CHOCOLATE AND HEALTH: FRIEND OR FOE?

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PUBLISHED IN: Frontiers in Nutrition and Frontiers in Immunology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88945-411-2
DOI 10.3389/978-2-88945-411-2

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CHOCOLATE AND HEALTH: FRIEND OR FOE?

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Criollo cocoa beans from Perù and heart health. Image: Mauro Serafini.

In the ancient past, cocoa has been appreciated as a high-calorie food to boost energy in soldiers and for its undefined medicinal and mystical properties. During other times, chocolate has been considered as the forbidden "food of God": a treasure of pleasure for the mind and the soul. The overall perception of the consumer for chocolate was of a "charming" and appealing food with lots of negative aspects related to high sugar content leading to consider chocolate as "junk food" for its "obesigen" calories.

Recently, in association with the renewed interest of nutrition science in alternative source of health-promoting foods and ingredients, a large body of research has been conducted to unravel the pro and cons of cocoa in relation to human health. Epidemiological evidences indicate that cocoa consumption helps preventing cardiovascular disease for its high content in bioactive flavonoids. Clinical trials show that chocolate consumption might improve vascular function, decreasing platelet aggregation and display an antioxidant and anti-inflammatory effect.

The putative protective action of cocoa seems to be multi-factorial and involving different aspects of vascular, antioxidant and endothelial function. However, the mechanism(s) that account for the benefits of cocoa it is still unclear.

The aim of this Research Topic is therefore to provide the reader with an objective picture of the state of art on the association between cocoa and health, mainly through the evidences of human trials; overwhelmingly considered the golden standard for nutritional science. The Research Topic will cover the analysis of the manufacturing processes of the chocolate and the

antioxidant effects in humans as well as the majority of the putative health effects of chocolate and cocoa, such as anti-inflammatory properties, effect on immunity, platelet aggregation, blood pressure, endothelial function and cognitive behavior. Unraveling the functional properties of cocoa will help to understand if the 'food of God' is a primordial gift for the health of mankind.

Citation: Serafini, M., Jirillo, E., eds. (2018). Chocolate and Health: Friend or Foe? Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-411-2

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frontiers in Nutrition

EDITORIAL
published: 22 December 2017
doi: 10.3389/fnut.2017.00067

Editorial: Chocolate and Health: Friend or Foe?

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Keywords: cocoa, chocolate, humans, antioxidants, immunity, inflammation, blood pressure, flavonoids

Editorial on the Research Topic

Chocolate and Health: Friend or Foe?

In the ancient past, cocoa has been appreciated as a high-calorie food to boost energy in soldiers and for its undefined medicinal and mystical properties, granting to chocolate the forbidden title of "food of God," primordial gift for the health of mankind. Later on, as a common place, the overall perception of chocolate consumers was that of a "charming" and appealing food with the negative aspects related to high caloric content, ultimately, leading to consider chocolate as "junk food" for its obesogenic calories.

In the last years, in view of the renewed interest for nutrition science, in search of alternative sources of health-promoting foods and ingredients, a large body of research has been conducted to unravel the pros and cons of cocoa consumption in relation to human health. The supposed protective action of cocoa seems to be multifactorial, also involving different aspects of the functional arrays of physiological defenses of the body, and, in particular, the immune system. The major aim of this Research Topic (RT) is, therefore, to provide the reader with an objective picture of the state of art on the association between cocoa consumption and health. Special emphasis will be placed mainly on human trials, encompassing the effects on antioxidants of chocolate manufacturing processes, on the one hand, and, on the other hand, the evaluation of putative healthy activities exerted by both chocolate and cocoa, with special reference to immune responsiveness, cardiovascular function, and cognitive behavior.

The RT starts focusing on the effects of technology processes on the *in vitro* antioxidant activity of chocolate (De Mattia et al.). This paper provides a very informative figure on the impact of each processing steps on the antioxidant content from raw bean to conched chocolate, highlighting the massive loss of redox ingredients during the food chain. De Mattia et al. reviewing the body of evidence about the antioxidant role of chocolate in long-term intervention trials, highlight the lack of studies on the effects of processing *in vivo*, also suggesting the importance of optimizing technological process linked with more pieces of evidence from human studies. This is in order to advice consumers about the "optimal" dose of chocolate.

Strengthening the findings by De Mattia et al. about the importance of testing chocolate in subjects characterized by an ongoing oxidative stress rather than in "not stressed" subjects, Ioannone et al. clearly show that chocolate polyphenol extract was more effective in inhibiting oxidative burst in human neutrophils and monocytes isolated from obese and overweight subjects, characterized by a more enhanced oxidative/inflammatory stress, than that observed in lean subjects. This work suggests the potential role of cocoa and chocolate's ingredients to modulate, through a redox mechanism, a key aspect of the cell-mediated immune response. In this direction, the manuscript from Camps-Bossacoma et al. expands such a perspective, reviewing the role of cocoa as a dietary modulator of the immune system, mainly in terms of antibody response, at

OPEN ACCESS

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This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Nutrition

Received: *16 November 2017*
Accepted: *12 December 2017*
Published: *22 December 2017*

Citation:
Serafini M and Jirillo E (2017) Editorial: Chocolate and Health: Friend or Foe? Front. Nutr. 4:67. doi: 10.3389/fnut.2017.00067

systemic and mucosal level, as well as of cytokine release and receptor expression. Results document that the effects of cocoa are exerted at multiple steps, from antigenic presentation and cytokine production by T helper cells to the intestinal homing of activated cells, thus, providing evidence for the ability of cocoa to play a role as immune-modulator. The last part of the RT discusses the role of cocoa on cardiovascular and cognitive functions, central nervous system, and gut health. Ludovici et al. provide a comprehensive overview on interventional studies in humans looking at the effects of chocolate on blood pressure and endothelial function. A detailed and clear picture of the mechanisms of action of cocoa's flavonols in improving markers of cardiovascular function and of all the variables involved is provided in figure 1. In their conclusions, authors comment on the importance of proper technological process (in agreement with De Mattia et al.) to have a high content of flavonoids in commercially available chocolate in order to maximize the cardiovascular benefit minimizing sugar and energy content. The fascinating and still unclear role of chocolate in neuromodulation and neuroprotective actions in humans is critically discussed by Socci et al. suggesting an array of potentiality for chocolate in protecting human cognition and counteracting cognitive decline in age-related neurological disorders. The manuscript by Magrone et al. discusses through a broad overview the different mechanisms of actions of cocoa's polyphenols, involving cellular transcription factors, specific kinases and signal transduction pathways from biological setting to clinical applications such as vascular and neurological dysfunctions during aging, obesity, and neurological disorders. Finally, the manuscript of the RT (Petyaev and Bashmakov) highlights the need to establish and improve a strict and fruitful connection between food industry and medical sciences to fill up certain gaps such as the absence of clinically justified recommendations.

To summarize, the collection of review and research articles presented under the RT provide a comprehensive set of information on the importance of cocoa and chocolate as a functional food able to modulate different aspects of human's physiological response to stress, such as immunity, and to optimize cardiovascular and cognitive functions. Despite many scientific efforts, the "optimal" dose of cocoa sufficient to display a protective effect is still object of debate. One can envisage that the extremely high content of bioactive ingredients makes conceivable a functional effect at low doses (around 5–7 g/day) without affecting much the daily caloric intake. We hope that this RT will prompt a critical "thinking" in the context of the scientific community on the association between chocolate and health, providing clues for further research developments.

AUTHOR CONTRIBUTIONS

The authors confirm being the only contributors of this work and approved it for publication.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the support of Frontiers in Nutrition staff for the continuous passionate and technical support.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

MINI REVIEW
published: 29 September 2017
doi: 10.3389/fimmu.2017.01207

From Cocoa to Chocolate: The Impact of Processing on *In Vitro* Antioxidant Activity and the Effects of Chocolate on Antioxidant Markers *In Vivo*

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Chocolate is a product processed from cocoa rich in flavonoids, antioxidant compounds, and bioactive ingredients that have been associated with both its healthy and sensory properties. Chocolate production consists of a multistep process which, starting from cocoa beans, involves fermentation, drying, roasting, nib grinding and refining, conching, and tempering. During cocoa processing, the naturally occurring antioxidants (flavonoids) are lost, while others, such as Maillard reaction products, are formed. The final content of antioxidant compounds and the antioxidant activity of chocolate is a function of several variables, some related to the raw material and others related to processing and formulation. The aim of this mini-review is to revise the literature on the impact of full processing on the *in vitro* antioxidant activity of chocolate, providing a critical analysis of the implications of processing on the evaluation of the antioxidant effect of chocolate in *in vivo* studies in humans.

Keywords: cocoa, chocolate, processing, polyphenols, antioxidant activity, chronic intervention studies

OPEN ACCESS

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Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Immunology

Received: *19 June 2017*
Accepted: *12 September 2017*
Published: *29 September 2017*

Citation:
Di Mattia CD, Sacchetti G, Mastrocola D and Serafini M (2017) From Cocoa to Chocolate: The Impact of Processing on In Vitro Antioxidant Activity and the Effects of Chocolate on Antioxidant Markers In Vivo. Front. Immunol. 8:1207. doi: 10.3389/fimmu.2017.01207

INTRODUCTION

Chocolate, thanks to its unique structure and flavor, is a food usually consumed for pleasure that has been recently reconsidered as a source of healthy compounds. Chocolate is rich in polyphenols such as flavanols, which possess antioxidant and anti-inflammatory properties and have a protective effect against degenerative diseases (1–6). Procyanidin and flavanol polymers also contribute to chocolate taste by affecting bitterness and astringency (7, 8). The polyphenol content of chocolate depends on many factors, some related to the raw material, and others related to processing (9, 10).

The majority of published reviews aim at analyzing the impact of processing on the polyphenol content of cocoa more than on its functional properties, focusing only on selected processing steps deemed to have a major impact on phenolic content, and, sometimes, without a specific discussion of all the single steps (9–11).

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; MRPs, Maillard reaction products; TE, trolox equivalents; TEAC, trolox equivalent antioxidant capacity; TPC, total phenolic content; ORAC, oxygen radical antioxidant capacity; TRAP, total radical-trapping antioxidant parameter; NEAC, non-enzymatic antioxidant capacity; NASH, non-alcoholic steatohepatitis; CVD, cardiovascular disease.

This mini-review aims at revising the literature on the impact of full processing on the *in vitro* antioxidant properties of chocolate providing a critical analysis of the implication of processing on the antioxidant effect of chocolate in *in vivo* studies in humans.

CHOCOLATE PROCESSING IN BRIEF

Chocolate-making consists of a multistep process. At harvest, cocoa fruit contains about 30–40 seeds covered by a mucilaginous pulp removed by yeast and bacteria during fermentation, which is a key step for the development of the chocolate flavor, since it produces aroma precursors. After fermentation, a drying step is required to reduce the water content to 5–7%; this ensures product stability before further processing. Dried cocoa beans or nibs (i.e., beans without the outer shell) are then roasted to further develop the chocolate flavor. The next step in cocoa processing involves nib grinding to convert the solid nibs into a liquid paste (liquor).

For the production of dark chocolate, the basic ingredients are cocoa liquor, sugar, cocoa butter, and emulsifiers. Milk and other ingredients may be added, mixed and then refined to reduce the particle sizes of solids. After refining, the conching operation, which consists of the agitation of the chocolate mass at high temperatures, and finally tempering, which consists in a heating, cooling and mixing process, are required for the development of the final texture and flavor.

PHENOLIC ANTIOXIDANTS IN CHOCOLATE

Polyphenols are the main class of antioxidants in unfermented cocoa beans, and they account for approximately 2% w/w (12). Cocoa contains several classes of phenolic compounds among which, flavanols (37%), proanthocyanidins (58%), and anthocyanins (4%) (11).

Flavanols, and, in particular, flavan-3-ols, are the most studied compounds in cocoa. The main flavan-3-ols, are (−)-epicatechin and (+)-catechin, which have an antioxidant activity of 2.4–2.9 trolox equivalents (TE) using the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay and 2.2 TE using the ferric reducing antioxidant power (FRAP) assay, but they can be epimerized into (+)-epicatechin and (−)-catechin during processing into chocolate (5, 13).

Flavan-3-ols may group together to form dimeric, oligomeric, or polymeric combinations of units that are denominated proanthocyanidins, among which we can include procyanidins (oligomers of epicatechin). Oligomeric and polymeric proanthocyanidins are present in raw beans but could further polymerize during processing (14–16). The procyanidin dimers (B1, B2, B3, and B5) and trimer C1, as well as oligomers, up to decamers, have been reported in cocoa and chocolate (12, 17–19). The average antioxidant activity of procyanidin dimers is about 6.5, and that of trimers is 7–8 TE using the ABTS assay. Monomers, dimers, and trimers account for almost 33% of the antioxidant activity of cocoa. The antioxidant activity of procyanidin polymers seems to increase depending on the degree of polymerization even though polymerization decreases the concentration of polyphenols; the relative contribution of decamers to the total antioxidant activity is low (14).

Esters of catechins, such as gallocatechins and epigallocatechins, can be found in raw beans (20) but could also be formed during processing, in particular, during roasting (16), whereas esters of epigallocatechins, such as epigallocatechingallate, have only been reported in chocolate (21).

Anthocyanins that have been reported in fresh beans (22) are degraded during fermentation due to hydrolysis and further polymerization in condensed tannins (20).

Minor phenolic compounds are also present (i.e., flavonols, phenolic acids, simple phenols and isocoumarins, stilbenes, and their glucosides), but their content is low and their contribution to total antioxidant activity is limited.

Apart from polyphenols, chocolate contains other process-derived antioxidants such as Maillard reaction products (MRPs) that form during high temperature processing, among which drying, roasting, and conching.

EFFECT OF COCOA PROCESSING ON ANTIOXIDANT ACTIVITY

The evaluation of the antioxidant (i.e., phenolics) content and activity much depends on the extraction solvent and procedure (9), which is not standardized throughout literature on cocoa, so data are difficult to compare. In the colorimetric assays of the total phenolic content (TPC), discrepancies may arise due to the phenolic compounds used as reference for the standard curve as well as to the presence of reducing compounds, interfering with the assay. Regarding antioxidant activity, comparison of results could be problematic due to the large number of heterogeneous tests used. The most common assays [ABTS, 2,2-diphenyl-1-picrylhydrazyl (DPPH), oxygen radical antioxidant capacity, total radical-trapping antioxidant parameter (TRAP), and FRAP] are based on different reaction mechanisms (single electron transfer, hydrogen atom transfer, or mixed mechanisms) and could give discordant results depending on the most abundant antioxidant molecules in the system and their interactions.

Cocoa Beans

Cocoa beans are the seeds of the tropical *Theobroma cacao* L. tree. There are four types of cocoa: Forastero, which comprises 95% of the world production of cocoa and is the most widely used; Criollo, which is rarely grown because of disease susceptibility; Trinitario, which is a more disease-resistant hybrid of Criollo and Forastero; and Nacional, which is grown only in Ecuador (20, 23). The concentration of phenolic compounds in cocoa beans is highly variable and depends primarily on genetics, and then on many other factors such as geographical regions of cultivation, agronomical practices and climatic conditions (20).

Generally, Criollo cocoa beans have a lower phenolic content compared to the Forastero variety (10). Unfortunately, few studies on the phenolic content and antioxidant properties of unfermented beans are available and most results refer to beans that have undergone fermentation, drying or both these processes. When unfermented beans are considered, the total phenolic

content results in a range between 67 and 149 mg/g (24) or 120 and 180 mg/g (25). Large differences in the content of total polyphenols and individual phenolic compounds in unfermented ripe seeds of Forastero, Trinitario, and Criollo cocoa of six different origins were reported (22). Antioxidant activities of 709 ± 17 μM and 240–490 mmol TE/g were reported when the DPPH test was used (26, 27); however, the tests differed as regards the experimental conditions adopted. Values of 1.29–2.29 mmol TE/g and 600–800 mmol TE/g_{dw} were found with the ABTS method (14, 27) while reducing activities in the range 713–930 mmol Fe^{2+}/g_{dw} were obtained when using the FRAP method (14).

Fermentation

Fermentation of the pulp surrounding the beans represents the first important step for the development of chocolate flavor and taste since it produces aroma precursors. During fermentation, which can last from 5 to 10 days, the combination of endogenous and microbial enzymatic activities, along with the rise of temperature to about 50°C, and the diffusion of metabolites into and out of the cotyledons, allow polyphenols to polymerize and react with other compounds to form complexes. Fermentation is thus considered responsible for the decrease of the flavan-3-ol content, (−)-epicatechin in particular.

The level of polyphenol reduction is proportionate to the degree of fermentation (25, 28–30). Significant differences can be detected in the TPC content after fermentation as determined by the Folin–Ciocalteu's reagent: a range between 120–140 mg/g was found by Di Mattia et al. (14); a similar range (90–120 mg/g) was reported by Niemenak et al. (24) and Afoakwa (20). Higher levels (220 mg/g) were detected by Ryan et al. (31) while lower contents were determined by do Carmo Brito et al. (32). The antioxidant activity, as determined by the ABTS, DPPH, and FRAP methods, generally followed the same fate of the phenolic content, with reduction levels of 20–40% (14, 32). In the work by Suazo et al. (26), a reduction of about 80% was determined in the DPPH values while an increase in the total antioxidant capacity (+50–160%), evaluated using DPPH and ABTS methods, was observed in cocoa varieties after spontaneous fermentation (27).

Drying

The aim of cocoa drying is to remove water so as to reach moisture content below 7% and is usually carried out by sun heating in static conditions but heating dryers are also used.

Sun drying reduces the polyphenol content to different extents: Camu et al. (29) reported a reduction from 77 to 44%, Di Mattia et al. (14), a 72% reduction, Hii et al. (11), a 30% reduction, and finally, de Brito et al. (28), a 26% reduction. The reduction of polyphenols depends on climatic conditions (29), and reduction levels ranging from 77 to 44% were reported for the same cocoa sample dried in different seasons.

Sun drying not only affects the polyphenol content but also the antioxidant activity of cocoa beans, and a reduction of about 70% of TPC and 80% in flavan-3-ols was shown to determine a decrease of 70 ± 5% in antioxidant activity depending on the method used (14).

Experimental data on air drying are scarce; an industrial process carried out on a batch of 1,600 kg of cocoa beans for 11 days at a temperature of 60°C, decreased the content of TPC (52%) and flavan-3-ols (66%) inducing a 60 ± 5% decrease of antioxidant activity, depending on the assay (14). Hot air drying of cocoa beans has also been studied in laboratory scale conditions (11, 33–36), and the mean reduction of total polyphenols was about 45%, but this could dramatically change depending on process conditions.

Roasting

Roasting determines the formation of the characteristic color, aroma, taste, and texture of roasted cocoa beans (37). Roasting temperatures of 120–150°C and times of 5–120 min are used (37, 38), and under these conditions, a decrease of flavanols and TPC has been observed.

During roasting, monomeric flavanols are reduced from 0 to 95% depending on the cultivar and the roasting temperature (16, 18, 19). High roasting temperatures improve the rate of polyphenol degradation, but in some cases a lower degradation was observed at high temperatures due to reduced processing times (16). Roasting temperature being equal, polyphenol degradation could be reduced by about 20% by adopting "high" relative humidity (5%) roasting conditions (18).

Roasting generally depletes the antioxidant activity of cocoa. Arlorio et al. (39) reported a decrease between 37 and 48% after pre-roasting at 100°C and roasting different varieties of cocoa at 130°C. Hu et al. (40) reported a decrease of antioxidant activity between 44 and 50% during roasting at high temperature (190°C) for short times (15 min) regardless of the assay used to test it. Ioannone et al. (16) observed a decrease of antioxidant activity during the first part of the roasting process and an increase during roasting time due to the formation of MRPs (16, 41). They reported a FRAP decrease of 51 and 45% at 125 and 145°C, respectively, as well as a TRAP increase of 7% at 125°C and a TRAP decrease of 20% at 145°C at the end of roasting. Dramatic differences between FRAP and TRAP values could be explained by considering MRP formation during roasting (41) since MRPs show a high chain-breaking activity despite their low reducing potential (42). A low roasting temperature (125°C) led to higher TRAP values but lower FRAP values than a high roasting temperature (145°C).

Conching

Conching is a unit operation based on the agitation of chocolate mass at high temperatures (above 50°C); it is an essential step for the development of proper viscosity and the attainment of final texture and flavor (23, 43). Different time/temperature combinations are selected according to the final product to be manufactured. In dark chocolates, temperatures ranging from 70 to 90°C can be used; variations in conching time and temperature combinations modify chocolate texture and flavor (44–46). Little attention has been paid to conching and its effect on polyphenol content and antioxidant properties. However, the conching process does not impair the phenolic content and pattern, as well as antioxidant activity since small yet not significant variations (3%) were found, regardless of the time/temperature combination applied (47–49). The same results were reported by Di Mattia et al. (15) for the TPC; however, authors reported a significant

increase of trolox equivalent antioxidant capacity (+16% on average) after conching.

Complete Process

The content and antiradical activity of cocoa beans, nibs, cocoa mass, and finished dark chocolate obtained from fermented beans from different geographical origins have been studied (50). Generally a progressive decrease of the phenolic content was observed upon processing, with roasting playing a major role. Nonetheless, the most significant losses in both phenolic content and antioxidant activity emerged in the final steps of processing, and in particular between the conched and non-tempered chocolate and the dark chocolate. The authors remarked that the results were ascribable to a dilution and even to an antagonistic effect produced by the addition of other ingredients. However, it is not clear if the authors considered the recovery of phenolic compounds on the basis of the amount used in the recipe (40% of cocoa mass).

Despite few attempts, the concurrent evaluation of the changes of polyphenol content and antioxidant activity upon all the processing steps is actually lacking and further investigations are needed. A general trend of the variation of antioxidant activity during processing is shown in **Figure 1**, obtained by taking into account the losses reported in works where single manufacturing steps were considered.

ANTIOXIDANT EFFECT OF CHOCOLATE *IN VIVO*

As far as chronic intervention studies in humans are concerned, there are no published studies that consider the effect of processing on the antioxidant properties of chocolate. This is a big gap in literature that deeply impairs the massive amount of work performed on chocolate processing optimization.

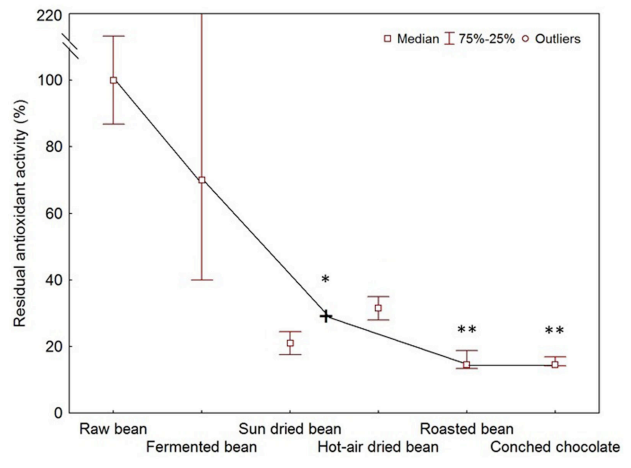

FIGURE 1 | Residual antioxidant activity of cocoa processed products after each processing step. *Mean of sun drying and hot air drying data; **data calculated on the mean of sun drying and hot air drying data. The top of the error bar of the second point on the *x*-axis overlaps with the figure frame.

Literature data from 10 human chronic intervention studies investigating the effect of chocolate intake on plasma and urinary levels of markers of antioxidant function, isoprostanes, and non-enzymatic antioxidant capacity (NEAC) were reviewed, and the results are presented on **Table 1**, where type of chocolate, number of intervention days, number of subjects, dose/day, effect on isoprostanes, effect on NEAC, and effect on polyphenols were described. Plasma/serum/urine isoprostanes, plasma NEAC, and polyphenols were assessed in nine, six, and seven studies, respectively.

On the basis of existing data, only one study showed an effect of chocolate on markers of antioxidant functions in humans. An increase in plasma polyphenol levels, namely, epicatechin, catechin, epicatechin-3*O*-methylether, and total phenolics, following a cocoa-based product supplementation period was detected in three studies out of seven. Increases were not correlated to any changes in markers of antioxidant function except for Loffredo et al. (57).

Although, from this analysis, it could be inferred that antioxidant networks do not respond very well to dietary supplementation with chocolate, some considerations are required. First of all, we need to consider the high heterogeneity of the reviewed studies, involving not only very different chocolate sources and doses of supplementation but also different size power, type of subjects, and duration of the supplementation; all variables that might affect the outcome of the trial.

It seems that all the different formulations that were used in the studies, such as tablets and chocolate drinks, failed to display any significant effect. Moreover, in agreement with previous evidences *in vivo* (1), milk chocolate does not produce any significant antioxidant effect in humans, and it has been utilized as control (57) in the only study where an effect was detected with dark chocolate.

The outcome of a study may also depend on the kind of subjects involved, namely, on their health condition. As previously stated, elevated levels of isoprostanes have been reported in individuals with diseases, or related risk factors, in which oxidative stress is involved; these subjects are supposed to have a higher requirement of antioxidants and, thus, to better respond to dietary intervention. In this respect, it is interesting to highlight that the only study where chocolate displayed an antioxidant effect in humans was conducted on subjects with non-alcoholic steatohepatitis diseases characterized by a non-physiological condition of oxidative stress. When oxidative stress is ongoing, endogenous antioxidants are not able to inhibit the production of free radicals efficiently; therefore, the contribution of exogenous antioxidants in diets may be crucial to support the endogenous redox system providing a clear effect on antioxidant status markers in humans (59–61). This aspect might explain the lack of effect observed for chocolate products, since all the studies, except the one where chocolate was effective, were conducted on healthy subjects characterized by a physiological equilibrium of free radicals and antioxidants. A systematic review (62) and a meta-analysis (63) support this hypothesis by showing that plant food, as well as chocolate supplementation, displays a better efficiency on antioxidant defense markers when the trials are conducted on subjects with oxidative stress-related risk factors

TABLE 1 | Chronic intervention studies in humans providing cocoa-based products: effect on F_2-IsoP, NEAC,[a] and PP.[a]

Food	Days	Subjects	Dose/day	F_2-IsoP	NEAC[a]	PP[a]	Reference
Flavonoid-rich dark chocolate	14	11	46 g	↔ Plasma	↔	↑ EC	(2)
Cocoa tablets	28	13	6 Tablets	↔ Plasma	↔	↑ EC, C	(51)
Dark chocolate and cocoa powder drink	42	25	36.90 g of dark chocolate and 30.95 g of cocoa powder drink	↔ Urine	↔	↔ Total phenols	(52)
Dark chocolate	21	15	75 g	↔ Plasma	↔		(53)
Polyphenols-rich dark chocolate	21	15	75 g	↔ Plasma	↔		(53)
Polyphenols-rich dark chocolate	126	22 with prehypertension or stage 1 hypertension	6.3 g	↔ Plasma		↔ EC, C, procyanidin B2, procyanidin B2 gallate	(54)
PP-rich milk chocolate	14	28	105 g		↔	↔ C, EC	(55)
Flavonoid-rich dark chocolate	14	20	45 g	↔ Serum			(56)
Dark chocolate	14	19 NASH 1	40 g	↓ Serum		↑ ECMet, TP	(57)
Milk chocolate	14	19 NASH 1	40 g	↔ Serum		ECMet,[b] ↔ TP	(57)

[a]*Plasma and/or serum measurements.*

↑, increase; ↔, no change; ↓, decrease; F_2-IsoP, F_2-isoprostanes; NEAC, non-enzymatic antioxidant capacity; PP, polyphenols; NASH, non-alcoholic steatohepatitis; EC, epicatechin; C, catechin; ECMet, epicatechin-3O-methylether.

[b]*Discrepancy between table and text. Modified from Petrosino and Serafini (58).*

rather than on healthy subjects. Moreover, in a large clinical trial on subjects characterized by cardiovascular disease risk factors, the PREDIMED study, it was shown that the efficiency of the supplementation of Mediterranean diet with antioxidant rich foods for 1 year was correlated with the baseline levels of antioxidant defenses (64). Subjects starting from lower levels of plasma NEAC showed a higher increase in NEAC compared to subjects starting from higher baseline levels of antioxidants, highlighting the importance of the redox "condition" of the subject on the efficiency of antioxidant supplementation.

