gallate inhibits nitric oxide-induced apoptosis in rat PC12 cells. Neurosci Lett. 2007;411:222–227.