CONCLUSION

Chocolate processing affects the content of total polyphenols as well as the antioxidant activity of chocolate and proper technology could "optimize" polyphenol retention and the *in vitro* antioxidant activity of chocolate. This work highlights the need to provide evidence of chocolate functionality in human beings to identify a proper technological process for chocolate processing. This is a necessary step to suggest to consumers the "optimal" doses of chocolate, which optimizes the functional effect by avoiding potential side effects, such as a high-energy load.

Human trials should be conducted mainly on subjects characterized by oxidative stress conditions, sharing a common requirement for dietary antioxidants, to increase the chance of observing an antioxidant effect *in vivo*.

AUTHOR CONTRIBUTIONS

CM, GS, and DM contributed to draft the section related to food chemistry and technology; MS contributed to draft the section related to nutrition; and CM, GS, DM, and MS contributed to data analysis and interpretation.

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Conflict of Interest Statement: The authors declare that the research project was conducted in the absence of any commercial or financial relations that could be construed as a potential conflict of interest.

frontiers in Nutrition

ORIGINAL RESEARCH
published: 09 June 2017
doi: 10.3389/fnut.2017.00023

Effect of Dark Chocolate Extracts on Phorbol 12-Myristate 13-Acetate-Induced Oxidative Burst in Leukocytes Isolated by Normo-Weight and Overweight/Obese Subjects

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OPEN ACCESS

Edited by:
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Reviewed by:
Douglas W. Bigwood, DBE Technologies, United States
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Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Nutrition

Received: *07 March 2017*
Accepted: *16 May 2017*
Published: *09 June 2017*

Citation:
Ioannone F, Sacchetti G and Serafini M (2017) Effect of Dark Chocolate Extracts on Phorbol 12-Myristate 13-Acetate-Induced Oxidative Burst in Leukocytes Isolated by Normo-Weight and Overweight/Obese Subjects. Front. Nutr. 4:23. doi: 10.3389/fnut.2017.00023

Oxidative and inflammatory stress represents a major risk factor for cardiovascular disease (CVD) in overweight and obese subjects. Between the different plant foods, chocolate has been shown to decrease CVD risk due to its antioxidant and anti-inflammatory properties. However, as we recently showed in epidemiological studies, meta-analyses, and human trials, dietary antioxidants resulted more effective in subjects characterized by an ongoing oxidative stress, than in healthy people. Aim of this work was to investigate the effect of different concentrations of chocolate phenolic extract (CPE) on *in vitro* free radical production, stimulated by phorbol 12-myristate 13-acetate (PMA), in leukocytes extracted from blood of normo-weight and overweight/obese subjects. Neutrophils from overweight/obese group had a significantly higher free radical production compared to the normo-weight group. In neutrophils, the lowest CPE concentration significantly reduced free radical production in overweight/obese group only, and higher CPE concentrations were effective in both groups. In monocytes, the CPE concentration that was significantly effective in reducing free radical production was lower in overweight/obese subjects than in normo-weight subjects. Chocolate polyphenol extracts inhibit oxidative burst in human neutrophils and monocytes with a higher efficiency in subjects characterized by an unphysiological oxidative/inflammatory stress, such as overweight and obese. Results of this study provide further evidence about a differential role of dietary antioxidant strictly related to the "stress" condition of the subjects.

Keywords: chocolate, polyphenols, antioxidant activity, oxidative stress, inflammation, obesity

Abbreviations: BMI, body mass index; CPE, chocolate phenolic extract; DHR123, dihydrorhodamine 123; EDTA, ethylene diamine tetraacetic acid; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalent; HPLC, high-performance liquid chromatography; MCF, mean channel fluorescence; MS, mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate buffer saline; PMA, phorbol 12-myristate 13-acetate; PMNs, polymorphonuclear leukocytes; ROS, reactive oxygen species; TPC, total polyphenols content; TPI, total phenolics index; WC, waist circumference.

INTRODUCTION

Oxidative stress, imbalance between reactive oxygen species (ROS) production and the neutralizing capacity of antioxidant mechanism, represents a prepathological status involved in the development of majority of degenerative diseases (1). A raising number of evidence is showing how obesity status is characterized by chronic oxidative and inflammatory stress condition playing a role in the initiation and progression of cardiovascular disease (CVD) (2). Several possible mechanisms that generate oxidative stress in obesity have been identified (3), between them, hyperglycemia (4–6), elevated tissue lipid levels (7, 8), inadequate antioxidant defenses (7, 9), chronic inflammation (10, 11), and endothelial ROS production (12, 13).

The tight link between oxidative and inflammatory stress, in the mechanisms of body's defenses against stress, is highlighted in the oxidative burst of leukocytes, the innate immune response involving the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and myeloperoxidase yielding to a massive production of ROS and reactive nitrogen species (14). Polymorphonuclear neutrophils and monocytes are the primary effector cells in the host response to injury and infection. During activation, neutrophils and monocytes generate and release extremely high amounts of ROS through NADPH oxidase system. When there is an excessive and uncontrolled ROS and cytokines production, a condition defined as "low-grade chronic inflammation" takes place and it has been associated with prepathological conditions and degenerative diseases (15, 16). Obesity is associated with an alteration of immune function and a high oxidative burst activity (17). The ROS production upon oxidative burst have been suggested to be modulated by dietary antioxidants that can scavenge free radicals and also exert indirectly their activity by inhibiting enzymes involved in ROS production. Recently, therapeutic tools such as functional foods and nutraceuticals were proposed to treat inflammatory diet-related disease, among which is obesity (18).

Cocoa and, among its principal products, dark chocolate are functional foods that have been shown to display an antioxidant role in humans (19). The antioxidant action of chocolate is due to its high content in flavonoids, such as epicatechin (EC), catechin, and proanthocyanidins, naturally occurring in cacao and cocoa (20) and also to compounds, such as Maillard reaction products, which are formed during cocoa and chocolate processing (21–24). Besides their antioxidant effect, cocoa and dark chocolate have been shown to decrease low-density lipoprotein oxidation and platelet activation, to enhance serum lipid profile, to lower blood pressure, and to promote endothelium-dependent relaxation, supporting the beneficial role of cocoa and dark chocolate in the cardiovascular system (25–30). Cocoa flavonoids has also exhibited promising regulatory effects on immune cells involved in innate and acquired immunity (31) and a recent review pointed out that the immune-modulatory effect of flavonoids is most pronounced in subjects with inflammatory stress than in healthy people (32).

The aim of the present study was to investigate the ability of polyphenol extracts from dark chocolate rich in flavonoids to protect *in vitro* neutrophils and monocytes, isolated from normoweight and overweight/obese subjects, from oxidative damage and to identify the correlation between body mass indices and oxidative stress.

MATERIALS AND METHODS

Chocolate Samples

Different types of dark chocolate bars rich in polyphenols and having a cocoa percentage between 60 and 70%, identified in previous studies, were analyzed in order to choose the sample with higher antioxidant (i.e., polyphenols) content and antioxidant activity.

Preparation of Chocolate Extract

Sample extraction was carried out according to the procedure described by Adamson et al. (33). Briefly, chocolate (4 g) was extracted with 25 mL of hexane to remove the lipids. The extract was centrifuged at 2,000 × *g* for 10 min, and the hexane was decanted. Hexane was evaporated at room temperature overnight. The defatted sample was extracted with a solvent mixture (acetone:water:acetic acid, 70:29.5:0.5 v/v/v). After the addition of solvent, the tube was vortexed for 30 s and eventually subjected to sonication at 37°C for 10 min. At the end of extraction, the tube was centrifuged at 2,000 × *g* for 15 min. The supernatant, representing the chocolate phenolic extract (CPE), was collected and used for further analyses.

Polyphenols Determination

Flavanols and proanthocyanidins determination was carried out by high-performance liquid chromatography (HPLC) analysis according to Ioannone et al. (23). The sample (20 μL) was injected onto a Phenomenex 5 μm normal-phase Luna Silica column, 100 A, 250 mm × 4.6 mm (inside diameter), at 25°C; the column was equipped with a SecurityGuard Cartridges Silica 4 mm × 3.0 mm (inside diameter). Separation of proanthocyanidins was carried out at a flow rate of 1 mL min^{-1} with a linear gradient from A (dichloromethane) to B (methanol) and a constant 4% level of C (acid acetic and water, 1:1 v/v) according to Counet and Collin (34). Gradient elution was as follows: from 14 to 28% B from 0 to 30 min, from 28 to 50% B from 30 to 60 min, from 50 to 86% B from 60 to 65 min, and isocratic from 65 to 70 min. Separation of the compounds was previously made according to retention times by HPLC analysis and then, the compounds were collected according to Counet and Collin (34) and submitted to mass spectrometric (MS) analysis for their identification.

The MS analysis of the HPLC fractions (P1–P10) has been carried out by means of a triple quadrupole mass spectrometer API 2000 from AB-Sciex (Toronto, ON, Canada) equipped with a TurboIon-Spray source. The spectra were acquired by injecting each solution at a flow of 10 μL min^{-1} by a syringe pump; all the analytes were detected in negative ionization with a capillary voltage of −4,500 V, nebulizer gas (air) at 0.21 N mm^{-2}, curtain gas (nitrogen) at 0.21 N mm^{-2}. The declustering potential was set at −22 V for $m/z < 1{,}000$ amu; −80 V for $m/z > 1{,}000$ amu. For the MS/MS experiments, the collision gas was set at 3 (in a scale 0–6) and the collision energy was −20 eV. The spectra were acquired using the AB-Sciex Analyst Software 1.5.

The quantification of single proanthocyanidins was carried out by HPLC analysis using diode array detection. Since proanthocyanidins show a similar absorption coefficient (34), a calibration curve made with (−)-EC was used for their quantification and the results for each proanthocyanidins class were expressed as milligrams of EC equivalents per gram of chocolate. The concentrations of the different classes of phenolic compounds were added to compute the total polyphenol content.

Total Phenolics Index and Ferric Reducing Antioxidant Power (FRAP) of Chocolate

The total phenolics index (TPI) was determined according to Singleton and Rossi (35). A total of 20 μL of diluted CPE was pipetted into a 96-well plate. A total of 100 μL of Folin–Ciocalteu reagent diluted 1:10 with water and 75 μL of 10% (w/v) sodium carbonate solution were added to each well, and the plate was placed for 2 h at room temperature in dark. Absorbance at 740 nm was then measured using a Sunrise absorbance plate reader (Tecan, Segrate, Italy). Total phenolic index was calculated by a calibration curve, obtained with increasing concentrations of gallic acid, and results were expressed as milligrams of gallic acid equivalents per gram of sample. The reducing power of extracts was measured by the FRAP assay (36), which is based on the reduction of the ferric-tripiridyltriazine (Fe^{3+}-TPTZ) complex to the ferrous form at low pH. Briefly, 160 μL of FRAP reagent, prepared daily, was mixed with 30 μL of water and 10 μL of diluted CPE; the absorbance at 595 nm was recorded after a 30 min incubation at 37°C with the Sunrise absorbance plate reader. FRAP values were calculated using a calibration curve obtained with increasing concentrations of Fe^{2+} and expressed as micromoles of Fe^{2+} per micromoles per gram of sample.

Subjects

Eight normo-weight [four men and four women aged 46 ± 9 years, body mass index (BMI) = 20.54 ± 0.94 kg m^{-2}] and seven overweight and obese subjects (four men and three women aged 47 ± 11 years, BMI = 27.21 ± 3.52 kg m^{-2}) were recruited for the study. Criteria for inclusion were based on physical examination and BMI. Exclusion criteria are diabetes mellitus, CVD, gastrointestinal tract disease, pulmonary disease, psychiatric disorder, alcohol and drug dependence, history of organ transplant, surgery within 12 months, positive test results for immunodeficiency virus, evidence for hepatitis B virus or hepatitis virus C infection, chronic liver disease or nephropathies, cancer, organ failure, taking lipids-lowering, anti-inflammatory or other medications, taking vitamins, minerals, polyphenols or other supplements, regular consumer of fruits and vegetables of more than four servings per day, following caloric restriction diet, and unbalanced intake of macro and micro nutrients. Waist circumference (WC) was measured in subjects who were categorized as normal or overweight on the BMI scale. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

Oxidative Burst

Venous peripheral bloods from health volunteers were collected in vacutainer tubes containing ethylene diamine tetraacetic acid (EDTA). All the tubes were stored at room temperature and were immediately used as a source of polymorphonuclear leukocytes (PMNs) for oxidative burst generation assay. PMNs were isolated after bulk erythrocyte lysing. After 10 min of incubation with lysis buffer (1 L of distilled water, 8.02 g of ammonium chloride, 0.84 g of sodium bicarbonate, and 0.37 g of EDTA), the cells were washed twice with phosphate buffer saline (PBS). The method adopted for the measurement of oxidative burst in neutrophils use dihydrorhodamine 123 (DHR123) as probe and phorbol 12-myristate 13-acetate (PMA) for activation (37, 38). DHR123 is an uncharged non-fluorescent probe that passively diffuses across cell membranes and is converted upon oxidation to the fluorescent membrane-impermeant rhodamine 123 (37, 39). Leukocytes (500 cells/μL) staining was performed in PBS with DHR123 (20 μM) for 15 min at 37°C. Leukocytes were split into two different aliquots containing PMA or not (unstimulated sample). Lasting time was 15 min at 37°C, after which cells were stored in ice, to stop reactions, and further rapidly analyzed. Unstimulated cells were used as blank, and trolox was used as standard.

To investigate the *in vitro* ability of dark chocolate to protect neutrophils and monocytes, leukocytes were split into different aliquots containing PMA and different concentration of CPE (previously dried under nitrogen flow and then suspended in dimethyl sulfoxide): 5, 10, 20, 50, and 100 μg mL^{-1}. To test whether polyphenols in chocolate extract preparation were responsible for the inhibitory effect of oxidative burst, the effect of EC, one of the major polyphenols in cocoa, was studied at a concentration of 100 μM. Flow cytometric analyses were carried out using a FACScan flow cytometer (BD Biosciences, Milan, Italy). Lymphocytes, monocytes and neutrophils were sorted out using the cytometer by segregating them in gates using forward angle light scatter and side scatter. ROS production was quantified by mean channel fluorescence (MCF) of DHR123 green fluorescence histogram (FL1). Free radicals production (stimulation index) was obtained by dividing the MCF of PMA-stimulated granulocytes by the MCF of unstimulated granulocytes (37).

Statistical Analyses

All determinations were carried out at least in triplicate. Experimental data are presented as mean values ± SD. Differences between means were compared using the Student's *t*-test for independent (unpaired) samples. Statistical analyses were performed using the Microsoft Excel software.

RESULTS

The antioxidant (i.e., polyphenols) content, the total phenolic index, and antioxidant activity of six commercial dark chocolate samples with 60–70% cocoa were determined. **Table 1** shows the content of total polyphenols, the total phenolics index, and the FRAP of the selected chocolate samples.

Sample 1, showing the highest content of total polyphenols as well as the highest antioxidant capacity, was characterized for its phenolic profile (**Table 2**) and was used for an *ex vivo* study aimed to investigate the effect of chocolate phenolic extract (CPE) addition on PMA-induced oxidative burst of leukocytes isolated by normo-weight and overweight/obese subjects.

TABLE 1 | Total polyphenols content (TPC), total phenolics index (TPI), and ferric reducing antioxidant power (FRAP) of selected chocolate samples.

Sample	TPC (mg g^{-1})	TPI (mg of gallic acid equivalents g^{-1})	FRAP (μmol g^{-1})
Chocolate 1	3.81 ± 0.68	20.34 ± 0.24	213.4 ± 11.5
Chocolate 2	3.38 ± 0.63	19.05 ± 0.16	181.9 ± 42.1
Chocolate 3	3.73 ± 0.63	20.11 ± 0.12	201.2 ± 25.5
Chocolate 4	2.81 ± 0.53	17.33 ± 0.10	150.3 ± 35.4
Chocolate 5	1.41 ± 0.39	12.88 ± 0.11	74.8 ± 22.8
Chocolate 6	2.43 ± 0.52	16.15 ± 0.16	101.4 ± 24.5

TABLE 2 | Monomeric flavanols and proanthocyanidins content of chocolate 1 (mean ± SD).

Compound	mg g^{-1}
Epicatechin + catechin	1.57 ± 0.07
Proanthocyanidin dimers	0.70 ± 0.02
Proanthocyanidin trimers	0.44 ± 0.02
Proanthocyanidin tetramers	0.35 ± 0.02
Proanthocyanidin pentamers	0.35 ± 0.01
Proanthocyanidin hexamers	0.14 ± 0.02
Proanthocyanidin heptamers	0.10 ± 0.01
Proanthocyanidin octamers	0.08 ± 0.00
Proanthocyanidin nonamers	0.08 ± 0.00

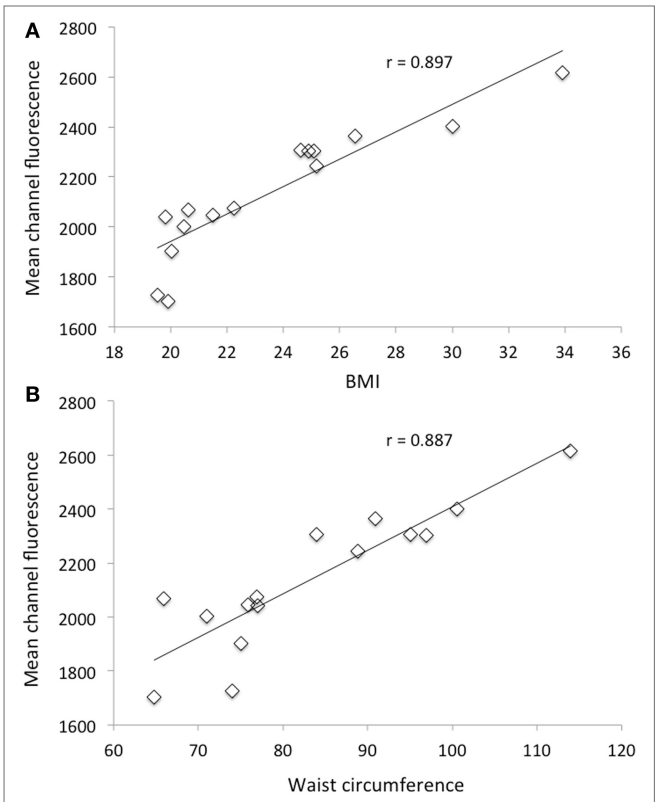

FIGURE 1 | Relationship between mean channel fluorescence (MCF) after phorbol 12-myristate 13-acetate (PMA) stimulation of neutrophils and body mass index **(A)** and MCF after PMA stimulation of neutrophils and waist circumference **(B)** for all selected subjects (n = 15).

In order to evaluate relationship between BMI and oxidative stress, the response to PMA for all subjects in both neutrophils and monocytes was first compared. Eventually, the effect of chocolate polyphenols was evaluated by comparing the monocytes and neutrophils activation between the two groups of subjects after adding different concentrations of chocolate extract.

In **Figure 1**, the relationship between MCF of neutrophils after PMA stimulation and BMI (**Figure 1A**) as well as WC (**Figure 1B**) for all subjects (normo-weight and overweight/obese volunteers) is shown. In both cases, there was a highly significant ($P < 0.001$) positive correlation ($r = 0.897$ and 0.887 for BMI and WC, respectively) between the considered morphometric indices and the response of neutrophils after PMA stimulation. No correlation was found between the same morphometric indices and the response of monocytes after PMA stimulation (data not shown).

Neutrophils isolated from overweight and obese individuals showed a significantly higher ($P < 0.05$) intracellular ROS generation when compared with those from healthy normo-weight subjects since the mean of fluorescence in normal individuals was 1,954 ± 61 and in overweight/obese ones was 2,353 ± 78 (**Figure 2**).

In neutrophils, as shown in **Figure 2**, all the tested concentration of CPE resulted significantly effective in reducing fluorescence of cells of overweight/obese subjects. The lowest cocoa polyphenol concentration (5 μg mL^{-1}) significantly reduced fluorescent intensity ($P < 0.01$ vs. PMA) in overweight/obese subjects only, with an inhibition of radical production of 27% respect to PMA. A 10 μg mL^{-1} chocolate concentration fraction showed a significant reduction ($P < 0.01$ vs. PMA) of fluorescent intensity for overweight/obese subjects and a less significant reduction ($P < 0.05$ vs. PMA) in normo-weight subjects (ROS inhibition production respect to PMA 31 and 19%, respectively). Higher chocolate polyphenol concentration (from 20 to 100 μg mL^{-1}) showed a more significant reduction ($P < 0.001$ vs. PMA) in fluorescence intensity than low concentration in both groups (65 and 62% for 100 μg mL^{-1}, 49 and 55% for 50 μg mL^{-1}, and 41 and 37% for 20 μg mL^{-1} for normo-weight and overweight/obese, respectively). EC, used as positive control, exerted a significant ($P < 0.001$ vs. PMA) antioxidant effect in normo-weight and overweight/obese subjects, at a concentration of 100 μM.

Mean channel fluorescence of monocytes from normo-weight and overweight/obese subjects subjected to PMA-induced burst, in the same experimental conditions previously reported for neutrophils, is shown in **Figure 3**. A concentration of 100 μg mL^{-1} of chocolate polyphenol extract significantly reduced ($P < 0.01$) fluorescence intensity (MCF = 37 ± 12) in overweight/obese subjects group respect to PMA fluorescence (MCF = 72 ± 25), while in normal weight group, the reduction of fluorescence intensity (MCF = 35 ± 11) with respect to PMA (78 ± 14) was present but less significant ($P < 0.05$). The concentration of 50 μg mL^{-1} resulted the second most effective polyphenol concentration in reducing fluorescence both for normo-weight and overweight/obese subject groups ($P < 0.05$ vs. PMA). At 20 μg mL^{-1} polyphenol concentration, a significant reduction

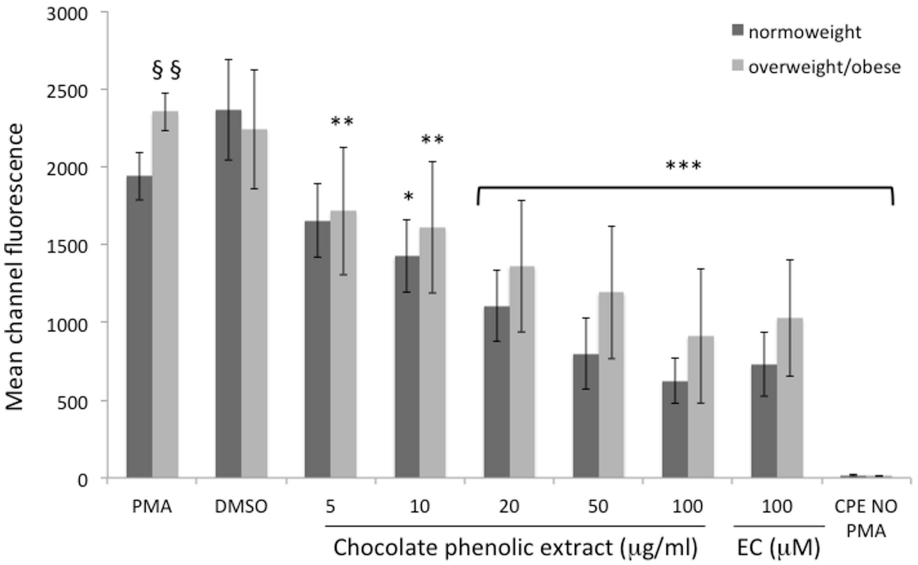

FIGURE 2 | Mean channel fluorescence of neutrophils from normo-weight and overweight/obese subjects in different treatments. Results are expressed as mean ± SD with $n = 8$ for normo-weight and $n = 7$ for overweight/obese groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$: significance of the difference between sample and phorbol 12-myristate 13-acetate (PMA)-stimulated control. $^{§§}P < 0.05$: significance of the difference between PMA normo-weight group and PMA overweight/obese group.

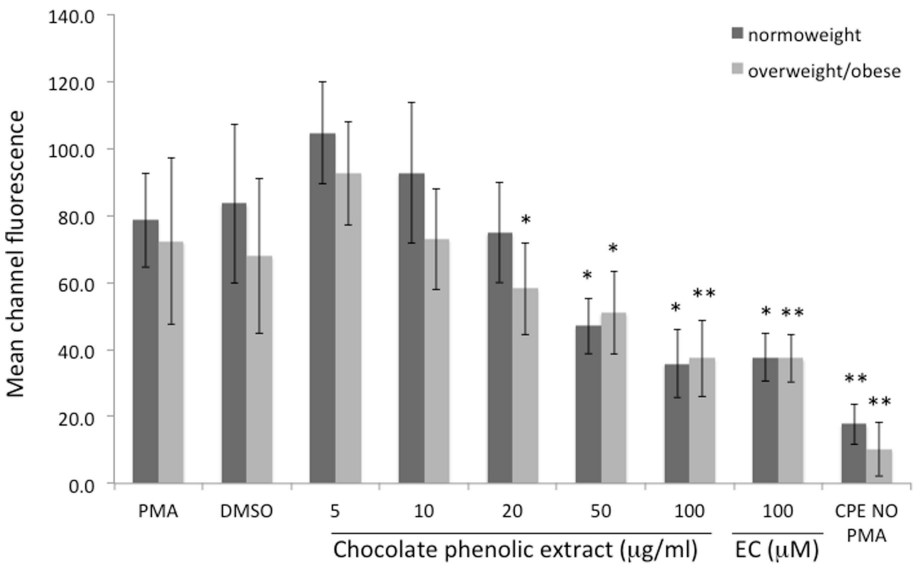

FIGURE 3 | Mean channel fluorescence of monocytes from normo-weight and overweight/obese subjects in different conditions. Results are expressed as mean ± SD with $n = 8$ for normo-weight and $n = 7$ for overweight/obese groups. $^{*}P < 0.05$, $^{**}P < 0.01$: significance of the difference between sample and phorbol 12-myristate 13-acetate-stimulated control.

was observed only for overweight/obese subjects ($P < 0.05$ vs. PMA). The lowest chocolate polyphenol concentration (10 and 5 μg mL^{-1}) did not show any significant activity in monocytes. EC of 100 μM determined a significant fluorescence reduction for normal weight ($P < 0.05$ vs. PMA) and overweight/obese subjects ($P < 0.01$). A significant correlation was observed between percentage of inhibition of ROS in neutrophils and monocytes and dark chocolate concentrations of phenolics in the normo-weight (**Figure 4**).

DISCUSSION

Results of this study clearly indicate that PMA-induced oxidative burst from human neutrophils display a significant correlation

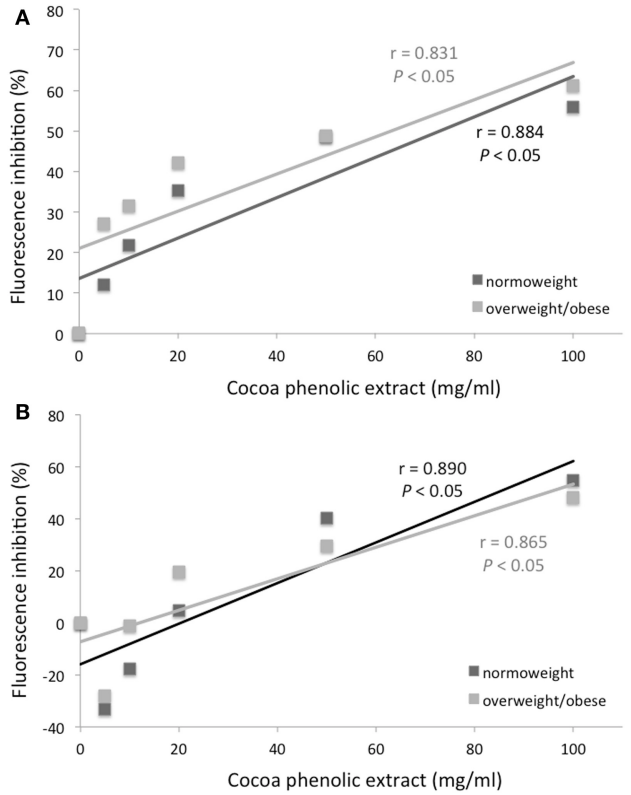

FIGURE 4 | Mean channel fluorescence inhibition (%) of neutrophils **(A)** and monocytes **(B)** from normo-weight and overweight/obese subjects after phorbol 12-myristate 13-acetate stimulation as a function of the concentration of chocolate phenolic extract.

with BMI and WCs of the subjects. Neutrophils isolated from overweight and obese individuals showed a higher intracellular ROS generation compared to cells from normo-weight subjects highlighting the presence of oxidative stress associated with excess body fat. We also showed that CPE was effective in reducing the PMA-induced burst of neutrophils and monocytes in a dose-dependent way. Moreover, the inhibitory effect of CPE against ROS production is more evident in leukocytes of subjects characterized by an unphysiological oxidative/inflammatory stress, such as overweight and obese subjects, rather than in normo-weight subjects.

It is known that priming agents able to induce a weak ROS production could enhance the ROS production of neutrophils after exposure to a stimulus that induces an oxidative burst characterized by a strong ROS production (40). Thus, the higher ROS production observed after PMA stimulation of neutrophils from overweight/obese subjects suggest that obesity, similar to other pathological conditions, is associated with an unbalanced redox status, shifting vs. pro-oxidant conditions, which enhance neutrophils response to PMA (41). This suggestion is supported by literature data indicating that obese individuals demonstrate elevated levels of free radicals (42) and a marked decrease in antioxidant defenses (43).

The first evidence of an inhibitory effect of flavonoids on the respiratory burst of neutrophils was by Pagonis et al. (44), who showed that all the studied flavonoids inhibited neutrophils hydrogen peroxide generation. The antioxidant activity of flavonoids against the respiratory burst of neutrophils from healthy human was later confirmed also by other authors (45) displaying a dose-dependent decrease of neutrophil ROS production upon exposure to polyphenol extracts. Cocoa's flavonoids were proven to moderate oxidative burst derived from a first stimulation of neutrophils with lipopolysaccharide followed by formyl-methionyl-leucyl-phenylalanine aimed to induce strong ROS formation (46). Since the polyphenolic profile of the chocolate used in this study was characterized, the weighed average molecular weight of the phenolics contained in the extract was computed as 786.7 g mol^{-1}; thus, the tested concentration of polyphenols was calculated between 6.4 and 127 μM. In neutrophils, the lowest tested concentration (6.4 μM) was effective in reducing significantly ROS production and a 50 μg mL^{-1} (69 μM) chocolate extract determined a protection against ROS formation not significantly different from the 100 μg mL^{-1} (147 μM) catechin positive control. Since the chocolate phenolic extract contains catechin, EC, and polymeric proanthocyanidins, it is possible to affirm that polymeric proanthocyanidins, that are present in a certain amount in chocolate, could show an inhibitory effect of ROS production even higher than catechin.

At low dosages, the CPE was more effective in inhibiting the oxidative burst of neutrophils than that of monocytes. Most of the studies on oxidative burst concern analysis of neutrophils and not monocytes. In fact, these two types of cells differ in structure, time of activation, and behavior during the inflammation response. In a typical acute inflammatory response, there is a well-defined sequence: neutrophils accumulate first and usher monocytes into sites of inflammation where the latter accumulates second (47). Probably, for this reason, neutrophils are most responsive toward the chocolate polyphenol extract than monocytes.

Postprandial stress, arising from the consumption of unbalanced high caloric meals, has been associated with an increased risk for atherosclerosis and obesity (48). Indeed, after the consumption of unbalanced meals, the susceptibility of the organism toward oxidative damage is increased and metabolic and transcriptional pathways are activated leading to a massive increase in the production of free radicals and pro-inflammatory cytokines, through an increase of neutrophil numbers (49, 50). Flavonoid-rich foods have been shown to reduce the onset of oxidative stress derived from postprandial stress condition (51). In this work, we clearly showed, for the first time, that the antioxidant effect of cocoa extract is more evident in neutrophils extracted from subjects characterized by oxidative stress such as overweight and obese. This evidence is in agreement with previous evidences showing a more efficient action of dietary antioxidant when an oxidative stress is present. In a systematic review, Serafini et al. (52) showed that, in dietary intervention studies with plant foods, the percentage of efficiency through an increase of plasma antioxidant defenses was of 58 and of 70%, respectively, on healthy subjects and in subjects characterized by oxidative stress risk factors. Moreover, in the meta-analyses by Lettieri-Barbato et al. (53), the overall action of fruit, vegetables, chocolate, wine, and tea

in increasing antioxidant defenses after ingestion was three times higher in subjects with oxidative stress conditions than in healthy subjects. In this study, the marked antioxidant effect of chocolate extracts in neutrophils from overweight and obese subjects suggest that, when a chronic postprandial stress is recurring, leading to excess body fat and to increased neutrophils activation, the presence of exogenous antioxidant might efficiently counteract cellular free radical production, reducing the onset of oxidative stress development.

CONCLUSION

Dark chocolate polyphenols showed an inhibitory effect on PMA activated oxidative burst in white blood cells, in a concentration-dependent manner. This inhibitory effect is significantly more evident in subjects characterized by an unphysiological oxidative/inflammatory stress, such as overweight and obese subjects. Although antioxidant capacity of dark chocolate in human is deeply related to the low bioavailability of the flavonoids contained in the foodstuff, our results suggest that chocolate polyphenols might efficiently display a higher antioxidant/anti-inflammatory effect in overweight and obese subjects, characterized by an ongoing oxidative and inflammatory stress, than in normo-weight subjects. Results of this study provide further evidence about a differential role of dietary antioxidant related to the "stress" condition of the subjects. In future human trials, one criteria of selection of the subjects should be the presence of oxidative stress condition in order to maximize the chance of success of the antioxidant intervention. Specifically, more evidences in human trials are needed in order to unravel the role of cocoa and polyphenols in reducing oxidative and inflammatory stress in overweight and obese subjects.

ETHICS STATEMENT

Due to the nature of the study, simple collection of blood specimens, the involvement of ethical committee was not necessary. All subjects remain anonymous and gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

MS designed the experiment; FI and GS conducted the experiments; MS and GS contributed to data analysis and interpretation; FI, GS, and MS contributed to the manuscript drafting.

FUNDING

The work of FI was funded by the Project P.O.R. FESR 2007–2013 financed by the Region Abruzzo and by the Project QualiFu—IDF financed by MiPAF (Italian Ministry of Agricultural and Forestry Policies).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

REVIEW
published: 27 June 2017
doi: 10.3389/fnut.2017.00028

Cocoa Diet and Antibody Immune Response in Preclinical Studies

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The ability of cocoa to interact with the immune system *in vitro* and *in vivo* has been described. In the latter context, a cocoa-enriched diet in healthy rats was able to modify the immune system's functionality. This fact could be observed in the composition and functionality of lymphoid tissues, such as the thymus, spleen, and lymph nodes. Consequently, immune effector mechanisms, such as antibody synthesis, were modified. A cocoa-enriched diet in young rats was able to attenuate the serum levels of immunoglobulin (Ig) G, IgM, and IgA and also the intestinal IgM and IgA secretion. Moreover, in immunized rats, the intake of cocoa decreased specific IgG1, IgG2a, IgG2c, and IgM concentrations in serum. This immune-regulator potential was then tested in disease models in which antibodies play a pathogenic role. A cocoa-enriched diet was able to partially prevent the synthesis of autoantibodies in a model of autoimmune arthritis in rats and was also able to protect against IgE and T helper 2-related antibody synthesis in two rat models of allergy. Likewise, a cocoa-enriched diet prevented an oral sensitization process in young rats. In this review, we will focus on the influence of cocoa on the acquired branch of the immune function. Therefore, we will focus on how a cocoa diet influences lymphocyte function both in the systemic and intestinal immune system. Likewise, its potential role in preventing some antibody-induced immune diseases is also included. Although further studies must characterize the particular cocoa components responsible for such effects and nutritional studies in humans need to be carried out, cocoa has potential as a nutraceutical agent in some hypersensitivity status.

Keywords: gut-associated lymphoid tissue, IgA, IgG, IgM, immunoregulator, lymph nodes, spleen, tolerance

OPEN ACCESS

Edited by:
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Reviewed by:
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Alberto Finamore,
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Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Nutrition

Received: *27 March 2017*
Accepted: *06 June 2017*
Published: *27 June 2017*

Citation:
Camps-Bossacoma M, Massot-Cladera M, Abril-Gil M, Franch A, Pérez-Cano FJ and Castell M (2017) Cocoa Diet and Antibody Immune Response in Preclinical Studies. Front. Nutr. 4:28. doi: 10.3389/fnut.2017.00028

INTRODUCTION

Antibody response is a kind of acquired immune response produced by complex interactions between several types of immune cells after the entry of an antigen into the body. In brief, when dendritic cells come into contact with an antigen in the skin or the mucosa, they become antigen-presenting cells and will be in charge of finding specific helper (Th) cells in order to trigger an acquired immune response (1). Activated specific Th cells will differentiate into effector T cells that, by means of different patterns of cytokines, will enhance the function of cells, such as B lymphocytes, macrophages, natural killer (NK) cells, cytotoxic T (Tc) lymphocytes, mast cells, or eosinophils. The activation of B cells, mainly related to Th2-immune response, will produce the formation of plasma cells that will eventually synthesize antibodies against the triggering antigen (1). In addition, inside the germinal centers of the secondary lymphoid tissues, another kind of antigen presentation occurs. Follicular dendritic cells (FDC) retain the native antigen that could prime

B cells to synthesized specific antibodies (2, 3). The FDC can form immune complex-coated bodies known as iccosomes that could also affect B cell activation, maturation, and maintenance (4). Whereas antibodies will neutralize or facilitate antigen destruction, sometimes, such as in hypersensitivity and autoimmune reactions, they could have a harmful effect on the body.

Although the earliest evidence for the medical use of cocoa was found in Mesoamerican civilizations (5), nowadays, the healthy properties of cocoa and its derivatives are re-emerging. In addition to the effects of cocoa on cardiovascular health (6, 7), the nervous system (8), and cancer (9–11), cocoa also has an effect on the immune system. The immunomodulatory properties of cocoa include its potential anti-inflammatory role, demonstrated in both *in vitro* and *in vivo* studies (12–14). However, only few clinical studies with this aim have been carried out, and recently it was suggested that there is scarce evidence of the anti-inflammatory effects of cocoa consumption in humans (15). Nevertheless, researchers in this field have joined on several occasions to discuss in depth the effects of chocolate and cocoa on medicine and have demonstrated the increasing emergence of cocoa as a diet compound able to prevent some diseases, or even being a coadjuvant in some therapies (16, 17). In this review, we will focus on the influence of cocoa on the acquired branch of the immune function. Therefore, we will focus on how a cocoa diet influences lymphocyte function both in the systemic and intestinal immune system. Likewise its potential role in preventing some antibody-induced immune diseases is also included.

COCOA INFLUENCES SYSTEMIC ANTIBODY SYNTHESIS

Preclinical studies performed 10 years ago showed for the first time the *in vivo* influence of a cocoa diet on the immune system (14, 18). These studies were carried out in young rats that were fed a diet containing 10% cocoa or in rats that were orally administered with a dose equivalent to 4% cocoa in food intake for 3 weeks. Results showed that the 10% cocoa-enriched diet, but not the 4% dose, was able to decrease serum immunoglobulin (Ig) G, IgM, and IgA concentrations (18) (**Table 1**). A further analysis of IgG isotypes showed that 3-week-old rats fed a 10% cocoa diet for 3 weeks resulted in attenuated levels of IgG2b antibodies but increased levels of IgG2a (19) (**Table 1**). However, in a study in which the cocoa diet was given later, at 6 weeks of age, the 10% cocoa-enriched diet was associated with lower values of serum IgG2a but higher serum IgG2c concentrations than those present in animals fed the standard diet (20) (**Table 1**). Moreover, it was observed that the minimum dose to achieve such an effect was 5% cocoa in the diet (20) and, at any rat age, a 5 or 10% cocoa diet attenuated the serum levels of IgM and IgA (19, 20), the effects being clearer when animals were younger and the diet lasted longer. Therefore, these studies in rats showed that a cocoa diet influences systemic immunoglobulin production but the effect depends on the antibody isotype, the age of the animal, and the length of the cocoa intervention.

TABLE 1 | Summary of the effects of cocoa diet in serum immunoglobulins and specific antibodies in healthy rats.

Strain	Initial age (weeks)	Cocoa dose	Length of the study (weeks)	Results	Reference
Wistar rats	3	4% by oral gavage	3	=IgG =IgM =IgA	(18)
		10% in the food	3	↓IgG ↓IgM ↓IgA	
Wistar rats	6	2% in the food	3	=IgG1, IgG2a, IgG2b, IgG2c =IgM =IgA	(20)
		5% in the food	3	=IgG1, IgG2b, ↓IgG2a, ↑IgG2c =IgM =IgA	
		10% in the food	3	=IgG1, IgG2b, IgG2c, ↓IgG2a ↓IgM =IgA	
Wistar rats	4	10% in the food	7	=IgG1, IgG2a, IgG2c, ↓IgG2b ↓IgM ↓IgA	(19)
Wistar rats	3	4% in the food	9	=Specific IgG2a, IgG2b ↓Specific IgG1, IgG2c ↓Specific IgM	(21)
		10% in the food	9	↓Specific IgG1, IgG2a, IgG2c ↑Specific IgG2b ↓Specific IgM	

Arrows indicate increases or decreases, equals sign means no changes.
Ig, immunoglobulins.

Apart from the cocoa's influence on basal serum immunoglobulin levels, it was interesting to shed some light on the antibody response in rats after a specific challenge, i.e., in immunized rats. In these conditions, animals were fed cocoa before and during an immunization process, and the overall synthesis of specific antibodies was also lowered (21) (**Table 1**). Specifically, the accurate analysis of antibodies revealed that the most attenuated isotypes were specific IgM, IgG1, IgG2a, and IgG2c antibodies, whereas specific IgG2b concentrations held steady or even increased with the 10% cocoa diet. As IgG rat isotypes can be associated with Th1 (IgG2b) or Th2 (IgG1 and IgG2a) immune response (22), these results may suggest a regulatory effect of cocoa in Th2-immune responses. This lowering effect on specific IgG1 and IgG2a, and therefore on Th2-related response, could be associated with cocoa polyphenols given that other polyphenols, such as genistein, chrysin, and apigenin (23, 24), and those from apple or soybean caused similar results (23, 25).

After establishing cocoa's influence on immunoglobulin synthesis, the reason why this diet produced such an effect remained to be studied.

COCOA INFLUENCES COMPOSITION AND FUNCTIONALITY OF PRIMARY AND SECONDARY LYMPHOID TISSUES

To ascertain the mechanisms induced by cocoa on the antibody immune response, lymphoid tissue composition and lymphocyte functionality were then determined. In addition, as cocoa intake can interact with gut-associated lymphoid tissue (GALT), several investigations were carried out to ascertain the influence of cocoa in this particular compartment of the immune system. Preclinical studies carried out in rats demonstrated that a cocoa diet modifies lymphoid tissue composition and function (13). Lymphoid tissues are considered as primary or secondary depending on whether they are devoted either to the formation of the lymphocyte repertoire or to the development of the immune response, respectively (26). Thymus is a primary lymphoid tissue where T-cell maturation takes place, whereas lymph nodes, spleen, and mucosal lymphoid tissue belong to the secondary lymphoid tissue category (27).

Cocoa and Systemic Lymphoid Tissue Composition

It was demonstrated that Wistar rats receiving a 10% cocoa diet for 3 weeks accumulate cocoa polyphenol metabolites in immune tissues, such as the thymus, lymph nodes, and spleen (28). In particular, the highest accumulation was in the thymus, where phenotypic changes were found due to the diet. In particular, cocoa intake resulted in an enhancement of the progression of immature thymocytes (those with low expression of the αβ T-cell receptor—TCR $\alpha\beta^{-}$, and expressing or non-expressing the clusters of differentiation CD4 and CD8, i.e., $TCR\alpha\beta^{low}CD4^{-}CD8^{-}$ or $TCR\alpha\beta^{low}CD4^{+}CD8^{+}$) toward more mature stages ($TCR\alpha\beta^{high}CD4^{+}CD8^{-}$) (29) (**Table 2**). In spite of this increase in $CD4^{+}$ (Th) cells in the thymus, the analysis of a secondary lymphoid tissue, such as the spleen, revealed that a 10% cocoa diet in young rats for 3 weeks increased the proportion of spleen B cells and decreased that of Th lymphocytes (18) (**Table 2**).

Lymph nodes were also affected by a cocoa diet. In particular, in mesenteric lymph nodes, a cocoa-enriched diet for 3 or 4 weeks in rats increased the proportion of innate cytotoxic lymphocytes, such as cells expressing γδ T-cell receptor ($TCR\gamma\delta^{+}$) and NK cells, and also that of the Tc lymphocytes and B cells, whereas the proportion of Th cells decreased (30, 31) (**Table 2**). These effects were only produced by a 10% cocoa diet whereas a 4% cocoa dose was insufficient to influence the phenotype of mesenteric lymph nodes (30). Similarly, the intake of a 10% cocoa-enriched diet given to rats for 6 weeks decreased the proportion of $TCR\alpha\beta^{+}$ cells but did not modify that of regulatory T cells (Treg) in inguinal lymph nodes in rats (32) (**Table 2**).

A more in-depth analysis of lymphocytes in mesenteric lymph nodes revealed that the increase of $TCR\gamma\delta^{+}$ cells was attributed to the presence of a higher amount of $CD8\alpha\alpha^{+}$ cells, a typical intestinal phenotype, which could be due to the migration of this cellular type from the intestine (34). The increase of Tc cells in mesenteric lymph nodes was accompanied by a higher proportion of activated cells ($CD25^{+}CD8^{+}$ cells) and cells expressing the αE-integrin ($CD103^{+}CD8^{+}$ cells) and a lower proportion of cells bearing L-selectin ($CD62L^{+}CD8^{+}$ cells) (31) (**Table 2**). CD103 is a subunit of the αE-integrin that can mediate cell adhesion and migration to the gut (35), whereas L-selectin is involved in lymphocyte rolling on the endothelium and the homing to secondary lymphoid tissues (36). These results could mean that the cocoa diet decreased the arrival of blood lymphocytes to mesenteric lymph nodes whereas it may favor intestinal cells entering. As cocoa compounds can reach the small intestine and even the colon (37, 38), they can affect the intestinal lymphocytes and promote their migration to mesenteric lymph nodes.

Overall, the increased proportion of $CD8\alpha\alpha^{+}TCR\gamma\delta^{+}$ cells, NK cells, and $CD103^{+}$ Tc cells in mesenteric lymph nodes could be involved in cocoa's influence on antibody immune response. $TCR\gamma\delta^{+}$ cells have been associated with an attenuating effect on the synthesis of antibodies (39), and NK cells could also contribute to the regulation of antibody synthesis (40). Moreover, $CD103^{+}$ cells have been associated with a regulatory function given that their proportion increased after treatment with immunosuppressive agents (41).

After feeding a cocoa-enriched diet, cocoa flavonoid metabolites are stored in the lymphoid tissues (thymus, lymph nodes, and spleen) as well as in the liver. In fact, epicatechin metabolites have been reported to be accumulated in concentrations twofold higher in the thymus, testes, and liver than in lymph nodes and spleen (28). With regard to the liver, the 10% cocoa intake in rats enhanced hepatic antioxidant capacity, without modifying hepatic superoxide dismutase and catalase activities (29).

Cocoa and Lymphocyte Function

The development of the acquired immune response implies the involvement of complex interactions between immune cells

TABLE 2 | Summary of the effects of cocoa diet in lymphocyte composition of lymphoid tissues.

Lymphoid tissue	Cocoa dose	Length of the diet (weeks)	Results (% cells)	Reference
Thymus	10% in the food	3	↓TCRαβ^{low}CD4$^-$CD8$^-$ ↓TCRαβ^{low}CD4$^+$CD8$^+$ ↑TCRαβ^{high}CD4$^+$CD8$^-$	(29)
Spleen	4% by oral gavage	3	No changes	(18)
	10% in the food		↑B ↓Th	
Lymph nodes	4% by oral gavage	3	No changes	(30)
	10% in the food		↑TCRγδ$^+$ ↓Th ↑Tc	
	10% in the food	4	↑NK ↑B ↓TCRαβ$^+$ ↑TCRγδ$^+$ (↑CD8αα$^+$) ↓Th (↓CD62L$^+$) ↑Tc (↑CD25$^+$, ↑CD103$^+$, ↓CD62L$^+$)	(31)
	10% in the food	6	↓TCRαβ$^+$ ↓Th ↑Tc =Treg	(32)
Peyer's patches	4% by oral gavage	3	No changes	(30)
	10% in the food		↑B ↓TCRαβ$^+$ ↑TCRγδ$^+$ ↓Th	
	10% in the food	4	↑TCRγδ$^+$ ↑NKT ↓Th (↑ CD25$^+$, ↑CD103$^+$, ↓CD62L$^+$) =Tc (↑ CD103$^+$)	(33)
Intestinal intraepithelium	10% in the food	4	↑TCRγδ$^+$ ↑NK ↓TLR4$^+$ ↑CD4$^+$CD103$^+$	(33)
Intestinal lamina propria	10% in the food	4	↓NKT ↓IgA$^+$	(33)

Arrows indicate increases or decreases, equals sign means no changes.

CD, cluster of differentiation; TCRαβ^{low}, cells with low expression of αβ T-cell receptor; Th, T helper lymphocytes; Tc, T cytotoxic lymphocytes; TCRγδ$^+$, cells with γδ T-cell receptor; NK, natural killer cells; NKT, natural killer T cells; Treg, T regulatory cells; TLR, toll-like receptor; Ig, immunoglobulin.

by means of particular surface molecules and the secretion of cytokines. The gene or protein expression of those molecules involved in the immune synapses, as well as cytokines and other molecules secreted by immune cells, can be evaluated.

In vitro studies carried out in lymphoid cell lines showed the ability of cocoa to reduce the synthesis of interleukin 2 (IL-2) involved in early T lymphocyte proliferation (42, 43). This cytokine is mainly produced by Th cells after antigen activation (44) and plays a crucial role in immune response, enhancing Tc cell, NK cell cytotoxic activities, T cell differentiation, and stimulating the proliferation and the antibody synthesis (45). These effects could be responsible for the cocoa downregulation of the antibody synthesis. However, the results obtained *in vivo* on IL-2 secretion or lymphocyte proliferation could not confirm such a mechanism (18, 21, 30) (**Figure 1A**). In particular, IL-2 secretion was not modified in spleen cells from rats fed 10% cocoa for 3 weeks, even though lymphocyte proliferation increased (18). On the other hand, higher or unmodified amounts of IL-2 secretion were detected after the stimulation of lymph node cells of rats fed a 10% cocoa diet for 3 or 9 weeks (21, 30). Therefore, the interaction of a cocoa diet in the initial phases of immune activation seems not to explain the attenuating effect on antibody synthesis. However, a recent study on the gene expression of mesenteric lymph node cells shows that certain molecules present on antigen-presenting cells (dendritic cells) were modified by this diet. In particular, a cocoa diet increased the gene expression of CD11c and OX40L (31) (**Figure 1A**). It has been suggested that, in a model of oral sensitization, a subset of dendritic cells (CD11c$^+$, CD103$^+$, and CD8$^+$) that migrates and activates in the mesenteric lymph nodes seems responsible for the Th2 polarization in this model (46). OX40L–OX40 interaction has been related to follicular Th cells and promotes the generation of Th2 response during antigen presentation (47, 48), and it was increased in an oral sensitization process (31). Despite these results, the cocoa

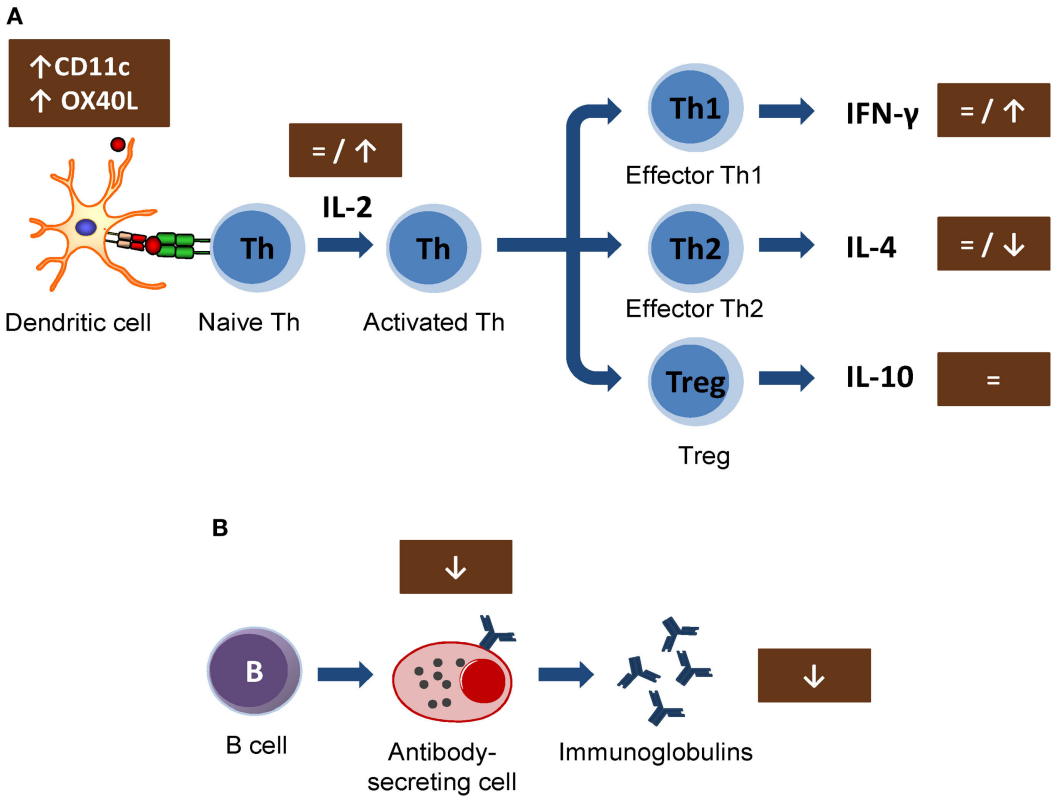

FIGURE 1 | Summary of the mechanisms involved by cocoa diet on lymphocyte function: **(A)** Cocoa effect on the induction of acquired immune response, involving from antigen presentation until the development of effector T cells. **(B)** Cocoa effect on the B-cell activation and antibody production. Arrows indicate increases or decreases, equals sign means no changes. Th, T helper cells; Treg, T regulatory cells; IL, interleukin; IFN, interferon; CD, cluster of differentiation.

diet attenuated the antibody synthesis and, therefore, this diet must interact with downstream pathways of the Th2-immune responses that would eventually inhibit antibody synthesis.

In general, the cytokine pattern secreted by activated lymphocytes reveals the stimulation of Th1, Th2, Th17, or Treg cells (49). Interferon γ (IFN-γ) is the most representative cytokine in Th1 activation (50). No changes were detected either in serum levels of IFN-γ in rats fed cocoa for 4 weeks (32) or in the secretion of IFN-γ by activated splenocytes or lymph node cells from rats fed a cocoa diet for 3 weeks (18) or 4 weeks (31). Nevertheless, an increase of IFN-γ was observed in lymph node lymphocytes from rats fed a cocoa diet for 8 weeks (21) (**Figure 1A**). Therefore, it seems that a cocoa intake over longer periods promotes Th1 immune response.

More interesting results were found in IL-4, the most representative Th2 cytokine (51). A reduction of IL-4 was found in activated lymph node cells from rats fed cocoa for 3 weeks (30) and in splenocytes from rats fed cocoa for 3 or 8 weeks (18, 21). However, no changes in IL-4 were found after 4 weeks of nutritional intervention (31). On the other hand, this downregulation on IL-4 secretion did not match with previous data *in vitro* (42, 43, 52) but it partially explains the down-modulatory role of the cocoa diet on antibody synthesis (**Figure 1A**). IL-4 promotes IgE upregulation and increases intestinal permeability (53, 54); therefore, the decrease in IL-4, along with the TCRγδ$^+$ cell increase induced by the cocoa diet, may be beneficial in reducing certain stages of hypersensitivity, such as food allergy. However, some reports focused on IL-10, a regulatory cytokine (55), did not detect any modification by the 10% cocoa diet (30, 31).

The effects of cocoa lowering IL-4 secretion in some lymphocytes populations agree with those found when the specific antibody-secreting cells after an immunization were enumerated. A significant decrease in the specific IgG-secreting cell numbers was reported by 5 and 10% cocoa diets, either in spleen or lymph node tissues, although no changes were observed in specific IgM-secreting cells (21) (**Figure 1B**). In summary, a cocoa-enriched diet plays an immune-regulatory role in the antibody immune response to an antigen that involves a lower number of specific antibody-secreting cells and, therefore, a decrease in antibody synthesis.

COCOA INTAKE INFLUENCES INTESTINAL IMMUNE SYSTEM

Cocoa Intake and Intestinal Immunoglobulins

Several years ago, Ramiro-Puig et al. first demonstrated that a cocoa-enriched diet influences the GALT by means of the

modulation of the intestinal secretory IgA (S-IgA) (30). Feeding just a 4% cocoa-enriched diet caused a decrease in the fecal S-IgA levels in the second week of the diet, but they were restored at the end of the third one. The 10% cocoa intervention caused lower fecal S-IgA secretion throughout the study (30), and this effect remained when the diet was maintained for 7 weeks (19). However, when a dose–effect study was performed with diets containing 2, 5, or 10% cocoa, the 2% diet was not enough to modify intestinal immunoglobulins (20). With regards to the gut wash—a typical sample used to evaluate intestinal immunoglobulins that consists of incubating the intestine with saline buffer at 37°C in a shaker for a few minutes to allow the release of the mucosa-linked antibodies—a lower secretion of S-IgA and S-IgM was detected (19, 20, 30, 56). These results evidence a lack of the S-IgM compensatory mechanism in certain states of S-IgA deficiency (57), probably because cocoa is also acting on S-IgM. Other studies have confirmed the previous attenuating effect of a 10% cocoa diet on S-IgA levels both in fecal samples and in gut washes (31, 58). Moreover, the immunoglobulin content has also been determined in intestinal tissues, such as Peyer's patches (PP) and mesenteric lymph nodes; and, in both tissues, the 10% cocoa diet for 3 weeks was able to decrease the levels of IgA and IgM (56).

The downregulation of intestinal immunoglobulins produced by a cocoa diet may be due to the influence of some cocoa compounds on the complex immune response developed in the GALT. This immune compartment includes inductive sites (PP and mesenteric lymph nodes) and effector sites [lamina propria (LPL) and intraepithelial lymphocytes (IEL)] (59). As explained, cocoa intake induced some changes in mesenteric lymph nodes, but the cocoa effect is not only restricted to that particular compartment. Therefore, further studies were then focused on looking in more depth the effect of a cocoa diet on PP as well as LPL and IEL.

Cocoa Intake and Lymphocyte Composition in Small Intestine and Colon

The attenuation of serum or intestinal immunoglobulin synthesis may be the result of multitude pathways, but the reduction of mucosal IgA observed after cocoa dietary intervention may possibly involve specific mechanisms located at the intestinal site.

The rat intake of 10% cocoa for 4 weeks modified the composition of lymphocytes in the PP and in the intraepithelial compartment whereas no modifications were seen in LPL (33) (**Table 2**). With regard to PP, cocoa-enriched diets were able to reduce the proportion of TCRαβ$^+$ T cells and to increase the proportion of B lymphocytes and TCRγδ$^+$ cells (30, 33), results that agree with changes detected in the mesenteric lymph nodes (30, 31). Analyzing in depth TCRαβ$^+$ cells in the intestine, the cocoa diet decreased the proportion of Th cells and increased that of natural killer T cells (NKT). In addition, after cocoa intake, PP also had higher proportions of CD4$^+$CD25$^+$ cells, CD4$^+$CD103$^+$ cells, CD8$^+$CD103$^+$ cells, and CD4$^+$CD62L$^+$ cells. Apart from the influence of cocoa intake on PP composition, the intraepithelial compartment was also affected by this diet. In IEL from the small intestine of rats fed cocoa, there was a higher percentage of TCRγδ$^+$ cells (both CD8αα$^+$ and CD8αβ$^+$) and NK cells (33).

In summary, in the GALT, the lower production of intestinal antibodies was accompanied by a relative increase in B cell numbers and a relative decrease in TCRαβ$^+$ or Th cell numbers in the inductive sites (mesenteric lymph nodes and PP). These results suggest that the antibody synthesis in B cells might be depleted by a lower stimulation from Th cells and/or a higher regulatory effect induced by cells, such as TCRγδ$^+$, NK, NKT, CD4$^+$CD25$^+$, CD4$^+$CD103$^+$, CD8$^+$CD103$^+$, and CD4$^+$CD62L$^+$, which is in agreement with the role of some of these cells in the regulation of the antibody synthesis (25, 40, 60). In whatever way the activation and differentiation of intestinal B cells was attenuated, a depletion of the high-capacity IgA-secretory cells was produced as reported when they were counted by Enzyme-Linked ImmunoSpot in PP (30) or by an immunofluorescence analysis in the small intestine lamina propria (33). These results agree with a lower IgA gene expression in PP and small intestine seen after 4 and 7 weeks of cocoa intake (19, 33).

Effects of Cocoa Diet on T Cell-Dependent Intestinal Immune Function

The gene expression of molecules involved in the intestinal immune response can shed some light on the mechanisms induced by cocoa on the regulation of the intestinal immune system. In this context, the mRNA levels of IgA, transforming growth factor (TGF) β1, IL-6, CD40, C–C chemokine receptor (CCR) 9, retinoic acid receptor (RAR) α, and RARβ have been reported in GALT tissues, such as mesenteric lymph nodes, PP, and small intestine after 3 or 7 weeks of a cocoa diet (19, 20).

CD40 is involved in the interaction between B and Th cells to begin the antibody immune response (61), and cocoa intake did not modify the expression of this molecule in any of the tissues considered (19, 20) suggesting that cocoa had no influence in this phase of the antibody synthesis. The main pathway that brings differentiation of B cells into IgA-secreting cells takes place in PP or mesenteric lymph nodes (62) and depends on cytokines, such as TGF-β1 and IL-6, among others (63). The 10% cocoa diet significantly decreased the TGF-β1 expression in the small intestine after 3 and 4 weeks (20, 33), although no changes were found after 7 weeks of nutritional intervention (19) (**Figure 2**). On the contrary, the longest nutritional intervention, but not the shortest one, was able to downregulate the IL-6 synthesis in mesenteric lymph nodes (19, 20). Therefore, the effect on these two cytokines, TGF-β1 and IL-6, involved in the S-IgA secretion at different periods (64), might be partly responsible for the downregulatory effect of cocoa. Neither TGF-β1 nor IgA gene expressions were downregulated by the 5% cocoa diet (20), which also caused a reduction in intestinal S-IgA, indicating that additional mechanisms may be interfering in the intestinal S-IgA content.

The next stage occurs when the activated B cells leave the inductor sites (PP and mesenteric lymph nodes) and home to the effector sites (i.e., lamina propria), where the differentiation

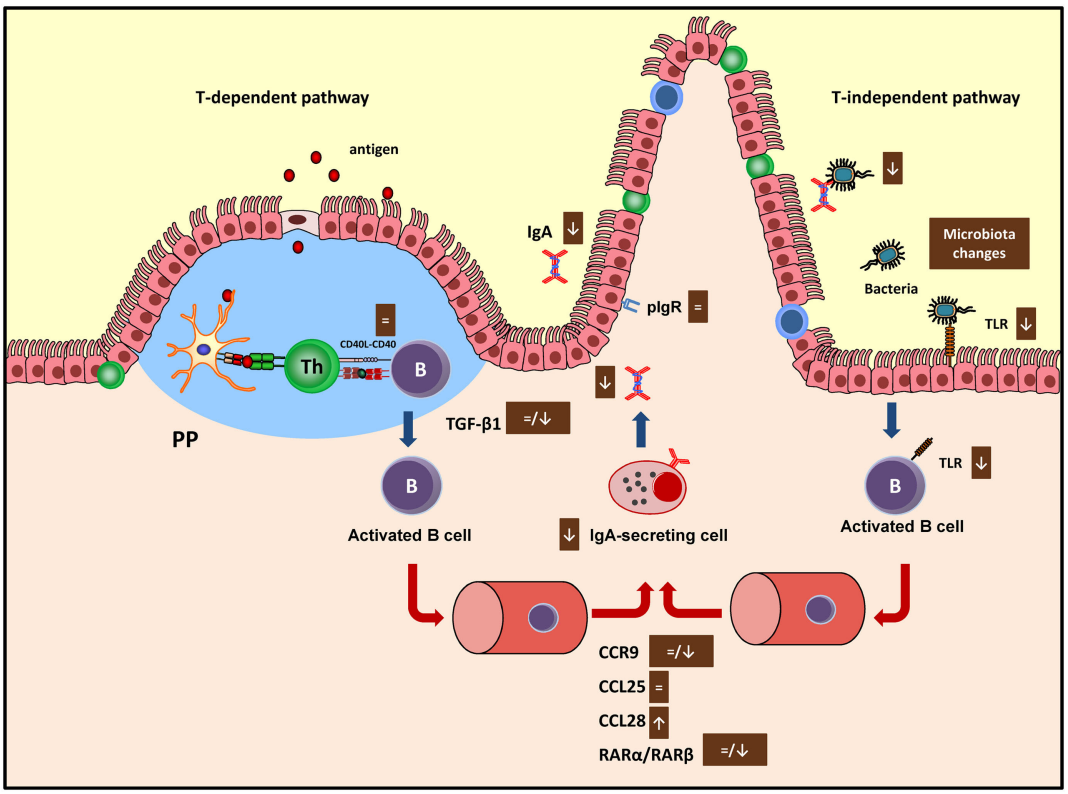

FIGURE 2 | Summary of the mechanisms involved by cocoa diet on intestinal immune function in both T-dependent and T-independent pathways. Arrows indicate increases or decreases, equals sign means no changes. Ig, immunoglobulin; PP, Peyer's patches; pIgR, polymeric immunoglobulin receptor; TLR, toll-like receptor; CCR, C–C chemokine receptor.

into IgA plasma cells takes place (65). Intestinal homing is regulated, among others, by chemokine-mediated interactions including the chemokine receptor CCR9, which binds to CCL25 and the CCL28 chemokines in the intestine (66). While 3 weeks of diet did not modify the expression of the CCR9 receptor nor the CCL25 but did increase the expression of CCL28 in the small intestine (20), 7 weeks of cocoa diet resulted in a downregulation of CCR9 and CCL28 gene expression in the same compartment (19) (**Figure 2**). In addition, retinoic acid produced by intestinal dendritic cells also plays a key role in gut homing (66) through the interaction with nuclear RAR (67). The gene expression of both RARα and RARβ was not modified in the intestinal tissue of rats fed cocoa for 3 weeks (20) but both decreased after 7 weeks (19) (**Figure 2**). Overall, these results could indicate that after being fed a cocoa diet over a long time there was an impairment of the arrival of IgA-secreting cells to the intestine because of the lack of gut-homing receptors observed. However, they do not explain the early decrease in S-IgA that was observed.

Finally, delivering IgA into the intestinal lumen depends on the transmembrane epithelial protein polymeric immunoglobulin receptor (pIgR) (68). This receptor was not modified by the cocoa intake (20), thus indicating that cocoa-induced S-IgA reduction did not occur as a consequence of a decreased transport across the epithelium.

Effects of Cocoa Diet on T Cell-Independent Intestinal Immune Function

Apart from these IgA-secreting mechanisms that depend on T-cell activation, IgA$^+$ B cells can be alternatively generated in a T-cell-independent manner involving toll-like receptor (TLR) signaling.

The gene and protein expression of other TLR has been modified both in the inductive sites of GALT (PP and mesenteric lymph nodes) and the effector sites (intestinal wall) after cocoa intake (19, 20, 33). In this context, among other components present in the cocoa, flavonoids have been suggested as dietary factors able to modulate TLR-mediated signaling pathways (69). TLR pathways can be modulated by flavonoids at different levels, and there are evidences of several flavonoids interfering at gene/protein expression level, in subsequent activation pathways such as the myeloid differentiation primary response 88 (MyD88), TIR-domain-containing adaptor protein inducing interferon beta (TRIF), and even downstream-associated signal transduction cascades (i.e., MAPK) (69). In this sense, alternative mechanisms in TLR regulation by cocoa flavonoids have been also suggested such as the direct modulation of their intracellular negative regulators such as the interleukin-1 receptor associated kinase (IRAK), toll interacting protein (TOLLIP), etc. To date, *in vitro* studies demonstrate the upregulation of IRAK-M by procyanidin dimer B2 (70), similarly to the effect described by other flavonoids

like epigallocatechin-galate in TOLLIP expression (70). Anyway, the synergistic action of cocoa on all these TLR-activating signaling could also contribute to the attenuation of S-IgA synthesis.

Cocoa intake for 4 weeks reduced the proportion of $TLR4^+$ cells in the IEL compartment (33) which agrees with a decrease of TLR4 mRNA in small intestine observed in previous studies (**Figure 2**). Nonetheless, higher TLR4 gene expression was found in PP (19). The TLR4 is the receptor of bacterial endotoxin lipopolysaccharide, and its signaling has implications for IgA production (71), becoming another pathway to attenuate intestinal S-IgA synthesis.

Toll-like receptors are expressed preferentially in tissues that are in constant contact with microorganisms (72, 73), and changes in the TLR expression induced by flavonoids could reflect changes in the intestinal microbiota and/or in its relation with intestinal immune cells (69, 74). Accordingly, several studies have shown that cocoa (58, 75, 76), cocoa flavonoids (58, 77), or cocoa fiber (78) induce changes in gut microbiota composition. Moreover, a lower proportion of IgA-coated bacteria have been observed after cocoa intake (79).

In summary, it could not be discarded that the influence of cocoa on GALT was partially mediated by its effect on the intestinal microbiota, which can lead to differential TLR activation and, therefore, may also influence the lowering IgA effect of cocoa.

Recently, an analysis of an untargeted ^{1}H NMR spectroscopy-based metabolomic approach in 24-h urine samples have been carried out in order to correlate urine cocoa metabolites with cocoa effects on immunity and the gut microbiota (80). The results of this analysis demonstrate that cocoa intake, besides affecting microbiota composition, also alters the host and bacterial metabolism concerning energy and amino acid pathways leading to a particular metabolic signature that correlates with the S-IgA lowering effect of cocoa. Accordingly, a different pattern of intestinal and serum short-chain fatty acids, with increasing amounts of butyric acid, has been reported (78).

Finally, and in order to have a broader view of the molecules involved in the intestinal immune response modulated by cocoa, the changes in colonic gene expression by a microarray analysis after a cocoa nutritional intervention has been carried out (81). This study shows that a cocoa diet downregulated an extensive number of genes, many of them involved in the biological processes related to the immune system and inflammation. Specifically, the most downregulated gene after cocoa intake was tachykinin 4 (81), described as the promoter of B lineage cells (82), which could explain the attenuating effect of cocoa on antibody synthesis, despite the fact that the proportion of B cells did not decrease but, on the contrary, increased in some lymphoid tissues. Moreover, other genes involved in pathways related to the mast cell-mediated immunity, its activation, and its degranulation were downregulated (81), pointing out the possible role of cocoa in inducing tolerance in allergic processes as observed in some studies next reported.

Cocoa Intake Also Influences Another Mucosal Lymphoid Tissue

The mucosal immune system is interconnected (59). Due to cocoa's influence on the intestinal immune system, it became of interest to know whether this effect was also extended to other mucosal compartments, such as the salivary glands. The IgA and IgM content in the salivary glands (submaxillary and parotid salivary glands) was quantified after a 10% cocoa intake in rats for 3 weeks. The cocoa diet induced a decrease in the IgA and IgM content in both glands (56). This attenuating effect was associated with a drastic reduction in the IgA gene expression together with a lower expression of some molecules involved in the maturation and differentiation of B cells, such as IL-6 and TGF-β1 (56), as previously observed in the small intestinal samples (19, 20). However, in agreement with what was detected at intestinal level, no changes were detected in pIgR gene expression in the salivary glands. Therefore, in conclusion, this study shows that cocoa intake not only has an influence on the gut intestinal compartment and the systemic immunity but also on other mucosal sites in rats.

EFFECT OF COCOA DIET ON ANTIBODY-MEDIATED DISEASES

Due to the attenuating properties of cocoa on immunoglobulin levels after cocoa intake in rats, it was of interest to test its impact on diseases in which antibodies play a harmful effect. Therefore, this nutritional intervention was tested on animal models of arthritis and allergy.

Effect of Cocoa Diet on Experimental Arthritis

Rheumatoid arthritis is a symmetric, polyarticular, systemic, and autoimmune inflammatory disease in which multiple factors, including genetic, immune, and environmental ones are involved (83). Diet components such as n-3 fatty acids, vitamins D and K, and antioxidants are protective compounds against rheumatoid arthritis (84). In this context, diets containing 5 or 10% cocoa were tested on adjuvant arthritis, a model of rheumatoid arthritis widely used for the screening of anti-inflammatory drugs (85). In this animal model, cocoa diet decreased the synthesis of antibodies against the pathology inducer (**Table 3**) and was also able to decrease the proportion of Th cells in both blood and regional lymphoid tissues (86). This latter effect is important because, as anti-CD4 therapy has been shown to prevent or ameliorate adjuvant arthritis (87, 88), the cocoa-induced decrease in Th cells could be beneficial to the arthritic process. Moreover, a 10% cocoa diet avoided the Th/Tc imbalance and the reduction of the proportion of NKT cells produced by the disease (86). However, the effect of cocoa on hind-paw inflammation was very poor (86), which did not agree with the protective effect of other flavonoids in a similar inflammatory model when given by oral (quercetin) or by intraperitoneal routes (quercetin, rutin, hesperidin, and morin) (89–91). Nevertheless, a cocoa extract inhibited mice ear edema (92) and acute paw edema in rat (93, 94). Moreover, cocoa flavonoids such as epicatechin, catechin, and procyanidin B2, among others, are able to attenuate the synthesis of inflammatory mediators, such as tumor necrosis factor (TNF)-α, monocyte chemoattractant protein-1, IL-6, and IL-8 (95–100).

The influence of a 10% cocoa diet was also analyzed in collagen-induced arthritis, another model of arthritis. This

TABLE 3 | Summary of the effects of cocoa diet in specific antibodies in rat models of arthritis and allergy.

Model	Strain	Cocoa dose	Results	Reference
Adjuvant arthritis	Wistar	5% in the food 10% in the food	↓Specific antibodies ↓Specific antibodies	(86)
Collagen-induced arthritis	Louvain	5–10% in the food	=Specific IgG1 ↓Specific IgG2a, IgG2b, IgG2c	(32)
Allergy induced by intraperitoneal route	Brown Norway	10% in the food	↓Specific IgG1, IgG2a =Specific IgG2b ↓Specific IgE	(22)
Food allergy induced by intraperitoneal and oral routes	Brown Norway	10% in the food	↓ specific IgG1, IgG2a =Specific IgG2b ↓Specific IgE	(101)
Oral sensitization	Lewis	10% in the food	↓Specific IgG1, IgG2b ↓Specific IgM	(31)

Ig, immunoglobulin.
Arrows indicate increases or decreases, equals sign means no changes.

inflammatory model requires T- and B-cell responses to autologous collagen (102). B cells from animals with collagen-induced arthritis produce a strong specific immune response against triple helical epitopes of collagen type II (103). Anti-collagen autoantibodies bind to the joint cartilage, activate the complement cascade, and mediate the inflammatory attack on the joints, thus contributing to the disease development (104). Susceptible Louvain rats were fed with a 10% cocoa diet for 2 weeks before arthritis induction and during the latency period (2 weeks after induction), and thereafter with a 5% cocoa diet until the end of the study (an additional 2 weeks). In this case, the cocoa-enriched diet was able to reduce the synthesis of specific antibodies against type II collagen, differentially according to their isotype (**Table 3**), decrease the Th lymphocyte proportion in regional lymph nodes, and reduce the release of inflammatory mediators from peritoneal macrophages. However, these immunomodulatory effects were not enough to reduce the hind-paw swelling in arthritic animals (32). It must be taken into account that the decrease in anti-collagen antibody concentration in that rat strain was only observed at the end of the study, and it was in a lesser extent and more slowly than that expected and observed in healthy rats as shown before. In a similar context, other authors reported the beneficial effect of isolated flavonoids in improving the paw swelling in animals in long-term studies (105–107). Otherwise a nutritional intervention with the flavonoid genistein had no success (108).

Effect of Cocoa Diet on Hypersensitivity Animal Models

Cocoa on Allergy Models

The effect of the consumption of a 10% cocoa diet over 4 weeks was studied in a model of allergy induced by an intraperitoneal (i.p.) injection of ovalbumin (OVA) and toxin of *Bordetella pertussis* in alum in young Brown Norway rats (22). The cocoa diet reduced the levels of anti-OVA IgG1 and IgG2a antibodies (**Table 3**), i.e., immunoglobulins related to Th2-immune response in rats, as previously mentioned. In addition, cocoa consumption decreased the serum concentrations of total and specific IgE (**Table 3**), which is the main immunoglobulin involved in allergic reactions. These results agree with studies performed in animal models of allergy treated with polyphenols, such as baicalein (109), quercetin (110), silibinin (111), sesamin (112), or an extract of *Kalanchoe pinnata* (*Crassulaceae*) containing several flavonoids such as quercetin (113).

To analyze the mechanisms involved in such action, cytokine secretion was quantified in mesenteric lymph nodes. Contrary to what was expected, cocoa diet increased the release of IL-4, a Th2 cytokine, and decreased that of IL-10, a cytokine related to immune-regulatory responses (22). In addition, cocoa intake induced a lower secretion of TNF-α, which has been described as a contributor to the development of Th2-mediated allergic inflammation by means of promoting the homing of Th2 cells to the site of allergic inflammation. These effects of IL-10 and TNF-α agree with those reported by other flavonoids in allergic conditions (113–115).

The influence of cocoa on the GALT makes it particularly interesting to test the effect of this nutritional intervention on a food allergy process. A model of food allergy using OVA as allergen was carried out in Brown Norway rats, combining an i.p. and oral administration of the allergen. The quantification of serum anti-OVA IgG1, IgG2a, and IgE antibodies revealed that the synthesis of these antibodies was completely prevented by the cocoa diet (101) (**Table 3**). In this study, a product that was richer in cocoa flavonoids was included, but it was not able to totally reproduce the same effects as the conventional cocoa-enriched diet. Therefore, it seems that cocoa flavonoids are only partially responsible for cocoa's anti-allergy properties.

In addition, after anaphylactic shock, the increase of the serum mast cell protease II was partially prevented in the allergic group fed a cocoa diet (101). Nevertheless, other markers of anaphylaxis were not modified by the cocoa intake (body temperature and motor activity), suggesting that its modifications were not enough to prevent the food allergy reaction induced (101).

In order to shed light on cocoa's anti-allergy properties, the expression of some small intestinal genes were quantified (101). The food allergy induction increased the IgA gene expression, an effect that was prevented by a cocoa diet. Moreover, the allergic animals fed a cocoa diet also had lower mRNA levels of high-affinity IgE receptors (FcεRI), mast cell protease-II, and TGF-β1 than reference animals, molecules which could be involved in the protective effect of cocoa on food allergy. Accordingly, the inhibitory effects of flavonoids on the FcεRI surface molecule or gene expression *in vitro* were described (116, 117), and the genetic analysis of colon from rats fed cocoa assessed by microarray analysis showed the downregulation of genes involved in pathways related to mast cell activation and degranulation (81). The cytokine production of food-allergic animals was also determined in mesenteric lymph nodes and spleen (101). In these tissues, the food allergy induction increased the secretion of Th2-cytokines, such as IL-4, IL-5, and IL-13. However, the cocoa diet prevented an increase in

IL-5 and IL-13 in lymph node cells and that of IL-4 and IL-13 in splenocytes.

In conclusion, in models of Th-2 immune response stimulation, the intake of cocoa prevents the secretion of typical Th2-cytokines, the synthesis of IgE involved in mast cell degranulation, and also downregulates the IgE receptors in mast cells and intestinal mast cell activation, which are the cells responsible for the most allergy symptoms. However, such effects were not able to totally prevent anaphylactic shock.

Cocoa on an Oral Sensitization Model

Although cocoa intake prevented the allergic sensitization in a model of food allergy induced by i.p. and oral allergen administration (101), it remained to find out what happened when the sensitization with the allergen was produced using only the oral route. Therefore, a 10% cocoa-enriched diet was given to 3-week-old Lewis rats submitted to an oral sensitization model induced by the oral administration of OVA together with the cholera toxin (CT) as adjuvant (118). The oral administration of OVA/CT, three times per week and for 3 weeks, was able to break down oral tolerance and induce the synthesis of specific antibodies after 4 weeks from the beginning of the sensitization protocol. Although this model did not induce detectable specific IgE synthesis, Th2-immune response related antibodies were produced (118) (**Table 3**). Feeding 10% cocoa from the beginning of the study and throughout 4 weeks attenuated the development of specific antibodies in sensitized rats fed the cocoa diet (31). In particular, the 10% cocoa diet prevented the production of anti-OVA IgG1, IgG2b, and IgM in agreement with the effect of cocoa in a food allergy model in Brown Norway rats (101).

In addition, although the IgA concentrations were not increased in this rat oral sensitization model, in contrast to other models using the same adjuvant (63, 119), the cocoa diet decreased the total IgA in both serum and intestinal compartments. As stated in previous sections, a cocoa diet influences the proliferation, differentiation, and gut homing of IgA^+ B cells (19, 31, 33), thus inducing a lower presence of these cells in the intestinal lamina propia (33) and, consequently, reducing the intestinal IgA development in line with what was reported in many studies (20, 79, 101). Additionally, the changes produced by the cocoa diet in both inductive and effector lymphoid tissues (see Cocoa Intake and Lymphocyte Composition in Small Intestine and Colon) might be responsible for the prevention of the oral sensitization. It is worth noting that the cocoa diet increased the proportion of $TCR\gamma\delta^+$ and NK cells in three intestinal compartments (mesenteric lymph nodes, PP, and IEL), suggesting their role in the tolerogenic process. In line with this, unripe apple polyphenols induced an increase in the proportion of $TCR\gamma\delta^+$ IEL in association with the inhibition of the development of an oral sensitization model (25), and it was also reported that the reduction of $TCR\gamma\delta^+$ cells by the anti-TCRγβ antibody favors an oral sensitization in mice (120). Furthermore, NK cells could have regulatory functions contributing to the avoidance of sensitization in line with the reported prevention of allergic disease (121, 122).

Other changes induced by a cocoa diet could contribute to its tolerogenic effect (31, 33). Such changes include a reduced proportion of Th cells in mesenteric lymph nodes, PP in IEL, an increase in the percentage of $CD103^+$ cells, a reduction of $CD62L^+$ cells, and an increase in the percentage of $CD25^+$ cells in PP. Cocoa intake also modulated the gene expression of several molecules both in mesenteric lymph nodes and in the small intestine (31, 33). In particular, cocoa consumption was associated with an increase in the gene expression of CD11c—a dendritic cell marker (123)—in mesenteric lymph nodes, whereas the mRNA levels of CD11c and CD11b were reduced in small intestinal samples; cocoa also upregulated the expression of OX40L in mesenteric lymph nodes (31)—mainly expressed on antigen-presenting cells (124). In this sense, the interaction of OX40–OX40L regulates cytokine production from T cells, antigen-presenting cells, NK cells, NKT cells, and cytokine receptor signaling (125). Additionally, cocoa decreased the gene expression of IL-1β—a potent pro-inflammatory cytokine (126)—in mesenteric lymph nodes, although no modifications were seen in the production of Th1 (IFN-γ and TNF-α), Th2 (IL-4), or Treg (IL-10) cytokines.

Overall, a cocoa intake, by means of its influence on the intestinal immune system, is able to avoid the sensitization to oral allergens, thus contributing to the downregulation of this hypersensitivity reaction.

Cocoa on an Atopic Dermatitis Model

Recently the role of a cocoa extract on atopic dermatitis has been published (127). The cocoa extract decreased the IgE levels induced by a *Dermatophagoides farinae* extract together with a reduction of atopic dermatitis symptoms. Particularly, the cocoa decreased the severity of the skin lesions, the loss of skin hydration and suppressed the infiltration of eosinophils and mast cells into the skin lesions. Moreover, an extract containing 0.25% cocoa downregulated IL-4 mRNA levels on the skin tissues, whereas an extract containing 1% cocoa decreased IL-5 gene expression at this level.

CONCLUSION

In this review, we summarize the effect of a cocoa diet on the immune system of rats, particularly in the antibody response, both in systemic and mucosal (intestinal and extraintestinal) compartments. The analyses of cells involved in such responses, as well as molecules, such as cytokines and receptors, demonstrate that the effects of a cocoa diet are exerted at multiple sites: in the antigenic presentation, in the cytokines produced by effector Th cells, and in the intestinal homing of activated cells. Eventually, these actions will reduce the synthesis of most antibody isotypes, in particular Th2-associated antibodies as IgE. The relative decrease of Th lymphocytes associated with an increase in $TCR\gamma\delta^+$ cells and NK cells detected in most lymphoid tissues studied suggest the involvement of these cells in the regulatory role of cocoa. The immunomodulatory potential of cocoa can be very beneficial in those diseases that involve hypersensitivity, such as allergy and autoimmune diseases. Nevertheless, although no signs of immunodeficiency were observed in the described studies, it must be considered that the attenuation of antibodies can be harmful when antibodies are needed to counteract a

pathogenic antigen, such as infections, and to induce antibody-dependent cytotoxicity, phagocytosis, and complement activation. Although further research must characterize the particular cocoa components responsible for such effects, and nutritional studies in humans need to be carried out, cocoa has potential as a nutraceutical agent in some hypersensitivity status.

AUTHOR CONTRIBUTIONS

MC-B, MM-C, MA-G, and AF were responsible for the manuscript preparation. MM-C and MA-G contributed to the manuscript draft. MC-B mainly wrote the manuscript. FP-C and MC contributed to its critical revision.

FUNDING

The present study was supported by a grant from the Spanish Ministry of Economy and Competitivity (AGL2011-24279) and a grant from the *Agencia Estatal de Investigación* (AEI, Spain) and European funds (Fondo Europeo de Desarrollo Regional, FEDER) (AGL2016-76972-R). MC-B is a recipient of a doctoral fellowship from the University of Barcelona (APIF2014).

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Conflict of Interest Statement: The authors declare that this study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

REVIEW
published: 02 August 2017
doi: 10.3389/fnut.2017.00036

Cocoa, Blood Pressure, and Vascular Function

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Cardiovascular disease (CVD) represents the most common cause of death worldwide. The consumption of natural polyphenol-rich foods, and cocoa in particular, has been related to a reduced risk of CVD, including coronary heart disease and stroke. Intervention studies strongly suggest that cocoa exerts a beneficial impact on cardiovascular health, through the reduction of blood pressure (BP), improvement of vascular function, modulation of lipid and glucose metabolism, and reduction of platelet aggregation. These potentially beneficial effects have been shown in healthy subjects as well as in patients with risk factors (arterial hypertension, diabetes, and smoking) or established CVD (coronary heart disease or heart failure). Several potential mechanisms are supposed to be responsible for the positive effect of cocoa; among them activation of nitric oxide (NO) synthase, increased bioavailability of NO as well as antioxidant, and anti-inflammatory properties. It is the aim of this review to summarize the findings of cocoa and chocolate on BP and vascular function.

Keywords: cocoa, endothelial function, blood pressure, arterial stiffness, flavonoids

OPEN ACCESS

Edited by:
Emilio Jirillo, Università degli studi di Bari Aldo Moro, Italy

Reviewed by:
Honglin Dong, Coventry University, United Kingdom
Nicolò Merendino, Università degli Studi della Tuscia, Italy
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Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Nutrition

Received: *06 April 2017*
Accepted: *10 July 2017*
Published: *02 August 2017*

Citation:
Ludovici V, Barthelmes J, Nägele MP, Enseleit F, Ferri C, Flammer AJ, Ruschitzka F and Sudano I (2017) Cocoa, Blood Pressure, and Vascular Function. Front. Nutr. 4:36. doi: 10.3389/fnut.2017.00036

INTRODUCTION

Cardiovascular disease (CVD) represents the most common cause of death in the Western world, with an estimated 17.5 million people dying from CHD (coronary heart disease or stroke) every year (1). A nutraceutical approach has been proposed to counteract the increasing burden of CVD. The consumption of polyphenol-rich foods has been related to a lower risk of cardiovascular events (cardiovascular mortality, myocardial infarction, and stroke) both in the general population and in patients with cardiovascular risk factors in several interventional and epidemiological trials (2–5). Polyphenols are believed to be largely responsible for this protective role. Characterized as compounds with phenolic structural features (6), they are a class of natural bioactive substances with numerous anti-atherogenic properties including anti-inflammatory, anti-aggregating, and vasodilatory effects, the ability to lower blood pressure (BP), to prevent oxidation of low-density lipoprotein (LDL), and to improve glucose and lipid profiles (7).

Fruits, vegetables, tea, chocolate, and wine contain a high amount of polyphenols. Among them, cocoa beans are one of the richest known sources of flavonoids (8), and their protective properties have been recognized and used by several cultures among centuries. The origins of chocolate are usually traced back to the pre-Columbian populations, which were probably the first to cultivate cocoa plants. The consumption of cocoa, appreciated for its invigorating and healthy effects, differed from today: they used to dissolve dried cocoa beans in water, adding cinnamon and pepper to enhance their strong and bitter taste. With its arrival in Europe in the sixteenth century, cocoa was processed in

a sweet soft beverage and rapidly became a luxury item. In the last century, the words cocoa and chocolate became intensely linked to hypertension, diabetes, overweight, and obesity (9). However, in the last two decades the salutary cardiovascular effects of this ancient medicinal food have begun to be reevaluated, and these properties have been related to cocoa's high content of flavonoids, members of the broader polyphenol class. The main constituents are flavanols, present as monomeric (−)-epicatechin and (+)-catechin, together with their dimers, oligomers, and polymers, the so-called proanthocyanidins, responsible for cocoa bitterness when complexing with salivary proteins (10). Although flavanols are likely to be responsible for cocoa's beneficial effects, they are lost during the conventional chocolate manufacturing process, so that the total flavanol content of commercial chocolate varies by more than 10-folds (11). Upon harvest, cocoa beans are usually fermented by environmental microbiota. This process creates flavor precursors that further develop during the roasting step, generating the peculiar cocoa flavors that define its quality (12). Fermentation and roasting significantly decreases the polyphenol and flavanol content of cocoa due to high temperature conditions and oxidation (13). Furthermore, alkalinization, used to modify cocoa color and give it a milder taste, results in a 60% decrease of total flavanol content (14). In humans, flavanol serum concentration increases in a dose-dependent manner after ingestion, reaching its peak usually 2–3 h after cocoa intake (15, 16). Flavanols are still detectable in plasma 8 h after consumption (17). Cocoa is also rich in theobromine, a 3,7-dimethylated xanthine alkaloid, and minerals such as potassium or magnesium (18).

Several epidemiological (19–21) and interventional studies strongly suggest that cocoa consumption, as well as vegetables and fruit intake, has numerous beneficial effects on cardiovascular health, including lowering of BP (22), improving vascular function (23), reducing of platelet aggregation and adhesion (24), having anti-inflammatory properties (25), and improving glucose and lipid metabolism (26). These positive effects have been found in healthy subjects (27) as well as in patients with risk factors (arterial hypertension, diabetes, and smoking) (28) or established CVD (coronary heart disease or heart failure) (29). Various potential mechanisms, including the increased bioavailability of nitric oxide (NO) and the anti-inflammatory and antioxidant effect, are supposed to be responsible for the protective properties of cocoa (30).

This review aims to summarize the effects of cocoa and chocolate on BP and vascular function.

EPIDEMIOLOGICAL EVIDENCE

After observing the Kuna Indians from Central America, researchers discovered how cocoa might be able to lower BP and extend life expectancy (31). Indeed, this population surprisingly had a very low incidence of hypertension when aging, despite having a high salt diet compared to other normotensive communities (32). Interestingly, this was not related to genetic factors, since the same population, migrated from the San Blas islands to Panama City for economic reasons, showed to have BP levels similar to other urban dwelling people (31). In addition, when compared to other American citizens, a marked reduction in cardiovascular mortality was noticed (33). To explain this difference, many environmental factors were investigated, such as differences in lifestyle or tobacco use, but ruled out as contributories. Finally, it was found that Island-Kuna, but not Mainland-Kuna, used to drink five cups of cocoa per day, which, moreover, was determined to be flavonoid-rich (approximate intake 900 mg per day, i.e., the suggested highest intake worldwide) (34).

Since then, many epidemiological studies confirmed the assumption that cocoa could be responsible for these findings. Flavonoid-rich foods intake, and particularly chocolate consumption, were associated with a lower risk of death due to CVD in the Iowa Women's Health Study (19). Subsequently, in the Zutphen Elderly Study, a similar reduction in cardiovascular mortality after chocolate consumption was reported. Comparing the groups with higher and lower chocolate intake, a reduction of 3.7 mmHg in systolic BP (SBP; 95% CI, −7.1 to −0.3 mmHg; $p = 0.03$) and a reduction of 2.1 mmHg in diastolic BP (DBP; 95% CI, −4.0 to −0.2 mmHg; $p = 0.03$) were observed. Higher chocolate intake was associated to significant reduction in cardiovascular mortality (adjusted relative risk 0.50, 95% CI, 0.32–0.78; $p = 0.004$) and all-cause mortality (adjusted relative risk 0.53, 95% CI, 0.39–0.72; $p < 0.001$) (20). In the Stockholm Heart Epidemiology Program, chocolate intake related to a reduced cardiovascular mortality after acute myocardial infarction (21). Furthermore, this reduction appeared to be dose dependent. Compared with those never consuming chocolate, the subjects consuming chocolate less than once, up to once or up to twice per week showed progressively decreasing hazard ratios for cardiac mortality [0.73 (95% CI, 0.41–1.31), 0.56 (0.32–0.99), and 0.34 (0.17–0.70), respectively]. However, chocolate consumption was weakly associated with a lower rate of total mortality and non-fatal outcomes (21). The same findings emerged in two cohort studies of middle-aged Swedish women and men, in which daily moderate chocolate intake had an inverse association with chronic heart failure hospitalization and death (35, 36). Furthermore, in the National Heart, Lung, and Blood Institute Family Heart Study, chocolate intake correlated inversely with prevalent coronary heart disease in a general United States population (37). The most recent epidemiological data coming from the analysis of the European Prospective Investigation into Cancer Norfolk cohort support the prior findings (38). When compared to those who consumed no chocolate, subjects in the highest quintile of chocolate intake (15.6–98.8 g per day) demonstrated a significantly reduced rate of stroke (HR 0.77, 95% CI, 0.62–0.96) and cardiovascular mortality (HR 0.75, 95% CI, 0.62–0.92). Similar results emerged from a meta-analysis of 9 separate studies involving 157,809 participants (38). However, it is important to underline that none of these epidemiological studies focused on the amount of cocoa intake. Thus, it is not possible to make efficient comparison between the abovementioned studies.

COCOA AND BP: INTERVENTIONAL STUDIES

Arterial hypertension is a major modifiable risk factor for cardiovascular and cerebrovascular disease (39). Every 10 mmHg

reduction in SBP significantly reduces the risk of major cardiovascular events, CHD, stroke, and heart failure, which leads to a significant 13% reduction in all-cause mortality (40). To date, several interventional studies have assessed the efficacy of cocoa in lowering BP both in healthy subjects and in patients with cardiovascular risk factors (**Table 1**).

Compared to cocoa butter chocolate, the regular consumption (14 days) of flavanol-rich milk chocolate (168 mg flavanols) was significantly linked with the reduction of SBP (−6 mmHg) and DBP (−5 mmHg), as well as with LDL cholesterol and oxidative stress markers in 28 healthy individuals (41). Similarly, short-term administration (7 days) of flavanol-rich dark chocolate significantly reduced SBP ($p < 0.05$) and insulin resistance ($p < 0.001$) in 20 healthy subjects (42). In 101 normotensive subjects, randomized to receive dark chocolate bars (397 mg flavanols), cocoa beverage (357 mg flavanols), or matching placebo for 6 weeks, the flavanol-rich chocolate consumption reduced SBP by 3.58 mmHg, with no significant effect on DBP (43). Furthermore, in 90 "healthy" elderly subjects, a statistically significant improvement in BP ($p < 0.0001$), insulin resistance ($p < 0.0001$), and lipid peroxidation ($p = 0.001$) were seen after 8 weeks in the high flavanol (993 mg, SBP: -7.83 ± 0.56 mmHg, DBP: -4.77 ± 0.37 mmHg) and intermediate flavanol (520 mg, SBP: -6.8 ± 0.59 mmHg, DBP: -3.2 ± 0.36 mmHg) intake groups in comparison to the low-flavanol group (48 mg, SBP: -1.6 ± 1.06 mmHg, DBP: -1.57 ± 0.61 mmHg). Moreover, flavanol consumption demonstrated a positive effect on cognitive performance (44). In the recently published Flaviola Health Study, 100 healthy subjects were enrolled and randomized to cocoa flavanol (CF) containing drink (450 mg) or CF-free drink for 1 month (27). CF intake decreased SBP and DBP by 4.4 mmHg (95% CI, 7.9–0.9 mmHg) and 3.9 mmHg (95% CI, 6.7–0.9 mmHg), improved endothelial function, and showed a positive effect on total and LDL cholesterol. By applying the

TABLE 1 | Studies investigating cocoa and blood pressure.

Reference	Year	Study design	Population	Duration (weeks)	Intervention	Reduction of SBP/ DBP active group (mmHg)	Reduction of SBP/DBP control group (mmHg)
Fraga et al. (41)	2005	Randomized crossover	28 normotensive	2	High-flavanol milk (168 mg)/white chocolate	−6/−5	−2/−1
Grassi et al. (42)	2005	Randomized crossover	15 normotensive	1	Dark (500 mg flavanols)/white chocolate	−7/−4.2	−0.5/−0.3
Crews et al. (43)	2008	Randomized double-blind parallel	101 normotensive	6	Dark chocolate (397 mg flavanol) and cocoa drink (357 mg flavanol)/low-flavanol chocolate and drink	−3.58/−0.5	−3.05/−0.57
Mastroiacovo et al. (44)	2015	Randomized double-blind parallel	90 normotensive	8	High (993 mg)/intermediate (520 mg)/low (48 mg) flavanol cocoa drink	−7.83/−4.77	−1.60/−1.57
Sansone et al. (27)	2015	Randomized double-blind parallel	100 normotensive	4	High (450 mg)/low-flavanol cocoa drink	−4.4/−3.9	−1/0
Grassi et al. (23)	2015	Randomized double-blind crossover	20 normotensive	1	High (800, 500, 200, 80 mg)/low (0 mg) flavanol cocoa	−4.8/−3.03	–/–
Murphy et al. (45)	2003	Randomized double-blind parallel	32 normotensive	4	High (234 mg flavanols and procyanidins)/low-flavanol chocolate	+2/−1	+3/0
Engler et al. (46)	2004	Randomized double-blind	21 normotensive	2	High (213 mg procyanidins, 46 mg epicatechin)/low-flavanol chocolate	−1/+0.9	−2.8/−0.1
Shiina et al. (47)	2009	Randomized single-blind	39 normotensive	2	Dark (550 mg flavanols)/white chocolate	+4.6/+6.6	+4/+5.2
Njike et al. (48)	2011	Randomized crossover	44 normotensive overweight	6	High/low-flavanol cocoa drink	+2.2/−0.5	−0.1/+0.8
Taubert et al. (49)	2003	Randomized crossover	13 hypertensive	2	Dark (500 mg flavanols)/white chocolate	−5.1/−1.8	+0.4/+0.3
Taubert et al. (50)	2007	Randomized single-blind parallel	44 pre-hypertensive/ hypertensive	18	Dark(30 mg flavanols)/white chocolate	−2.9/−1.9	+0.1/0
Grassi et al. (51)	2005	Randomized crossover	20 hypertensive	2	Dark (500 mg flavanols)/white chocolate	−11.9/−8.5	−0.7/−0.6
Muniyappa et al. (52)	2008	Randomized double-blind crossover	20 hypertensive	2	High (900 mg)/low-flavanol drink	−2/−3	−1/−4
Davison et al. (53)	2010	Randomized double-blind crossover	52 hypertensive	6	High (1,052 mg)/low-flavanol cocoa drink	−5.3/−3	−2.1/+0.1
Grassi et al. (54)	2008	Randomized crossover	19 hypertensive with IGT	2	Dark (1,008 mg polyphenols)/white chocolate	−3.8/−3.9	−0.1/−0.2
Davison et al. (55)	2008	Randomized parallel	49 normotensive obese or overweight	12	High (902 mg)/low-flavanol cocoa	−1.9/−1.8	+4.2/+2.8
Rostami et al. (28)	2015	Randomized double-blind	60 hypertensive diabetic	8	Dark/white chocolate	−5.9/−6.4	−1.1/+0.2
Monagas et al. (56)	2009	Randomized crossover	47 diabetics or more than 3 CV risk factors	4	Cocoa powder (495 mg polyphenols) with milk/milk	0.0/−2	−3/−3
De Palma et al. (57)	2016	Randomized crossover	32 patients with stable HF	4	High (1,064 mg)/low-flavanol dark chocolate	−1.8/−4.2	−0.9/+2.9

IGT, impaired glucose tolerance; HF, heart failure; CHD, coronary heart disease; CV, cardiovascular; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Framingham Risk Score, CF intake significantly lowered the 10-year risk for CHD, CVD, and cardiovascular death in so-far healthy people (27). Moreover, Grassi and colleagues recently demonstrated that 1 week of supplementation with either 80 or 200 mg total flavanols (17 or 42 mg epicatechin, respectively) significantly decreased BP in healthy individuals (23). In particular, they documented a decrease in BP (SBP: −4.8 ± 1.03 mmHg, $p < 0.0001$; DBP: −3.03 ± 1.07 mmHg, $p = 0.0011$), and an improvement in endothelial function (23). However, in four different studies, flavanol-rich cocoa (234, 259, 550, 805 mg flavanols per day, respectively) did not improve BP levels compared to placebo in normotensive subjects (45–48).

The effect of cocoa consumption on BP was also assessed in hypertensive patients. In a randomized crossover trial, 13 hypertensive patients were randomized to dark polyphenol-rich chocolate (500 mg polyphenols) or white chocolate. After 14 days, only polyphenol-rich chocolate decreased SBP by 5.1 mmHg and DBP by 1.8 mmHg; after consumption discontinuation, BP levels returned to pre-intervention values within 2 days (49). Moreover, in 46 mildly hypertensive patients, low chronic cocoa intake (30 mg flavanols per day for 18 weeks) reduced SBP by −2.9 ± 1.6 mmHg ($p < 0.001$) and DBP by −1.9 ± 1.0 mmHg ($p < 0.001$) compared to placebo (50). In a crossover study, 20 subjects with never treated essential hypertension were randomized to dark (500 mg flavanols) or white chocolate (flavanol free) for 15 days. Dark chocolate decreased ambulatory BP (24-h SBP: −11.9 ± 7.7 mmHg, $p < 0.0001$; 24-h DBP: −8.5 ± 5.0 mmHg, $p < 0.0001$) ($p < 0.0001$), serum LDL cholesterol ($p < 0.05$), and improved vascular function (51). However, in a different crossover study enrolling 20 hypertensive subjects, flavanol-rich cocoa intake (900 mg per day) did not improve BP after 2 weeks, compared to placebo (52). A subsequent study evaluated the minimum dose required for BP lowering. A population of 52 subjects with untreated mild arterial hypertension was randomized to receive cocoa beverage containing different doses of flavanols (33, 372, 712, or 1,052 mg per day, respectively). After 6 weeks, only the highest flavanol dose (1,052 mg per day) demonstrated a significant reduction in 24-h SBP (5.3 ± 5.1 mmHg; $p = 0.001$), DBP (3 ± 3.2 mmHg; $p = 0.002$), and mean arterial BP (3.8 ± 3.2 mmHg; $p = 0.0004$) (53).

Flavanol intake also demonstrated a positive effect in hypertensive patients with impaired glucose tolerance (IGT) and diabetes mellitus. In particular, 19 hypertensive patients with IGT were randomized to receive flavanol-rich dark chocolate or flavanol-free white chocolate for 15 days. Dark chocolate reduced both SBP and DBP (SBP: −3.82 ± 2.40 mmHg; 24-h SBP: −4.52 ± 3.94 mmHg; DBP: −3.92 ± 1.98 mmHg; 24-h DBP: −4.17 ± 3.29 mmHg), total and LDL cholesterol ($p < 0.0001$) as well as improved vascular function and insulin sensitivity ($p < 0.05$) (54). High-flavanol cocoa (902 mg) reduced SBP and DBP (SBP: −1.9 mmHg, DBP: −1.8 mmHg, $p < 0.05$) and improved vascular function among overweight and obese adults (55). A recent study conducted on 60 subjects affected by hypertension and type 2 diabetes, randomized to flavanol-rich dark chocolate or white chocolate for 8 weeks, confirmed that flavanol-rich chocolate is effective in decreasing BP, fasting blood sugar, and triglyceride levels in patients with cardiovascular risk factors (28). However, in a high-risk population (three or more cardiovascular risk factors), flavanol-rich cocoa intake did not reduce BP values after 4 weeks (56).

Furthermore, in 24 heart failure patients, a 4 weeks consumption of high-flavanol dark chocolate (1,064 mg flavanols per day) significantly reduced DBP and NT-proBNP levels, compared to low-flavanol dark chocolate intake (57).

Several meta-analyses evaluated the available evidence on this topic. Ried and colleagues analyzed 13 studies, revealing a BP reduction after cocoa consumption (mean BP change ± SE: SBP: −3.2 ± 1.9 mmHg, $p = 0.001$; DBP: −2.0 ± 1.3 mmHg, $p = 0.003$). The effect maintained statistical significance only for the studies evaluating hypertensive and pre-hypertensive patients (SBP: −5.0 ± 3.0 mmHg; $p = 0.0009$; DBP: −2.7 ± 2.2 mmHg, $p = 0.01$) (58). Desch and coworkers also provided a meta-analysis including 10 randomized controlled trials involving either healthy subjects or patients with prehypertension/stage 1 hypertension (297 individuals). Cocoa consumption was associated with a 4.5 mmHg reduction (95% CI, −5.9 to −3.2, $p < 0.001$) for SBP and a 2.5 mmHg reduction (95% CI, −3.9 to −1.2, $p < 0.001$) for DBP (59). In the same year, a Cochrane review showed that flavanol-rich chocolate and cocoa products may have a statistically significant effect in lowering BP by 2–3 mmHg in the short term [SBP (95% CI): −2.77 mmHg (−4.72 to −0.82), $p = 0.005$; DBP (95% CI): −2.20 mmHg (−3.46 to −0.93), $p = 0.006$] (22). A recent published Cochrane review confirmed these findings, observing a small (2 mmHg) decrease in BP in the short term, more pronounced in the pre-hypertensive/hypertensive population (60).

COCOA AND VASCULAR FUNCTION: INTERVENTIONAL STUDIES

Endothelium, the smooth, continuous inner lining of blood vessels, exhibits not only a barrier function but also synthetizes and releases a variety of vasoactive substances. Thus, the imbalance between vasodilating and vasoconstricting mediators results in endothelial dysfunction (61). The impairment of endothelial function is an early event in the development of atherosclerosis and is associated with CVD (62). Endothelial dysfunction of the forearm, as assessed by the flow-mediated dilation of the brachial artery (FMD), is recognized to be a powerful surrogate marker for cardiovascular events and cardiovascular mortality, both in healthy subjects (63) and in patients with CV risk factors (64). Thus, FMD has been used to evaluate the effects of different interventions on endothelial function. To date, various studies have subsequently assessed the effects of cocoa on vascular function (**Table 2**).

In 15 healthy subjects, CFs intake (1.4–10.9 mg/kg body weight) acutely improved FMD at 1, 2, 3, and 4 h after consumption. The improvement in vascular function was comparable to the one induced by nitrate intake (65). Flavanol-rich chocolate (213 mg procyanidins, 46 mg epicatechin) consumption significantly improved FMD in 21 healthy subjects also over a 2-week period ($p = 0.024$). Moreover, plasma epicatechin concentrations markedly increased after 2 weeks in the active treatment group,

TABLE 2 | Studies investigating cocoa and vascular function.

Reference	Year	Study design	Population	Duration	Intervention	Outcome
Rodriguez-Mateos et al. (65)	2015	Randomized double-blind crossover	15 healthy subjects	1,2,3,4 h	CF-rich drink (1.4–10.9 mg/kg body weight) vs. nitrate or nutrient-matched flavanol-free drink	Improvement in FMD after flavanol and nitrate intake
Engler et al. (46)	2004	Randomized double-blind placebo-controlled	21 heathy subjects	2 weeks	High flavonoid chocolate (213 mg procyanidins, 46 mg epicatechin) vs. low-flavonoid chocolate	Improvement in FMD, increased epicatechin concentrations
Sansone et al. (27)	2015	Randomized double-blind controlled parallel	100 healthy subjects	30 days	CF-containing drink (450 mg) or a nutrient-matched flavanol-free control bi-daily	Improvement in FMD, decreased SBP and DBP, decreased PWV
Grassi et al. (23)	2015	Randomized double-blind crossover controlled	20 healthy subjects	5 weeks	Five treatments with daily intake of 10 g cocoa (0, 80, 200, 500, 800 mg flavonoids)	Dose-dependent improvement in FMD, decreased PWV, and BP
Schroeter et al. (66)	2006	Randomized crossover	16 healthy subjects, isolated rabbit rings	2 h	Drink with high flavonoid content	Improvement in FMD, paralleled the appearance of flavanols in plasma
Heiss et al. (67)	2015	Randomized double-blind controlled parallel	42 healthy subjects	14 days	CF-containing drink (450 mg bid) vs. CF-free drink	Improvement in FMD, decreased PWV, and in total peripheral resistances
Shiina et al. (47)	2009	Randomized single-blind	39 healthy subjects	2 weeks	45 g commercially available dark chocolate vs. white chocolate	Improvement in coronary circulation as measured by coronary velocity flow reserve
Grassi et al. (51)	2005	Randomized crossover placebo-controlled	20 untreated hypertensive patients	15 days	100 g dark chocolate (21.91 mg catechin, 65,97 mg epicatechin) vs. flavanol-free white chocolate	Improvement in FMD, decreased BP and LDL cholesterol, increased insulin sensitivity
Grassi et al. (54)	2008	Randomized crossover placebo-controlled	19 hypertensive with IGT	15 days	100 g dark chocolate (36 mg catechin, 110 mg epicatechin) vs. flavanol-free white chocolate	Improvement in FMD, decreased SBP and DBP, decreased insulin resistance
Heiss et al. (69)	2005	Randomized double-blind crossover	11 smokers	2 h	100 ml cocoa drink with high (176–185 mg) or low (<11 mg) flavanol content	Improvement in FMD and increased circulating NO pool. Increased flavanol metabolites
Hermann et al. (70)	2006	Randomized placebo-controlled	20 smokers	2 h	40 g commercially available dark chocolate vs. white chocolate	Improvement in FMD, antioxidant status, and platelet function
Davison et al. (55)	2008	Randomized double-blind placebo-controlled parallel	49 obese and overweight patients	12 weeks	Dietary high (902 mg) vs. low (36 mg) flavanol intake	Improvement in FMD
Njike et al. (48)	2011	Randomized controlled crossover	44 overweight patients	6 weeks	Sugar-free cocoa beverage or placebo, sugar-sweetened cocoa beverage or placebo	Improvement in FMD, no change in weight
West et al. (71)	2014	Randomized double-blind crossover placebo-controlled	30 overweight patients	30 days	37 g dark chocolate plus sugar-free cocoa beverage (flavanols 814 mg) vs. low-flavanol chocolate bar and cocoa-free and sugar-free beverage	Unchanged FMD, increased basal diameter and peak diameter of the brachial artery, increased basal blood flow, in women decreased augmentation index
Balzer et al. (72)	2008	Randomized double-blind	41 diabetic patients	30 days	Flavanol-rich cocoa (321 mg flavanols × 3) or a nutrient-matched control (25 mg flavanols × 3)	Improvement in FMD
Mellor et al. (73)	2013	Randomized double-blind crossover controlled	10 diabetic patients	2 h	13.5 g of high vs. low-flavanol chocolate; 60 min later, a 75 g oral glucose load	Improved endothelial function assessed by reactive hyperemia peripheral artery tonometry
Heiss et al. (74)	2003	Randomized double-blind crossover	20 patients with at least 1 CV risk factor	2 h	Flavanol-rich cocoa drink (100 ml)	Improvement in FMD and increased levels of nitrosated and nitrosylated species
Heiss et al. (75)	2010	Randomized double-blind crossover controlled	16 CHD patients	30 days	Dietary high (375 mg bid) vs. low (9 mg bid) flavanol cocoa drink	Improvement in FMD and mobilization of endothelial progenitor cells
Flammer et al. (29)	2012	Randomized double-blind placebo-controlled	20 heart failure patients	2 h and 30 days	40 g commercially available dark chocolate vs. flavonoid-free placebo chocolate	Improvement in FMD of platelet function

(*Continued*)

TABLE 2 | Continued

Reference	Year	Study design	Population	Duration	Intervention	Outcome
Flammer et al. (76)	2007	Randomized double-blind	22 heart transplant patients	2 h	40 g commercially available dark chocolate vs. flavonoid-free placebo chocolate	Inducing coronary vasodilation, improvement in coronary endothelial function, and improvement in platelet function
Rassaf et al. (77)	2016	Randomized double-blind placebo-controlled	57 hemodialytic patients	30 days	CF-rich beverages (900 mg per study day) vs. flavanol-free beverages	Improvement in FMD decreased DBP. Ingestion of flavanols during HD alleviated HD-induced vascular dysfunction
Sansone et al. (68)	2017	Randomized double-blind crossover	47 healthy subjects		High (820 mg)/low-flavanol cocoa drink with high (220 mg)/low methylxanthines content	CFs with methylxanthines increased epicatechin serum concentration, increased FMD decreased PWV and DBP compared with flavanols alone

Modified from Ref. (116).

FMD, flow-mediated dilation; endoPAT, reactive hyperemia peripheral artery tonometry; PWV, pulse wave velocity; CV, cardiovascular; NO, nitric oxide; CHD, coronary heart disease; HD, hemodialysis; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low-density lipoprotein; CF, cocoa flavanol; IGT, impaired glucose tolerance.

suggesting that the effect on vascular function was flavanol-mediated (46). In the Flaviola Health Study, a 1-month CF intake increased FMD over control by 1.2% (95% CI, 1.0–1.4) and decreased pulse wave velocity by 0.4 m/s (95% CI, 0.8–0.04 m/s) (27). Furthermore, 20 healthy subjects were randomized to receive 5 treatments with daily intake of 10 g cocoa (0, 80, 200, 500, and 800 mg cocoa flavonoids per day) in5 periods lasting 1 week. A dose-dependent increase in FMD (from 6.2% to 7.3, 7.6, 8.1, 8.2% after the different flavonoid doses, respectively) was found (23). In healthy individuals, an improvement in vascular function after high-flavanol cocoa intake has been demonstrated in subsequent studies (66, 67). Flavanol-rich chocolate intake was also demonstrated to significantly improve coronary circulation in healthy adults, as assessed by coronary flow velocity reserve measurement with non-invasive transthoracic Doppler echocardiography (47). Interestingly, in a recently published randomized double-blind trial, an interaction between flavanols and methylxanthines, such as theobromine and caffeine contained in cocoa, has been identified. In particular, methylxanthines were demonstrated to increase epicatechin metabolites plasma levels, thus affecting flavanols absorption, and to enhance the positive vascular effect of flavanols (68).

Flavanol intake improved FMD in hypertensive patients with normal (51) as well as IGT (54). Flavanol-rich cocoa beverage also acutely improved endothelium-dependent vasodilation, platelet function, and circulating bioactive NO in smokers (69, 70). In 20 male smokers, dark chocolate, but not white chocolate, improved FMD after 2 h (7.0 vs. 4.4%, $p = 0.026$), and the effect lasted about 8 h after ingestion (70). Furthermore, a statistically significant reduction in DBP and mean BP, and a parallel improvement in FMD was shown after high-flavanol cocoa intake in obese and overweight subjects (55). Moreover, the positive effect of cocoa consumption on endothelial function was not associated with weight gain (48). Conversely, in a subsequent study, an increase in basal and peak brachial artery diameter, with no consequent change in FMD, was assessed in a group of 30 overweight patients after 30 days high-flavanol chocolate intake (71). In a diabetic population, high-flavanol cocoa consumption was associated with statistically significant improvement in vascular function both acutely (after 2 h) and chronically (30 days) in two different studies (72, 73). Flavanol-rich cocoa intake improved FMD (from 3.4 to 6.3%, $p < 0.001$) after 2 days in a population with at least one cardiovascular risk factor, including history of CHD, hypertension, hyperlipidemia, diabetes, or current tobacco use (74). Altogether, these studies highlight the ability of CFs in improving vascular function in patients with cardiovascular risk factors.

The efficacy of flavanol intake on vascular function has been assessed also in a population of 16 CHD patients, randomized to receive flavanol-rich or low-flavanol cocoa for 30 days. Results showed a significant improvement in endothelial function by 48%, a decrease in SBP (mean change in active group: -4.2 ± 2.7 mmHg), and increasing levels of circulating angiogenic cells in the active treatment group compared with controls (75).

In a study from our group, 20 chronic heart failure patients were randomized to receive commercially available flavanol-rich chocolate or control chocolate (29). Flavanol-rich chocolate significantly improved FMD from baseline levels of 4.98 ± 1.95, to $5.98 \pm 2.32\%$ ($p = 0.045$ and 0.02 for between-group changes) 2 h after intake, and to $6.86 \pm 1.76\%$ after 4 weeks of daily consumption ($p = 0.03$ and 0.004 for between groups). After flavanol-rich chocolate intake, platelet adhesion significantly decreased in the short term, but the effect was not sustained after 4 weeks. BP values and heart rate did not change. We also assessed the effect of flavanol-rich dark chocolate compared with control chocolate on coronary vascular function in 22 heart transplant recipients, patients characterized by severely impaired vascular function (76). Two hours after ingestion, flavanol-rich dark chocolate but not control chocolate induced significant coronary vasodilation ($p < 0.01$), improved coronary vascular function ($p = 0.01$), and decreased platelet adhesion.

Cocoa flavanols demonstrated to have a protective role also among patients with end-stage renal disease (ESRD). A population of 57 ESRD patients was randomized to receive either flavanol-rich beverage (900 mg) or placebo. Flavanol-rich cocoa improved endothelial function by 53% ($p < 0.001$) and alleviated hemodialysis induced endothelial dysfunction ($p < 0.001$) after acute ingestion, with no effect on BP and heart rate. After 4 weeks of treatment, cocoa improved vascular function by 18% and decreased DBP ($p = 0.03$) (77).

In a meta-analysis of 11 chronic and 11 acute studies, Hooper and colleagues found strong beneficial effects of cocoa on FMD, as well as reductions in DBP and mean arterial pressure. Chocolate or cocoa improved FMD regardless of the dose consumed (78).

Based on the epidemiological evidence and the results from interventional studies, the European Food Safety Authority (EFSA) published a health claim about the effect of polyphenols derived from cocoa on cardiovascular risk factors, assessing that CFs "help maintaining the elasticity of blood vessels, which contributes to normal blood flow." In order to obtain the claimed effect, they suggested "to consume 200 mg of cocoa flavanols per day, provided by 2.5 g of high-flavanol cocoa powder or 10 g of high-flavanol dark chocolate, in the context of a balanced diet" (79). Conversely, Vlachojannis and coworkers asserted that only cocoa products with 100 mg epicatechin or CF doses of around 900 mg were able to achieve positive effects on FMD and BP, questioning the 200 mg flavanols/46 mg epicatechin dose recommended by the EFSA (80).

Currently, the COcoa Supplement and Multivitamin Outcomes Study (COSMOS; NCT02422745) is ongoing to assess the capability of a cocoa extract supplement (600 mg per day flavanols/80 mg epicatechin) compared to a standard multivitamin supplement, to reduce the risk of CVD and cancer among men aged 60 years and older and women aged 65 years and older. Concomitantly, an ancillary study (COSMOS-Mind; NCT03035201) is being conducted to evaluate the effects of such supplements on cognitive function.

PUTATIVE MECHANISMS

The protective role of CFs intake on BP and endothelial function is likely to come from its vasodilatory effect; the underlying mechanisms are multiple and not fully understood (30).

In this context, the increased NO availability and the subsequent vasodilation may play a central role. In young spontaneously hypertensive rats, epicatechin delayed the occurrence of arterial hypertension and reduced locomotor hyperactivity; these results were both mediated by increased NO bioavailability and erythrocyte deformability (81). Taubert and colleagues (50) demonstrated that prolonged intake of small amounts of dark chocolate (6.3 g per day for 18 weeks) reduced BP and improved NO production in a population of 44 pre-hypertensive individuals. In particular, dark chocolate intake reduced mean SBP by 2.9 mmHg ($p < 0.001$) and DBP by 1.9 mmHg ($p < 0.001$); these results were accompanied by a sustained increase of S-nitrosoglutathione, a source of bioavailable NO, by 0.23 nmol/L ($p < 0.001$) (50). Flavanols, and particularly flavanol-rich cocoa, elevate NO bioavailability by both stimulating the NO synthase (eNOS) activity (82, 83) and increasing the availability of L-arginine (*via* reduction of its degradation by arginase) (84). Furthermore, in a rat model, flavanols prevented the elevation of BP induced by L-nitroarginine methyl ester (L-NAME), a powerful inhibitor of NOS (83). In addition, cocoa showed a similar inhibitor effect on endothelin-1 production, a powerful vasoconstrictor (85, 86). Flavanols proved capable to induce both NO-mediated and endothelium-derived hyperpolarizing factor-mediated relaxation in a large number of arteries including the coronary arteries (87). Since NO degradation is mediated by free radicals, the improvement in vascular function is also related to the anti-inflammatory and antioxidant properties of cocoa (88). In a systematic review of the literature on polyphenols and oxidative stress, four studies reported statistically significant improvements in markers of oxidative stress and inflammation after flavanol-rich cocoa intake (89). A significant reduction in oxidative stress occurred when dark chocolate was administered to smokers as opposed to milk chocolate (90). Furthermore, a high dose (472.5 mg) of flavonoids through cocoa powder led to reduction in all oxidative and inflammatory markers in type 2 diabetics, and to a parallel improvement in endothelial function (1.7 ± 0.1 vs. $2.3 \pm 0.1\%$, $p = 0.01$) assessed by reactive hyperemia peripheral artery tonometry (EndoPAT-2000) (73). After consumption of cocoa products, a decrease in markers of peroxidation was also observed in healthy subjects, as well as in obese, dyslipidemic, pre-hypertensive, and hypertensive patients (25). CFs directly scavenge ROS and nitrogen species (91); moreover, they modulate crucial enzymes related to oxidative stress, such as catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione transferase, xanthine oxidase, and lipooxygenase (92, 93). In line with this, the exposure of human endothelial cells to epicatechin inhibited endothelial NADPH oxidase, reduced superoxide and peroxynitrite levels, and consequently induced an increase in NO and cyclic guanosine monophosphate cellular levels (94). Furthermore, cocoa powder and epicatechin demonstrated to significantly decrease aortic oxidative stress and circulating markers related to impaired coagulation (von Willebrand factor, factor VIII, and fibrinogen) and inflammation (tissue necrosis factor-α, interleukin-6, interleukin-10, and C-reactive protein) (95). *In vitro* experiments show that CFs inhibit pro-inflammatory cytokines such as interleukin-2, interleukin-1β, and tissue necrosis factor-α, and positively modulate the expression of anti-inflammatory cytokines, such as interleukin-4 and transforming growth factor-β (92, 96–98).

An inhibition of angiotensin-converting enzyme (ACE) by cocoa constituents has been postulated. In a rat model, flavanol-rich cocoa powder significantly reduced BP in spontaneously hypertensive rats but did not exert a similar effect in normotensive Wistar-Kyoto rats. Interestingly, the effect of flavanol-rich cocoa (300 mg/kg) clearly mimicked that caused by captopril (50 mg/kg) (99). In 2006, Actis-Goretta and colleagues documented an *in vitro* interaction between cocoa flavonoids and ACE. They demonstrated that procyanidin-rich chocolate significantly inhibited purified ACE activity, whereas the inhibitory activity correlated with both the phenolic content ($p < 0.003$) and the flavanol content ($p < 0.001$). When incubated in membrane suspensions from rat kidney, chocolate [634 μM (+)-catechin equivalents] high in procyanidin inhibited ACE activity by 70% and such low in procyanidin [314 μM (+)-catechin equivalents] only inhibited ACE by 45% ($p < 0.001$). The inhibition of ACE in tissue membrane suspensions was also observed in rat testes and lungs (100). In a subsequent study, extract from powdered cocoa beans was demonstrated to dose-dependently inhibit *in vitro* ACE activity; it also showed a dose-dependent radicals scavenging ability (101). Persson and colleagues (102) demonstrated that the acute consumption of dark chocolate (75 g, 72% of cocoa) inhibited ACE activity *in vitro*—after incubation in human endothelial

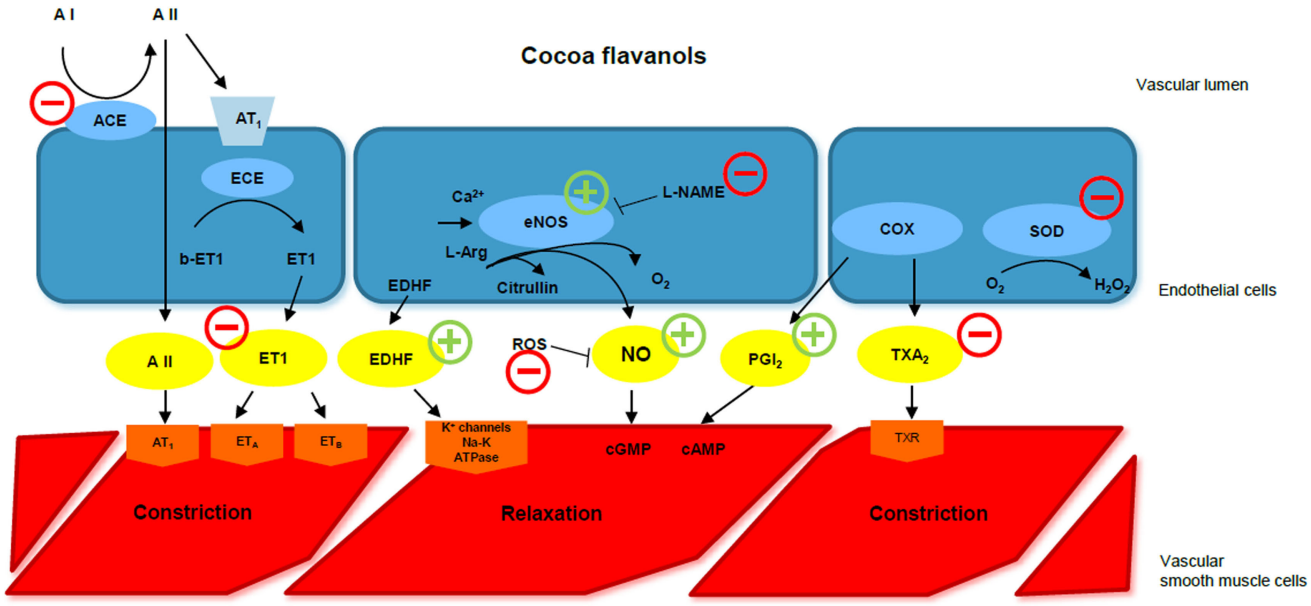

FIGURE 1 | Cocoa flavanols and endothelial function. ACE, angiotensin-converting enzyme; A I, angiotensin I; A II, angiotensin II; ECE, endothelin-converting enzyme; ET 1, endothelin-1; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial NO synthase; L-NAME, L-nitroarginine methyl ester; ROS, reactive oxygen species; NO, nitric oxide; cGMP, cyclic guanosine monophosphate; cAMP, cyclic adenosine monophosphate; COX, cyclooxygenase; PGI2, prostacyclin; TXA2, thromboxane A2; SOD, superoxide dismutase; CF, cocoa flavanol. CFs improve endothelial function *via* different pathways. They increase NO availability, stimulating eNOS function, preventing L-NAME-induced hypertension, and reducing ROS. They also stimulate EDHF-mediated relaxation, inhibit endothelin-1, and reduce ACE activity. Modified by Corti et al., Circulation 2009.

cells from umbilical veins (HUVEC) —and *in vivo* in 16 healthy volunteers. In HUVEC, a significant inhibition of ACE activity ($p < 0.01$) and an increase of NO levels ($p < 0.001$) were seen. In healthy subjects, dark chocolate significantly inhibited ACE activity (mean 18%) 3 h after oral intake, with no relevant changes in circulating NO levels. According to ACE genotype, significant inhibition of ACE activity emerged in individuals with genotype insertion/insertion and deletion/deletion (mean 21 and 28%, respectively) (102).

Recently, studies have begun to pay attention to the role of cocoa on mitochondria in cardiovascular health, as impaired mitochondrial function represents an early sign of endothelial dysfunction (103). The stimulation of mitochondrial function and biogenesis could ameliorate bioenergetic and metabolic status of cells, thereby improving vascular function and reducing CVD. Animal studies demonstrated that CFs are able to decrease cardiac post-ischemic damage *via* prevention of the mitochondrial permeability transition pore opening, and reduction in superoxide production (104); flavanols might also affect mitochondrial structure and function *via* stimulation of mitochondrial biogenesis (105). Patients, randomized to receive dark chocolate, showed increased maximal oxygen uptake and maximum work achieved, as well as increases in mitochondrial activity and glutathione levels, when compared to placebo (106). NO is suspected to mediate the effects of cocoa on mitochondria. In support of this thesis, a recent study demonstrated, *in vitro*, that flavanols are capable to stimulate mitochondrial function and biogenesis; effects disappeared with the inhibition of eNOS (107, 108).

Cocoa flavanols are also able to inhibit platelet activation, adhesion, and aggregation, mechanisms that play a central role in the development of endothelial dysfunction and atherosclerosis (109). Indeed, activated platelets secrete a number of adhesion molecules, such as P-selectin and C40 ligand, release inflammatory mediators into the local microenvironment (110), stimulate the chemotaxis of leukocytes to the site of inflammation (111), and generate ROS, reducing NO bioavailability and contributing to endothelial dysfunction and thrombosis (112).

Moreover, cocoa and its main flavanols may improve vascular function by regulating the glucose and lipid profile (113), crucial risk factors for vascular damage. There is evidence that CFs are able to modulate insulin secretion in β-pancreatic cells, target insulin-sensitive tissues, repress glucose production, enhance glucose uptake through the promotion of glucose transport, and improve lipid metabolism (114).

Altogether, these mechanisms might determine the antihypertensive and cardiovascular protective effects of flavanols *in vivo* (**Figure 1**).

CONCLUSION

Polyphenol-rich foods, such as fruit, vegetables, wine, olive oil, and cocoa, are able to reduce cardiovascular risk and prevent cardiovascular events and death (3–5, 115). Among them, cocoa beans have always been of particular interest, as they are one of the richest polyphenol sources. In this context, several epidemiological studies suggest a strong correlation between daily cocoa intake and better cardiovascular outcome in different population settings (19–21). Clinical interventional studies demonstrated a positive effect of flavanol-rich cocoa or chocolate intake on BP

reduction and improvement in microvascular and macrovascular function (116, 117). *In vitro* and *in vivo* studies identified increased NO availability, increased NO synthase activity, and inhibition of ACE as putative mechanisms of this beneficial effect (118).

Cocoa consumption has been demonstrated to improve endothelial function and to lower BP in healthy subjects, in patients with risk factors and hypertension, and in patients with coronary heart disease and heart failure. Furthermore, a 3-mmHg reduction in SBP has been estimated to lower the relative risk of CHD by 5% and the risk of global mortality by 4% (119). Thus, the introduction of a moderate amount of flavanol-rich cocoa in the daily diet may be a promising strategy to improve cardiovascular outcomes.

However, commercial chocolate with its high sugar and fat content may be undesirable in a population with increased cardiovascular risk. Furthermore, during the cocoa beans manufacturing process, the total amount of flavanols can be reduced more than 10-fold by fermentation or roasting (18). This results in an unpredictable content of polyphenols in most commercially available products. Moreover, the optimal dose of daily flavanol intake is still unclear. The EFSA recommends consuming 200 mg of CFs per day, provided by 2.5 g of high-flavanol cocoa powder or 10 g of high-flavanol dark chocolate in the context of a balanced diet, but this dose has recently been subject of discussion (80). Thus, a comparison among studies is difficult, because of the heterogeneity between trials, in terms of study population and design, flavanol doses in active and control groups, and study duration.

Further clinical studies are required in order to identify the correct dose and the right modality of manufacturing of flavanol-rich cocoa to be able to benefit from daily consumption of this natural medicinal product in the field of CVD.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception of this review. VL, JB, MN, and IS drafted the work; FE, CF, AF, and FR revised it critically for important intellectual content. All authors approved the final version of the paper.

FUNDING

The work was support by Swiss Heart Foundation (AF and IS).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

MINI REVIEW
published: 16 May 2017
doi: 10.3389/fnut.2017.00019

Enhancing Human Cognition with Cocoa Flavonoids

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Enhancing cognitive abilities has become a fascinating scientific challenge, recently driven by the interest in preventing age-related cognitive decline and sustaining normal cognitive performance in response to cognitively demanding environments. In recent years, cocoa and cocoa-derived products, as a rich source of flavonoids, mainly the flavanols sub-class, have been clearly shown to exert cardiovascular benefits. More recently, neuromodulation and neuroprotective actions have been also suggested. Here, we discuss human studies specifically aimed at investigating the effects of acute and chronic administration of cocoa flavanols on different cognitive domains, such as executive functions, attention and memory. Through a variety of direct and indirect biological actions, in part still speculative, cocoa and cocoa-derived food have been suggested to possess the potential to counteract cognitive decline and sustain cognitive abilities, particularly among patients at risk. Although still at a preliminary stage, research investigating the relations between cocoa and cognition shows dose-dependent improvements in general cognition, attention, processing speed, and working memory. Moreover, cocoa flavanols administration could also enhance normal cognitive functioning and exert a protective role on cognitive performance and cardiovascular function specifically impaired by sleep loss, in healthy subjects. Together, these findings converge at pointing to cocoa as a new interesting nutraceutical tool to protect human cognition and counteract different types of cognitive decline, thus encouraging further investigations. Future research should include complex experimental designs combining neuroimaging techniques with physiological and behavioral measures to better elucidate cocoa neuromodulatory properties and directly compare immediate versus long-lasting cognitive effects.

Keywords: chocolate, flavanols, neuroprotection, cognitive function, cardiovascular function

OPEN ACCESS

Edited by:
Mauro Serafini,
University of Teramo, Italy

Reviewed by:
Andrea Enzo Scaramuzza,
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Alberto Finamore,
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Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Nutrition

Received: *01 March 2017*
Accepted: *27 April 2017*
Published: *16 May 2017*

Citation:
Socci V, Tempesta D, Desideri G, De Gennaro L and Ferrara M (2017) Enhancing Human Cognition with Cocoa Flavonoids. Front. Nutr. 4:19. doi: 10.3389/fnut.2017.00019

Abbreviations: BOLD, blood oxygenation level-dependent; BDNF, brain-derived neurotrophic factor; CBF, cerebral blood flow; ERK, extracellular signal-regulated kinase; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; NIRS, near-infrared spectroscopy; NO, nitric oxide; PI3-kinase/Akt, phosphoinositide 3-kinase; SSVEP, steady-state visually evoked potentials.

INTRODUCTION

With the increase in general population aging, age-related cognitive decline has progressively become a major public health issue. Consequently, enhancing cognitive resources came to be a priority scientific challenge, mainly driven by the need to detect preventive agents for cognitive decline and dementia. At the same time, immediate cognitive enhancement is often desirable in cognitively demanding environments. Stimulants such as caffeine are indeed commonly used to sustain cognitive performance during periods of fatigue and prolonged wakefulness (1).

In the last years, the field of nutraceuticals and functional food has developed along with the interest in the potential modulatory effects of food constituents on human health. In this context, there has been increasing interest in the biological effects of flavonoids, a class of polyphenolic compounds, as potential nutraceuticals with several beneficial biological actions, including cardioprotection, neuroprotection, and neuromodulation. Particularly, cocoa bean has been recognized as a rich source of flavonoids, mainly the flavanols subclass in the form of epicatechin and catechin (2).

Chocolate also contains other functional ingredients, such as the methylxanthine caffeine and theobromine, with the potential to influence neurocognitive function. However, relative to total flavanols content, caffeine and theobromine concentrations in cocoa and chocolate have been considered lower than those required to exert significant pharmacological actions (3).

Epidemiologic studies suggest that a regular intake of flavonoids could be associated with better cognitive function (4), decreased risk of dementia (5) and cognitive decline (6), lower prevalence of cognitive impairment (7), better cognitive evolution over a 10-year period (8), and better dose-dependent cognitive performance in normal aging (9).

The specific mechanisms of action of flavonoids responsible for cognitive protection and modulation are not entirely elucidated. Nevertheless, increasing evidence supports the notion that cocoa and chocolate consumption provides several health benefits, including neurocognitive enhancement and neuroprotective effects. In this brief review, we discuss human studies specifically aimed at investigating the effects of acute and chronic administration of cocoa flavanols on cognition.

ENHANCING COGNITION WITH COCOA FLAVONOIDS: DIRECT VERSUS INDIRECT MECHANISMS OF ACTION

The beneficial effects of polyphenolic compounds have been first attributed to their ability to exert antioxidant actions (10). However, due to the very low concentration of flavonoids detectable in the brain, it is unlikely that direct antioxidant action can entirely account for cognitive effects *in vivo*. Instead, flavonoids' neurobiological effects are now believed to be mediated by a range of actions involving the ability to protect vulnerable neurons, enhance neuronal function, and stimulate regeneration *via* interaction with neuronal intracellular signaling pathways involved in neuronal survival and differentiation, long-term potentiation (LTP), and memory (11).

Flavonoids can counteract neuronal injury underlying neurodegenerative diseases such as Parkinson and Alzheimer diseases through their interaction with signaling proteins important in the pro-survival pathways (12). Interestingly, flavonoids and their metabolites cross the blood–brain barrier and have been localized in the brain, particularly in areas crucial for learning and memory such as hippocampus, cerebral cortex, cerebellum, and striatum (13–15). These structures are particularly vulnerable to the effects of aging and neurodegeneration, suggesting that flavonoids could exert direct neuroprotective effects (16). Direct interactions within several cellular signaling pathways have been described, such as mitogen-activated protein kinase, extracellular signal-regulated kinase, and phosphoinositide 3-kinase (PI3-kinase/Akt) signaling cascades (17), that are crucially involved in triggering gene expression and protein synthesis for LTP (18). In memory-related areas such as the hippocampus, flavonoids promote the expression of brain-derived neurotrophic factor (BDNF), that is crucial to adult neurogenesis, synaptic growth, and neuronal survival (19). Increasing evidence from animal models indicates that flavonoids can promote cognitive benefits through their ability to directly interact with the cellular and molecular architecture involved in memory function (2, 11, 12). To date, however, it is still not entirely clear whether and to what extent these direct biological actions also extend to the human brain.

Flavonoids could also display their neurocognitive effects indirectly, through their capability of inducing cardiovascular actions. In this regard, cardiovascular benefits of cocoa and chocolate consumption are now well established and include endothelium-dependent vasodilation, which contributes to the maintenance of normal blood flow, reduced platelet aggregation, and blood pressure improvement (20–26).

Importantly, flavanols such as epicatechin can improve endothelial function by increasing the nitric oxide (NO) bioavailability, a key regulator of vascular function, leading to improvements in vascular tone and blood pressure regulation (27, 28). These peripheral vascular changes may also extend to brain perfusion, leading to a more efficient cerebrovascular coupling during neuronal activation, which is considered crucial for the functional and structural integrity of the brain. Moreover, as increased cerebrovascular function promotes adult neurogenesis in the hippocampus (29), flavonoid-mediated vascular changes could be additionally relevant for memory function.

Coherently, increasing evidence highlights the potential of cocoa flavanols to influence cognitive abilities interacting with the cerebrovascular system. Flavanol-rich cocoa has been shown to significantly increase cerebral blood flow (CBF) 1–2 h post-intervention in humans (30–32), suggesting that flavanols' indirect, vasodilatatory actions could account for acute cognitive enhancement following a single administration (16). Conversely, cognitive benefits associated with long-term flavonoids intake are more likely to involve morphological changes induced by direct actions on neuronal signaling. However, indirect mechanisms of action can also contribute to long-term neuroprotective effects, as increases in NO levels can further impact on brain vascularization over time by promoting angiogenesis, thus resulting in better cerebrovascular communication.

EFFECTS OF COCOA FLAVANOLS DAILY INTAKE ON COGNITION: EVIDENCE FROM CHRONIC INVESTIGATIONS

In line with the evidence suggesting a favorable association between flavonoids' regular consumption and cognitive performance in elderly subjects (33), chronic and sub-chronic cognitive effects of cocoa flavanols administration have been addressed in normal aging and clinical populations (see **Table 1**).

In this respect, the daily consumption of flavanol-rich cocoa drink has been showed to positively affect cognition, leading to improvements in cognitive performance both in older adults with early memory decline (36) and in cognitively intact elderly subjects (39). Specifically, compared to the low-flavanol condition (48 mg), the chronic administration of intermediate (520 mg) and high (993 mg) cocoa flavanols content over an 8-week period was associated to improvements in processing speed, executive function, and working memory in subjects with mild cognitive impairment. At higher cocoa flavanols concentrations, significant improvements were also evident in a verbal fluency task. Interestingly, such cognitive beneficial effects were paralleled by improvements in blood pressure and insulin resistance, suggesting a role of endothelial function and glucose sensitivity in modulating cognitive function in these patients (36). More recently, similar findings were replicated in healthy aged participants. Subjects in the intermediate and high cocoa flavanols

TABLE 1 | Summary of the studies examining the effects of daily administration of cocoa flavanols on cognitive performance.

Reference	Participants	Flavanols amount	Cognitive measures	Principal findings
Francis et al. (31)	16 subjects (all females; 18–30 years)	Cocoa drinks containing 172 and 13 mg flavanols daily over a 5-day period	Task switching paradigm	Increased BOLD signal in response to the task switching paradigm after the higher flavanol drink, with no significant behavioral effect
Crews et al. (34)	101 subjects (41 males, 60 females; mean age: 69 years)	Chocolate bar containing 397 mg flavanols, chocolate drink containing 357 mg flavanols, or similar placebo	Selective Reminding Test, Stroop Test, Trail Making Test, Wechsler Adult Intelligence Scale-III (Digit Symbol-Coding Subtest), Wechsler Memory Scale-III (Faces I and Faces II Subtests)	No differences in cognitive performance between placebo and polyphenol groups
Camfield et al. (35)	63 subjects (40–65 years)	Cocoa drinks containing 500, 250, and 0 mg flavanols (placebo) over a 30-day period	Spatial Working Memory Test	SSVEP changes indicating improved working memory function after flavanol treatment, with no significant behavioral effect
Desideri et al. (36)	90 subjects with Mild Cognitive Impairment (43 males, 47 females; 64–82 years)	Cocoa drinks containing 993, 520, or 48 mg (low-flavanol) flavanols daily over an 8-week period	Mini-Mental State Examination, Trail Making Test (A–B), Verbal Fluency Test	Improvements in blood pressure, insulin resistance and Tail Making Test (A–B) performance for high and intermediate flavanol groups compared to the low-flavanol group. Improved verbal fluency performance in the high-flavanol group
Sorond et al. (37)	60 subjects with vascular risk factors (29 males, 31 females; mean age: 72.9 years)	Cocoa drinks containing 609 or 13 mg (flavanol-poor) flavanols daily over a 30-day period	Mini-Mental State Examination, Trail Making Test (A–B)	Improvements in neurovascular coupling and Trial Making Test B performance for the flavanols group, only in those with impaired neurovascular coupling at baseline
Brickman et al. (38)	37 subjects (13 males, 24 females; 50–69 years)	Cocoa supplement containing 900 or 10 mg (low-flavanol) flavanols daily over a 3-month period	Modified Benton Task (dentate gyrus-dependent memory task)	Correlation between increased cerebral blood volume in the dentate gyrus and improvements in the Modified Benton Task performance in the high-flavanol group
Mastroiacovo et al. (39)	90 subjects (37 males, 53 females; 65–85 years)	Cocoa drinks containing 993, 520, or 48 mg (low-flavanol) flavanols daily over an 8-week period	Mini-Mental State Examination, Trail Making Test (A–B), Verbal Fluency Test	Improvements in blood pressure, insulin resistance and Tail Making Test (A–B) performance for high and intermediate flavanol groups in comparison to low-flavanol group. Improved verbal fluency performance among all treatment groups
Neshatdoust et al. (40)	40 subjects (22 males, 18 females; 62–75 years, mean age: 68.3 years)	Cocoa drinks containing 494 and 23 mg (low-flavanol) flavanols daily over a 28-day period	Several cognitive tasks measuring executive functions, episodic memory, working memory, spatial memory, implicit memory, attention and processing speed	Higher BDNF serum levels and improvements in global cognition scores following high-flavanol treatment

BDNF, brain-derived neurotrophic factor; BOLD, blood oxygenation level-dependent; SSVEP, steady-state visually evoked potentials.

groups, after a daily consumption over an 8-week period, showed better performance in several cognitive domains compared to those in the low-flavanol group (39).

By contrast, in healthy older adults a 6-week cocoa flavanols intervention showed no significant effects on cognitive and cardiovascular outcomes (34). In this study, however, participants were administered chocolate bar or beverage containing 397 or 357 mg flavanols, respectively; insufficient flavanols content may therefore account for the negative finding.

In middle-aged (40–65) subjects, Camfield et al. (35) investigated the chronic (30-day intake) neurocognitive effects of 250 and 500 mg cocoa flavanols administration using a spatial working memory task and steady-state visually evoked potentials (SSVEP). Compared to placebo, participants receiving cocoa flavanols treatment showed changes in SSVEP average amplitude and phase across several posterior parietal and centro-frontal sites that indicated an increased neural efficiency in response to the working memory task. Hence, cocoa flavanols long-term intake could effectively improve cognition trough increased neural efficiency, although, in this healthy population, the observed changes in brain activation were not paralleled by concomitant improvements in spatial working memory accuracy. Similarly, a sub-chronic (5 days) daily intake of 172 mg cocoa flavanols was associated, in healthy young subjects, to increased blood oxygenation level-dependent signal in various brain regions in response to an attention switching task, without any behavioral effect (31).

It has been proposed that cocoa long-term cognitive protection could particularly affect populations at risk. In this regard, Sorond et al. (37) evaluated the effect of flavanol-rich cocoa on neurovascular coupling in older subjects with vascular risk factors. In patients with impaired cerebrovascular coupling at baseline, cocoa administration was associated with improvements in cerebrovascular function both acutely and after a 30-day period. Moreover, chronic flavanols intake also resulted in improvements in cognitive flexibility. Conversely, no significant effects of cocoa administration on neurovascular coupling and cognitive performance were observed in those with intact cerebrovascular functions (37).

Besides improving CBF and CBF velocity after daily intake (41, 42), cocoa flavanols could also specifically affect cerebral blood volume in memory-related brain areas (38). Using high-resolution functional magnetic resonance imaging, a 3-month intervention with 900-mg cocoa flavanols resulted in increased cerebral blood volume in the dentate gyrus of the hippocampus, a structure particularly affected by aging and potentially implicated in memory decline. Moreover, the increase in cerebral blood volume was highly correlated with improvements in performance in a dentate gyrus-dependent memory task. Cocoa flavanols regular intake could therefore improve human dentate gyrus function through global increase in blood flow or a more selective increase in capillary density.

More recently, Neshatdoust et al. (40) investigated the link between cognitive performance and BDNF serum levels in older participants following cocoa flavanols chronic administration over 12 weeks. Results showed that, in comparison to a low-flavanol control (23 mg total flavanols), the daily intake of a high-flavanol cocoa drink (494 mg total flavanols) was associated with higher BDNF serum levels and improvements in global cognition scores. Therefore, changes in serum BDNF levels induced by cocoa flavanols could additionally underpin the beneficial effect of chronic cocoa flavanols intake on cognitive functions.

Collectively, these findings seem to support quite consolidated epidemiological evidence indicating that regular cocoa flavanols intake possesses the potential to protect human cognition, particularly in aged populations (33). As suggested (43), cocoa flavanols long-term beneficial effects on cognition could also possibly explain the correlation between country levels of chocolate consumption and Nobel Prizes *per capita*.

ACUTE EFFECTS OF COCOA FLAVANOLS INTAKE ON COGNITION

Only few randomized controlled trials have investigated the possibility that human cognitive function may be improved following acute cocoa consumption, within 6 h post-ingestion (see **Table 2**).

In a double-blind crossover study, 30 healthy adults were administered chocolate drinks containing 520 and 994 mg flavanols and 46 mg matched control, with a 3-day washout between sessions (44). Cognitive assessment, designed to be cognitively fatiguing, consisted in a 10-min task battery administered in six consecutive repetitions, including a serial subtraction task and a rapid visual information processing task. Results showed that, compared to control, both 520 and 994 mg cocoa flavanols doses significantly improved working memory performance on serial subtractions.

Using a similar acute intervention, Field et al. (45) found that 2 ho after a 773-mg cocoa flavanols supplementation, young participants showed enhanced performance on the accuracy measure of a spatial working memory task, as well as performance improvements on a choice reaction time task. Also, visual function was improved, with better visual contrast sensitivity and time to detect motion direction (45).

By contrast, Pase et al. (46) failed to demonstrate any acute effect of 500 mg cocoa flavanols administration on cognition in older adults. Cognitive assessment included measures of memory and attention. However, as a limitation, participants had a standardized lunch break before testing session; it is therefore possible that flavonoids effects were masked by post-prandial factors (46).

Immediate cognitive enhancement induced by high-flavanol cocoa is generally supposed to be mediated by the capability of their metabolites to promote crucial changes in peripheral and central blood flow. Some investigations therefore included behavioral and cardiovascular measures to explore the association between acute post-intervention cognitive enhancement and changes in peripheral and central blood perfusion. In this respect, Massee et al. (47) showed improvements in a serial subtraction task after the acute administration, in healthy subjects, of a cocoa bar containing 250 mg of flavanols. However, this study failed to demonstrate significant concomitant cardiovascular improvements after treatment. Conversely, a pilot study in healthy young

TABLE 2 | Summary of the studies examining the effects of acute administration of cocoa flavanols on cognitive performance.

Reference	Participants	Flavanols amount	Cognitive measures	Principal findings
Scholey et al. (44)	30 subjects (13 males, 17 females; 18–35 years)	Dairy based cocoa drinks containing 994, 520, and 46 mg flavanols (control)	Rapid Visual Information Processing Task, Serial Threes Subtraction, Serial Sevens Subtraction	Improvements in Serial Threes performance following 994 and 520 mg flavanols compared to 46 mg. Improved visual information processing performance after 994 mg flavanols
Field et al. (45)	30 subjects (8 males, 22 females; 18–25 years)	Dark chocolate containing 773 mg flavanols and white chocolate (control)	Choice Reaction Time, Visual Spatial Working Memory Test	Improvements in spatial working memory performance and choice reaction time in the flavanol condition
Pase et al. (46)	71 subjects (40–65 years; 34% males)	Chocolate drinks containing 500, 250, or 0 mg flavonoids (placebo)	Mood assessment, Cognitive Drug Research Battery measuring working memory, episodic memory, speed of memory and attention	No differences in cognitive performance between placebo and treatment groups
Massee et al. (47)	40 subjects (18–40 years; 16% males)	Cocoa bars containing 250 or 0 mg flavanols (placebo)	Mental fatigue assessment, Serial Threes and Serial Sevens, Rapid Visual Information Processing Task	Improvements in serial sevens subtraction performance and self-reported mental fatigue in the flavanol group. No significant effect for cardiovascular measures
Decroix et al. (48)	12 subjects (all males; mean age: 30 years)	Cocoa drinks containing 903 and 15 mg flavanols (placebo)	Stroop task	Increased cerebral blood oxygenation (NIRS) during the task with no significant behavioral effect
Grassi et al. (27, 28)	32 subjects (16 males, 16 females; mean age: 25.3 years)	Chocolate bars containing 520 mg (flavanol-rich) and 88.5 mg flavanols (flavanol-poor)	Psychomotor Vigilance Task, 2-back task	Improvements in 2-back task accuracy after flavanol-rich treatment following a night of total sleep deprivation, in females. In the sleep condition, correlation between 2-back accuracy and FMD for the whole sample

FMD, flow-mediated dilation; NIRS, near-infrared spectroscopy.

participants showed that a single higher dose of flavanol-rich cocoa (516 mg) caused a significant global increase in CBF 2 h post-treatment (31). This result suggests an increased brain perfusion mediated by NO-dependent vasodilation, after cocoa consumption (31, 49).

In line with this result, in a recent near-infrared spectroscopy study, the administration of 903 mg cocoa flavanols increased cerebral oxygenation during the Stroop task performance in comparison to placebo, again suggesting increased NO-mediated vasodilation. Conversely, cocoa flavanols had no acute effects on serum BDNF levels (48). Therefore, these data support the idea that flavanols-induced acute changes in brain perfusion could underpin immediate cognitive-enhancing effects. However, the increased cerebral blood oxygenation was not paralleled by concomitant effects on cognitive performance (48). The young age of participants may have contributed to the lack of behavioral effects. This could be particularly relevant in this study, as participant performed a very short, relatively simple cognitive task. Since cocoa has been shown to affect cognitive performance in healthy individuals undergoing sustained effortful cognitive processing (44), these results open the possibility that, at least in healthy young adults, a high level of cognitive demand is needed to uncover the subtle immediate behavioral effects of flavonoids.

Accordingly, it could be argued that flavanols may be more effective in sustaining performance in highly demanding contexts or in presence of impaired physiological functions (e.g., cardiovascular risk factors). In this respect, we recently evaluated, in healthy young subjects, the effects of acute flavanol-rich chocolate administration on cardiovascular and cognitive function known to be specifically impaired by lack of sleep (27, 28). To this purpose, participants underwent two baseline sessions, performed after undisturbed nocturnal sleep, and two experimental sessions, after one night of total sleep deprivation. Subjects were randomly assigned to consume, 90 min before each testing session, flavonoid-rich dark chocolate (520 mg total flavanols) or flavonoid-poor chocolate (88.5 mg total flavanols). Cognitive assessment included the Psychomotor Vigilance Task, as a measure of behavioral alertness, and a 2-back working memory task. A single flavanol-rich cocoa administration was effective at counteracting the vascular impairment observed after a night of total sleep deprivation. Moreover, in the female subsample, cocoa flavanols also counteracted cognitive impairment specifically induced by sleep loss, leading to improvements in the accuracy measure of the working memory task. This result supports the idea of a specific effectiveness of cocoa flavanols in sustaining higher order cognitive performance during sustained effortful processing in healthy individuals. Interestingly, in the whole sample higher flow-mediated dilation levels, indicative of improved endothelial function, were related to better performance accuracy. Thus, although direct CBF measurements were not included, the results indicate that a single flavanol-rich chocolate administration could exert beneficial effects on cognitive performance through the capability to induce acute changes in peripheral and central blood flow *via* NO-dependent vasodilation.

Altogether, investigations on acute cognitive-enhancing effects of cocoa flavanols support the effectiveness of cocoa at sustaining cognitive performance, although with mixed results. Quantity and bioavailability of the consumed cocoa flavanols, together

with the length and cognitive load of the cognitive assessment, represent crucial factors that may significantly impact on the experimental outcomes.

CONCLUSION

In recent years, in the context of an increased interest in the modulatory effects of food constituents on human health, cocoa flavanols have been suggested to display a variety of beneficial biological actions, including neuroprotection and cognitive modulation.

At present, the limited number of studies investigating cocoa flavanols intake and cognitive performance has produced mixed results. Indeed, physiological responses to flavonoid supplementation such as vasodilation, both at peripheral and central levels, have been consistently replicated; conversely, cognitive findings are not as unequivocal. When trying to account for such discrepancies, several methodological differences should be considered in dose, form, and timeframe of the cocoa flavanols administration, as well as in length and cognitive load of the experimental tasks. All these variables could have a remarkable impact on physiological and behavioral results and are likely to partly explain discrepancies among results.

Nevertheless, the evidence accumulated so far suggests that cocoa flavanols administration can be effective at sustaining cognitive performance, leading to improvements in measures of general cognition, attention, processing speed and memory. Beneficial cognitive effects of regular flavanols intake, particularly in patients at risk, are presumably mediated by direct neuroprotective actions as well as improvements in cerebrovascular and metabolic functions. Furthermore, acute administration of cocoa flavanols could result in immediate cognitive-enhancing effect, sustaining performance particularly in cognitively demanding conditions, including fatigue and sleep loss.

Altogether, research on the effects of cocoa and chocolate on human cognition, although at its preliminary stage, converges at pointing to cocoa as a new interesting nutraceutical tool to protect human cognition and counteract different types of cognitive decline, thus encouraging further investigations. Future research should be addressed to the identification of sensitive experimental measures capable of detecting flavanol-induced subtle changes in cognitive performance. Moreover, the characterization of appropriate dose, timing, and form of flavanols intervention required to reach beneficial effects in different populations, as well as the inclusion of fully matched placebo controls, are needed. Undefined remain also the effects of both acute and chronic administration of cocoa flavanols on chronic sleep deprivation and shift work, situations that may have a pronounced clinical impact. To elucidate immediate and long-term neuromodulatory properties of cocoa, future investigations should be ideally designed to include neuroimaging techniques in conjunction with cognitive and physiological measures.

AUTHOR CONTRIBUTIONS

VS wrote the manuscript. VS and MF conceived and organized the structure of the review. DT contributed to write the first draft of the manuscript. DT, GD, LG, and MF contributed to the critical revision of the paper and approved the final manuscript for the publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 09 June 2017
doi: 10.3389/fimmu.2017.00677

Cocoa and Dark Chocolate Polyphenols: From Biology to Clinical Applications

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It is well known that cocoa and dark chocolate possess polyphenols as major constituents whose dietary consumption has been associated to beneficial effects. In fact, cocoa and dark chocolate polyphenols exert antioxidant and anti-inflammatory activities switching on some important signaling pathways such as toll-like receptor 4/nuclear factor κB/signal transducer and activator of transcription. In particular, cocoa polyphenols induce release of nitric oxide (NO) through activation of endothelial NO synthase which, in turn, accounts for vasodilation and cardioprotective effects. In the light of the above described properties, a number of clinical trials based on the consumption of cocoa and dark chocolate have been conducted in healthy subjects as well as in different categories of patients, such as those affected by cardiovascular, neurological, intestinal, and metabolic pathologies. Even if data are not always concordant, modifications of biomarkers of disease are frequently associated to improvement of clinical manifestations. Quite interestingly, following cocoa and dark chocolate ingestion, cocoa polyphenols also modulate intestinal microbiota, thus leading to the growth of bacteria that trigger a tolerogenic anti-inflammatory pathway in the host. Finally, many evidences encourage the consumption of cocoa and dark chocolate by aged people for the recovery of the neurovascular unit.

Keywords: anti-inflammatory activity, cocoa, dark chocolate, flavanols, nitric oxide, polyphenols, reactive oxygen species, transcription factors

OPEN ACCESS

Edited by:
Lorraine M. Sordillo, Michigan State University, United States

Reviewed by:
Margarida Castell, University of Barcelona, Spain
Nicolò Merendino, Università degli Studi della Tuscia, Italy

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Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Immunology

Received: *04 April 2017*
Accepted: *24 May 2017*
Published: *09 June 2017*

Citation:
Magrone T, Russo MA and Jirillo E (2017) Cocoa and Dark Chocolate Polyphenols: From Biology to Clinical Applications. Front. Immunol. 8:677. doi: 10.3389/fimmu.2017.00677

INTRODUCTION

Polyphenols represent a class of natural products that are very spread in the plant kingdom. Mostly, fruits, vegetables, and cereals are considered as major sources of dietary polyphenols, which human beings assume with food. In this context, Mediterranean diet (MED) represents an healthy nutritional regimen based on the consumption of extra virgin olive oil, fruits, vegetables, cereals, legumes, nuts, and seeds plus moderate intake of red wine (1, 2). It has been reported that MED is highly protective against chronic low-grade inflammation, and, in the case of atherosclerosis, stabilizes atheromatous plaques (3). Another study has emphasized the important role played by resveratrol, a non-flavonoid compound contained in red wine, to induce formation of sirtuins (Sirt) which, in turn, exert potent anti-aging effects (4). The MOLI-sani project has documented that in a large prospective cohort study of 24,325 Italian people MED reduced levels of glucose, lipids, C reactive protein (CRP), blood pressure (BP), and 10-year cardiovascular risk (5). Quite interestingly, Morabito and associates (6) have demonstrated that polyphenols contained in fruit juices prevent the post-prandial metabolic stress in humans as well as inflammatory disease outcome.

Taken together, all these are general consideration on dietary polyphenols effects and for more details on their chemical structure and functions, readers are referred to Ref. (7, 8).

With special reference to cocoa, polyphenols are constituents of the beans and their derivatives from the *Theobroma cacao* tree. Cocoa liquor is the paste derived from cocoa beans, the so-called nibs, and it is composed by non-fat cocoa solids and cocoa butter (9). Instead, cocoa powder is obtained by getting rid of some of the cocoa butter from the liquor. Finally, chocolate results from the combination of cocoa liquor with cocoa butter and sugar.

With regard to lipids, cocoa butter contains both monounsaturated and saturated fatty acids (FAs) (10). Oleic acid is the major monounsaturated FA that is present in similar amounts to those contained in the olive oil (10). Conversely, palmitic and stearic acids represent the main saturated FAs. However, stearic acid has been found to be anti-atherogenic, also accounting for one-third of the lipids contained in cocoa butter (11).

Fibers are present in cocoa beans, and their consumption has been shown to improve the low density lipoprotein (LDL):high density lipoprotein (HDL) ratio (12), also reducing risk of type 2 diabetes (13).

Among minerals, magnesium, copper, potassium, and iron are present in cocoa and chocolate in significant amounts (14). Magnesium, copper, and potassium exert a cardio protective role (15–17), while iron, mainly present in dark chocolate, contributes to the 25% of the U.S. recommended dietary allowance for middle-aged man, thus preventing anemia outcome (18).

Finally, with regard to polyphenol composition, catechins, anthocyanins, and proanthocyanidins are the most abundant class of compounds contained in cocoa powder (19). In particular, flavanols are presented as monomers, e.g., monomers (+)− and (−)− isomers of catechin and epicatechin (epi), and, in addition their derivatives are build-up of epi subunit polymers (proanthocyanidins) (19–21). Minor components are represented by phenolic acids, flavonols, and their glycoside, some stilbenes, simple phenol, and isocoumarin (22–24). Among anthocyanins, cyanidins-3-α-L-arabinoside and cyanidin-3-β-D-galactoside are the most represented compounds (18). (−)− epi accounts for the 35% of the total phenolic content, while (+)− catechin, (+)− epigallocatechin and gallocatechin are minor constituents. Procyanidins are present as dimers, trimers, and oligomers of flavan-3, 4-diols, linked by 4 → 8 or 4 → 6 bounds (20, 25, 26).

As far as bioavailability of cocoa is concerned, monomeric and polymeric flavanols are rapidly absorbed in the small intestine upon ingestion with a maximal plasma concentration after 2 h from intake (27). Elimination of flavanols is completed after 6 h from ingestion (28). However, absorption not only depends on flavanol chemistry but also on their structural isomerism and stereoisomerism (29). Also, the range of polymerization seems to determine their bioavailability (30). Once absorbed under form of monomers, flavanols are transformed into metabolites detectable in plasma and urine, such as (−)− epi as sulfate, glucuronides, or methyl conjugated forms (31, 32). On the other hand, polymers and monomers of unabsorbed flavanols undergo colonic microbiota catabolism, and valero lactones and valeric acids represent the so-called first-step microbiota-derived catabolites (33, 34). Instead, a number of phenolic acids constitute intermediate and last-step catabolites (33, 35–37). Of note, a part of unabsorbed flavanols is excreted into the feces (33, 38, 39). In this framework, it is worthwhile emphasizing that microbiota-derived metabolites of ingested polyphenols in view of their healthy effects are object of intensive investigation (40, 41). For instance, with special reference to consumers of cocoa polyphenols, a comparison between regular consumers of chocolate and low consumers has clearly shown a significant difference in terms of metabolite profiles (42).

This review will illustrate the major effects of cocoa and dark chocolate consumption in health and disease and possible cellular and molecular mechanisms of action involved also in relation to putative therapeutic implications.

EFFECTS OF COCOA AND DARK CHOCOLATE ON THE CARDIOVASCULAR SYSTEM

The cardioprotective effects exerted by polyphenols have been published long ago (43, 44). Since then, a series of studies supported the protective effects of cocoa and chocolate intake on the cardiovascular system. First of all, there is robust evidence that consumption of flavanol-rich cocoa leads to beneficial effects in healthy individuals. A study has documented that vasodilation was the main effect observed as a consequence of nitric oxide (NO) release following cocoa ingestion (45). In this connection, improvement of endothelial function was higher in older (>50 years) than in younger (<50 years) healthy individuals, as assessed by flow-mediated dilation (FMD) measurement (46). In this context, *ex vivo* flavanol-induced relaxation of preconstricted rabbit aortic rings, as well as *in vivo* increase in FMD were abrogated by inhibition of NO synthase, thus supporting the role of NO in the amelioration of endothelial function (21).

In an acute study, the effects of dark chocolate and white chocolate were evaluated in healthy participants monitoring variations of FMD and BP (47). Actually, dark chocolate was more effective than white chocolate in lowering the above mentioned parameters. In the second phase of the study, sugar-free but not sugared cocoa consumption led to a significant reduction of both systolic and diastolic BP in comparison with placebo (48). In similar trials, the effects of consumption of solid dark chocolate on endothelial function of healthy individuals were determined (49). A significant increase in FMD was observed in high-flavonoid intakers of dark chocolate (46 g) when compared to low flavonoid intakers once a day for 2 weeks. Shiina et al. (50) reported in healthy individuals an increase of coronary flow velocity reserve following consumption of 45 g of flavonoid-rich dark chocolate in comparison to flavonoid-free white chocolate. All these evidences are confirmed by studies conducted in Kuna islanders who commonly ingest higher amounts of cocoa than mainlanders (51, 52). In fact, in the former urinary flavanol metabolites were more elevated than in the latter, and this evidence correlates with low rate of cardiovascular disease (CVD), diabetes, and cancer in islanders.

On the other hand, in subjects at risk for CVD, consumption of cocoa led to results of clinical value, such as increase in nitrosylated and nitrosated species and FMD (53). Same results were

obtained in smokers who consumed high flavanol cocoa beverages for 7 days (54). FMD increase was maintained on each day after a washout of 1 week. Also in diabetics, chronic consumption of cocoa three times a day for 30 days, containing 321 mg of flavanols, led to higher increase in FMD in comparison to the low-flavanol cocoa group (55). Conflicting results have been obtained in patients with coronary artery disease (CAD). For instance, in a study involving 40 CAD patients who consumed a chocolate bar and cocoa beverage, containing 444 mg of flavanols for 6 weeks, no significant differences were seen in terms of endothelial function measurement and high-sensitivity CRP, oxidized LDL, lipids, glucose, and insulin determination in comparison to placebo-treated patients (56). Conversely, in another research, 16 CAD patients were divided into 2 groups, one receiving high flavanol cocoa (375 mg) and another one consuming low flavanol cocoa (9 mg) 2 times a day for 30 days, randomly (57). More significant results were observed in the high flavanol group in comparison to the low flavanol group in terms of increase in both FMD and mobilization of circulating angiogenic cells and decrease in BP. Furthermore, other two studies have clearly demonstrated the effects of daily chocolate consumption on coronary circulation. In heart transplanted individuals, intake of 40 g of dark chocolate led to increase in coronary artery diameters and endothelium-dependent coronary vasomotion 2 h after intake of flavonoid-rich dark chocolate with a significant decrease in platelet aggregation (58). Parallely, increase in serum epi was recorded.

With regard to the mechanisms of action of NO on endothelium function, there is evidence that it causes arterial vasodilation in healthy subjects, while in individuals at risk for cardiac disease NO response is decreased while oxidative stress is increased (59–61). Furthermore, NO exerts anti-inflammatory activity *in situ* by decreasing leukocyte recruitment and platelet aggregation (62). In this framework, our own studies have clearly demonstrated that human healthy peripheral monocytes are great producers of NO when *in vitro* stimulated with red wine polyphenols (63). Then, in addition to endothelial cells, which are another source of NO, also monocytes contribute to the NO-mediated vasodilation and cardioprotection.

Taken together, these evidences clarify why polyphenols, even including those from cocoa and dark chocolate, are able to improve endothelial function in health and disease *via* NO release.

With regard to the mechanisms of NO release, all polyphenols regardless of their sources are able to activate endothelial NO synthase (eNOS), thus leading to NO generation (64). The administration of pure (−)− epi seems to reproduce the effects of cocoa-induced synthesis of NO on human coronary artery endothelial cells through eNOS activation *via* phosphatidylinositide 3-kinases/protein kinase B, also known as AKT/protein kinase A and Ca^{2+}-calmodulin (CaM)/CaM K II pathway (64). Moreover, by inhibiting phospholipase C, evidence has been provided for the existence of a putative epi receptor on the cellular plasmalemma (64).

Once released, NO is able to activate the soluble guanylate cyclase in the smooth muscle cells and platelets with increase of cyclic guanosine monophosphate (cGMP) (65, 66). The subsequent inhibition of calcium flux and decrease of cytosolic calcium concentration give rise to smooth muscle cell relaxation and platelet aggregation inhibition (see also next paragraphs) (65, 66). Furthermore, cGMP is able to increase cyclic adenosine monophosphate (cAMP), which, in turn, activates prostacyclin (65–67). Quite interestingly, prostacyclin acts as a vasodilator in synergy with NO, thus contributing to protection from thrombosis. Furthermore, the anti-inflammatory and vasoprotective properties of prostacyclin are enhanced by its capacity to decrease plasma leukotrienes (68, 69).

Some of the major vasoprotective effects of cocoa and dark chocolate are illustrated in **Figure 1**.

Finally, NADPH oxidase seems to be another target of NO activity. In fact, cocoa polyphenols reduce levels of NADPH oxidase, which generates O_2^- that, in turn, scavenges NO. Therefore, its inhibition increases levels of NO (70, 71).

Another important target of polyphenol-rich cocoa is represented by platelets. First of all, platelets can *per se* release NO under influence of flavanols (72), thus contributing to vasodilation. Cocoa-mediated inhibition of platelet aggregation has been shown to depend on the decrease of thromboxan (TX) A2 synthesis

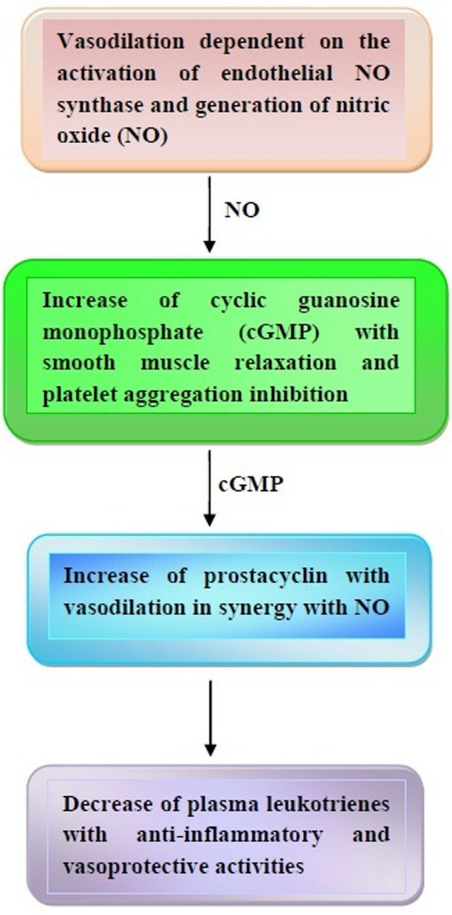

FIGURE 1 | Major effects of cocoa and dark chocolate on the cardiovascular system. In response to cocoa and dark chocolate ingestion, a cascade of events takes place based on the nitric oxide (NO) and cyclic guanosine monophosphate (cGMP)-induced vasodilation and prostacyclin-mediated anti-inflammatory effects. Other details are contained in the text.

and antagonism at TXA2 receptors (73–75). Furthermore, other possible mechanisms of action are represented by the inhibition of platelet–leukocyte interaction since cocoa flavanols are able to inhibit CD62P expression on activated platelets (76–78). Of note, CD62P binds P-selectin glycoprotein ligand-1 on leukocytes, thus mediating the platelet–leukocyte interaction.

A series of studies have demonstrated the cocoa's platelet inhibitory effects in healthy individuals and in heart transplant patients (79–81) who had consumed cocoa or dark chocolate. Taking into account that platelet activation greatly contributes to the inflammation and thrombosis in the progression of CVD, their inhibition by polyphenol-rich diets, even including consumption of cocoa and dark chocolate, is of clinical relevance.

The cocoa-mediated decrease of BP can be ascribed to several mechanisms. Increase in NO may explain the anti-hypertensive effects of cocoa (82). In addition, there is also evidence that flavanols and flavonol are able to *in vitro* inhibit angiotensin-converting enzyme (ACE) activity (83, 84). ACE, in turn, acts on the renin–angiotensin system, cleaving angiotensin I into angiotensin II with release of vasopressin or aldosterone and anti-diuretic hormone and increase in sodium and water retention. ACE also inhibits bradykinin and kallidin, which act as vasodilators (85).

In terms of effects of cocoa on serum lipid profile, a number of studies have clearly demonstrated that consumption of cocoa leads to increase in HDL while lowering LDL (86, 87). The same holds true also in the case of ingestion of high-polyphenol chocolate (88). Basically, same results were reported in individuals fed cocoa beverages containing only cocoa powder. Furthermore, a meta-analysis study confirmed the ability of cocoa to reduce LDL cholesterol and total cholesterol in subjects at high cardiovascular risk (89, 90). Also, inhibition of LDL oxidation is another effect of both cocoa and dark chocolate consumption (89–92). Conversely, other studies failed to demonstrate significant differences in serum lipids between consumers of high-flavonoid chocolate and consumers of low-flavonoid chocolate (49, 93). Similarly, in other three studies, no effects of cocoa beverages on serum lipids were observed (94, 95).

COCOA AND DARK CHOCOLATE EFFECTS ON THE CENTRAL NERVOUS SYSTEM (CNS) AND BEHAVIOR

The beneficial effects of polyphenols on the CNS have extensively been described in human and animal studies. The majority of research has been conducted with polyphenols derived from soy, berries, wine, tea, and curcuma and much less from cocoa and chocolate (96). Also, flavonoids extracted from *Ginkgo biloba* have been reported to retard memory loss, dementia, and Alzheimer's disease (AD) progression. However, data are still controversial (97, 98). In a series of researches, the anti-inflammatory activity exerted by polyphenols on the CNS has been documented. Curcumin extracted from *Curcuma longa* root was able to reduce the production of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and reactive oxygen species (ROS) from primary astrocytes *in vitro* stimulated with 1-methyl-4-phenylpiridinium ion (MPP+) (99). Moreover, curcumin increased levels of IL-10 and glutathione. Curcumin also decreased levels of toll-like receptor (TLR)-4, as well as of NF-κB, interferon regulatory factor 3, MyD88, and TIR-domain-containing adapter-inducing interferon-β otherwise enhanced by MPP+ (100). Similarly, epi and resveratrol have been found to exert neuroprotective activity modulating TLR-4/NF-κB/signal transducer and activator of transcription (STAT) signaling pathways (100).

Others have reported that polyphenols can interact with some signaling pathways, such as mitogen-activated protein and phosphoinositide-3-kinase (PI3-kinase)/AKT, thus leading to gene expression and protein synthesis for long-term potentiation and long-term memory occurrence (101). Flavonoids modulate transcription factors *via* protein kinase inhibition (102), while inducing the expression of brain-derived neurotrophic factor (BDNF). This factor contributes to neurogenesis, synaptic growth, and neuron survival in certain learning and memory brain regions, such as the hippocampus and subventricular areas (103, 104). Another mechanism is based on the generation of NO that leads to vasodilation and increased cerebral blood flow and blood perfusion in the context of the CNS as well as of the peripheral nervous system (105, 106). Such an increased blood flow is able to supply oxygen and glucose to neurons, also getting rid of waste metabolites in the brain and sensory organs (107, 108) while stimulating angiogenesis in the hippocampus (109). The effects of cocoa flavanols on the brain are represented in **Figure 2**.

Different cocoa flavonoid effects on Parkinson's disease (PD) have been reported. In PD, death of neurons in *substantia nigra* depends on the generation of 5-S-cysteinil-dihydrobenzothiazine ROS mediated-effects (110). Quite interestingly, neuronal damage mediated by 5-S-cys-DA is dramatically mitigated by quercetin, hesperetin, and caffeic acid, which are derivatives of catechin and epi (110). Neuroinflammation is another hallmark of PD pathogenesis (111). Microglia response plays the major role in the progression of neuronal degeneration and, consumption of cocoa flavonoids, e.g., quercetin, leads to anti-inflammatory effects (112). In particular, quercetin behaves as certain kinase inhibitors that exert anti-inflammatory effects on glial cells (112), likely preventing excitotoxic death in neurons (113). In relevance to the above cited anti-inflammatory effects, evidence has been provided that fermented grape marc (FGM) polyphenols have the capacity to reduce *in vitro* release of granzyme B from healthy peripheral human cytotoxic T cells, thus lowering their neurotoxic potential (114). By analogy, cocoa polyphenols may exert similar neuroprotective activity.

Alzheimer's disease is characterized by an increased production of amyloid (A)β oligomers, which activate microglia with release of inflammatory mediators and neuronal death (115). In an *in vitro* model of human AD, cocoa polyphenolic extracts have been shown to exert not only antioxidant effects but also to afford neuroprotection (116). This last effect has been attributed to the activation of BDNF survival pathway either on Aβ plaque-treated cells or on Aβ oligomer-treated cells, thus, ultimately, leading to reduction of neurite dystrophy. Resveratrol, a non-flavonoid component of polyphenols (117), exhibited neuroprotective effects in AD. In fact, it promoted non-amyloidogenic breakdown of the amyloid precursor proteins and removal of neurotoxic Aβ peptides. It is likely that also cocoa polyphenols may exhibit

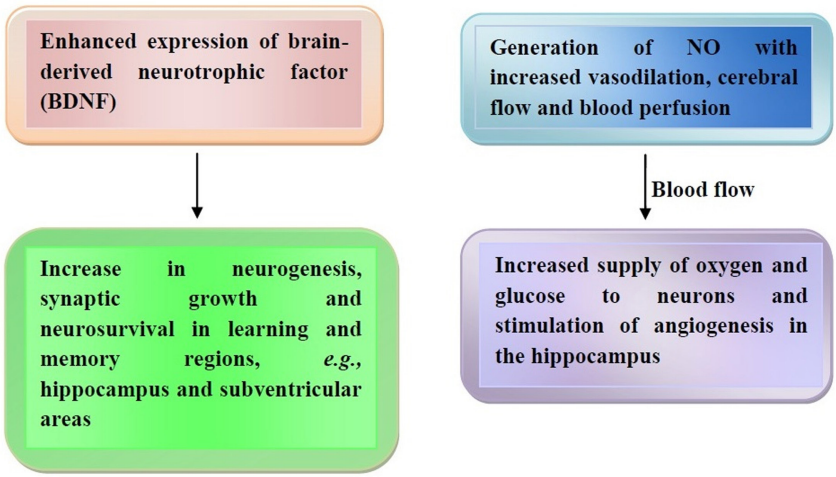

FIGURE 2 | Cocoa flavanol-mediated brain effects. Release of brain-derived neurotrophic factor (BDNF) with increased neurogenesis and neurosurvival (left panel) and nitric oxide (NO)-mediated increase of cerebral blood flow (right panel) are the major effects exerted by cocoa flavanols. Further details are illustrated in the text.

similar activities. Another protective mechanism mediated by cocoa polyphenols is the activation of NAD(+)-dependent histone deacetylase enzymes, termed Sirt (118). In particular, in the course of AD, reduced levels of Sirt1 upregulate NF-κB, which, in turn, trigger inflammation and enhances Aβ toxicity (119, 120).

Another experimental study based on the administration of dark chocolate to a non-transgenic AD obese model showed a reduction of hyperglycemia and cholinesterase activity in the hippocampal tissue homogenates and improvement of the cognitive performance (121).

Another neurotrophic effect of cocoa flavonoids is represented by their ability to increase cerebral blood flow in healthy young subjects, as assessed by functional magnetic resonance imaging (FMRI) (122). This effect was observed 3 h after cocoa consumption. Furthermore, such an increased blood flow to gray matter has been shown to account for angiogenesis as well as growth of new hippocampal neurons involved in the memory processing (110). In this context, evidence has been provided that increase in blood flow in the middle cerebral artery may account for protective effects in the course of dementia and stroke (123).

The effects of cocoa flavonols on PD and AD progression are represented in **Table 1**.

With special reference to the influence on behavior, a series of studies have demonstrated that palatable chocolate consumption is able to improve mood in a more significant manner than that performed by a non-palatable chocolate (124, 125). Palatability seems to be related to the chocolate-mediated release of opioids, such as β-endorphins in the hypothalamus (126), thus producing an analgesic effect (127).

Also, cognitive function has been shown to be improved by cocoa beverages with reduction of mental fatigue (128). However, others did not find any significant change of cognitive tests in comparison to placebo group in healthy old subjects who consumed cocoa-enriched beverages and dark chocolate (129).

Chocolate consumption seems to stimulate different brain areas, especially chemosensory areas, such as insula, prefrontal region, caudomedial and caudolateral orbitofrontal cortex (130). According to FMIR, a significant taste-related activation in the orbitofrontal and insular cortices was reported (131). Also, chocolate color modulates brain activity with significant reduction in theta activity. This implies reduced levels of attention and higher levels of distraction (132). Finally, the sight of chocolate generated more activation in chocolate cravers than non-cravers in the medial orbitofrontal cortex and ventral striatum (133).

TABLE 1 | Beneficial effects of cocoa flavanols on the progression of Parkinson's disease (PD) and Alzheimer's disease (AD).

PD	AD
Inhibition of 5-*S*-cysteinil-dihydrobenzothiazine-mediated neuronal damage (110)	Activation of brain-derived neurotrophic factor on amyloid (A)β plaque-treated cells or on Aβ oligomer-treated cells (116)
Anti-inflammatory effect mediated by quercetin on glial cells, behaving as certain kinase inhibitors, thus preventing excitotoxic death in neurons (112, 113)	Activation of NAD(+)-dependent histone deacetylase enzymes known as sirtuins (118)
	Reduction of hyperglycemia and cholinesterase activity in the hippocampus with improvement of cognitive functions (121)

EFFECTS OF COCOA AND DARK CHOCOLATE ON INTESTINAL INFLAMMATION

Over the past years, plant-derived polyphenols have been experimented in *in vitro* and *in vivo* models of intestinal inflammation in view of their anti-inflammatory potential (134, 135). Interesting results have been obtained *in vitro* treating Caco-2 cells with cocoa polyphenols (134). Such a treatment led to induction of prostaglandin E2 synthesis *via* cyclooxygenase (COX)-1 effect,

which may be involved in the maintenance of mucosal integrity. On the other hand, the murine model of dextran sulfate sodium (DSS)-induced colitis has been used for investigating the effects of polyphenol administration. For instance, administration of cocoa FGM-derived polyphenols to DSS-induced colitis mice led to a partial but significant abrogation of intestinal length reduction, while levels of TNF-α and IL-1β significantly dropped in inflamed colon homogenates in comparison to untreated colitis animals (136). Similar results have been documented by Pérez-Berezo and associates (137) in rats with DSS-induced colitis administered with a cocoa-enriched diet. Decrease of colonic cellular infiltrates was paralleled by reduction of serum TNF-α and colon inducible (iNOS) activity. However, despite the reported changes, no clinical improvement was recorded in rats. In a murine model of DSS-induced colitis, Andújar and associates (138) reported that administration of cocoa polyphenols mitigated symptomatology accompanied by reduction of neutrophil infiltration, NO generation, expression of COX-2 and STAT-1 and STAT-3 (138) as well reduction of IL-1β, IL-6, and TNF-α from peritoneal macrophages (138). These modifications of biomarkers were associated to improvement of colitis. However, no inhibitory effect of NF-κB was detected in the nuclear extract of colon. Conversely, cocoa consumption by healthy volunteers led to a significant reduction of NF-κB in peripheral blood mononuclear cells (PBMCs), thus suggesting an inhibitory effect on the release of pro-inflammatory cytokines (139).

In the light of these results, addition of polyphenols to enteral nutrition in patients with inflammatory bowel disease may be beneficial in view of their ability to induce phase II antioxidant and detoxifying proteins, thus preventing or improving the inflammatory status (140).

EFFECT OF COCOA AND DARK CHOCOLATE ON OBESITY

Evidence has been provided that cocoa administration to rats decreased visceral adipose tissue, thus changing the expression of genes, which are involved in the generation of enzymes and molecules for the occurrence of FA synthesis and thermogenesis in liver and white adipose tissue (141). In a study conducted in 12 females, dark chocolate smelling was assessed for evaluating appetite response (142). This led to a satiation response, which inversely correlated with ghrelin levels. Since ghrelin is involved in adiposity induction (143), one can conclude that chocolate may reduce appetite, preventing weight gain. Furthermore, evidence has been provided that flavonoids act on peroxisome proliferator-activated receptors (PPARs), thus behaving as agonists of PPAR-α and partial agonist of liver X receptor α (144–146). In addition, increased expression of PPAR-γ, which, in turn, increases expression of adiponectin and glucose transporter 4, is another mechanism elicited by cocoa flavonoid consumption (147). These events may lead to reduced lipogenesis, induction of lipolysis, and increase in adiponectin secretion. Adiponectin also reduces lipid deposition and insulin resistance, thus mitigating obesity.

These last mechanisms are depicted in **Figure 3**.

Another important function of cocoa flavanols related to obesity is the delay of LDL oxidation. For example, they decrease

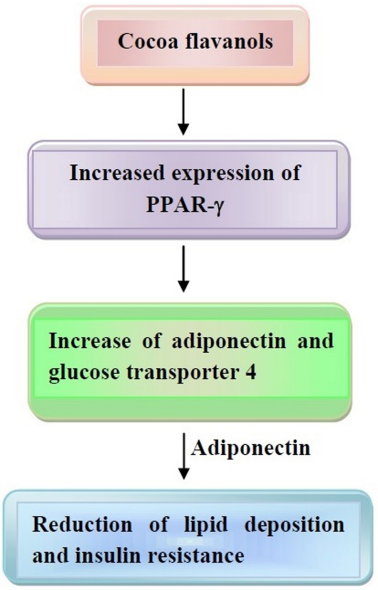

FIGURE 3 | Mechanisms of action of cocoa flavanols on obesity development. Increased expression of peroxisome proliferator-activated receptor (PPAR)-γ and adiponectin leads to reduction of lipid deposition and insulin resistance. Other details are present in the text.

F2-isoprostane levels, which represent *in vivo* markers of lipid peroxidation (148, 149). As result of LDL oxidation inhibition, decrease in atherosclerotic lesions in hypercolesterolemic rabbits treated with a diet enriched in cocoa powder for 24 weeks has been documented (150). Conversely, other researchers failed to confirm inhibition of LDL oxidation in rats treated with cocoa polyphenols for 2 weeks (151). In healthy human volunteers, evidence has been provided that cocoa consumption led to decrease of F2-isoprostane and thiobarbituric acid reactive substances, which are biomarkers of LDL oxidation and lipid peroxidation, respectively (152–154). Quite interestingly, in healthy humans, cocoa consumption increased plasma HDL cholesterol (92, 155), while decreasing plasma triglycerides (156–158). These results suggest the healthy benefits of cocoa consumption by changing the expression of genes involved in FA catabolism.

EFFECT OF COCOA AND DARK CHOCOLATE ON THE IMMUNE SYSTEM

Several studies of our group have been conducted on the effects of red wine or FGM-derived polyphenols on the immune cells. In murine models of asthma, FGM-derived polyphenols were able to mitigate symptomatology (159) when orally administered. In human studies, both red wine and FGM-derived polyphenols were able to induce *in vitro* activation of T regulatory (Treg) cells and release of IL-10, which, in turn, mediates anti-inflammatory activity (160, 161). FGM-derived polyphenols were also able to reduce the respiratory burst of healthy neutrophils and monocytes and abrogate basophil as well as rat mast cell degranulation *in vitro* (162, 163).

With special reference to cocoa flavanols, their *in vivo* administration to experimental animals has clearly demonstrated changes in the lymphoid organs. In rats, a diet based on 10% cocoa led to thymocyte differentiation and upregulation of thymic antioxidant defenses (164). Same dietary regimen increased splenic B cell percentage and decreased splenic T helper (h) cell frequency in rats (165, 166). In the gut of rats, changes in lymphomonocyte profile and Th cells frequency at Peyer's patches and mesenteric lymph node levels were noted following cocoa administration (165, 166).

The *in vitro* effects of cocoa on cytokine secretion are quite controversial. Increase in TNF-α, IL-1β, IL-6, and IL-10 from human PBMCs stimulated with flavanol fractions of cocoa have been reported (167). Conversely, following cocoa stimulation reduced production of TNF-α, monocyte chemoattractant protein-1, and NO by endotoxin-stimulated macrophages has been documented (168). In the same set of experiments, it was reported that cocoa polyphenols were able to modulate endotoxin activation of granulocytes (168). With special reference to Th cells, a cocoa diet in rats increased IL-4 production (a Th2 cytokine) from splenocytes (169). Secretion of interferon-γ from rat splenic Th1 cells was unmodified (166, 170), increased (171), or *in vitro* suppressed by cocoa extracts (172). Of note, cocoa diet did not modify rat IL-10 production (166, 173).

A series of experiments with procyanidin C1 using RAW 264.7 macrophages have clarified some important aspects of cocoa-mediated immunomodulation. In this respect, procyanidin C1 significantly enhanced levels of iNOS-mediated NO generation by activated macrophages (174). In addition, it increased the expression of the costimulatory molecules CD80 and CD86, thus potentiating antigen presentation to T cells (175). With regard to signaling pathways, procyanidin C1 was able to trigger phosphorylation of MAPKs, even including p38 and extracellular signal-regulated kinase as well as of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-α with subsequent activation of NF-κB. These findings were confirmed by using specific inhibitors of NF-κB and MAPK, which hampered pro-inflammatory cytokine production in the same experimental model.

Transforming growth factor (TGF)-β1 is a pleiotropic cytokine involved in tissue repair and regeneration (176, 177). Therefore, the effects of cocoa flavanols on the production of this cytokine were also evaluated in human subjects (178). Results pointed out that in healthy subjects cocoa consumption was able to regulate TGF-β1 production with an increase in low producers and a decrease in high producers (178). Of note, low levels of TGF-β1 were detected in patients with advanced atherosclerosis (178), while its excessive production has been shown to lead to cardiac fibrosis (179). Therefore, cocoa consumption by individuals with cardiovascular risk leads to modulation of TGF-β1 production, thus leading to protective functions.

Cocoa flavanols have been shown to regulate secretion of IL-5. Smaller molecular weight flavanol fractions were able to *in vitro* enhance IL-5 release by healthy human PBMCs, while larger molecular weight flavanol fraction decreased its release (180). The cocoa-induced increase of IL-5 may be indicative of a switch of the humoral immune response toward secretory IgA production, thus reducing the risk for caries and periodontal disease (180).

Finally, the effects of cocoa polyphenols on the composition of intestinal microbiota need to be mentioned. According to studies of Tzounis and associates (181, 182), Spencer and associates (183), and Massot-Cladera and associates (184), flavanol monomers and dimers are absorbed in the small intestine, while procyanidins are metabolized in the colon by the intestinal microbiota into a variety of phenolic acids, which are also absorbed. All absorbed products are metabolized in the liver and eliminated in the urine, and, partly, in the feces. In a human trial conducted on healthy volunteers, consumption of a high-cocoa flavanol beverage for 4 weeks, containing 494 mg flavanols, significantly increased the growth of *Lactobacillus* spp. and *Bifidobacterium* spp. in comparison to a low cocoa flavanol drink (182). Usually, these bacteria are able to maintain an anti-inflammatory status in the bowel with activation of Treg cells and production of IL-10 (185), thus suggesting that cocoa polyphenols may behave as prebiotics and trigger a tolerogenic pathway in the gut.

The effects of cocoa on microbiota are illustrated in **Figure 4**.

At the end of this section, one should mention the effects of (−)− epi, (+)− catechin, and dimeric flavonols on NF-κB, a transcription factor involved in immune cell activation.

The abovementioned compounds are able to inhibit NF-κB activation, and, in particular the phorbol mirystate acetate (PMA) DNA binding activity, thus resulting in IL-2 production decrease (185). Inhibition of binding activity is provoked by a blockade of the binding of active NF-κB to the DNA KB

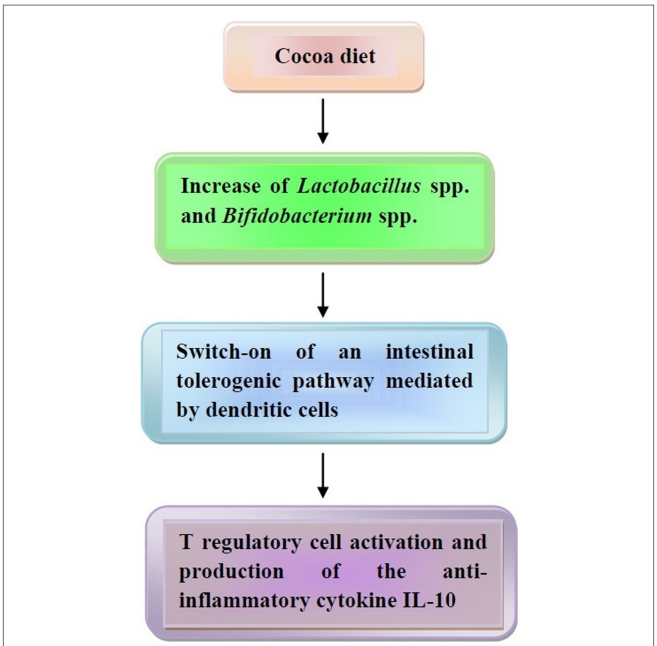

FIGURE 4 | The effects of coca-enriched diet on human microbiota. Cocoa diet modifies the intestinal microbiota, thus leading to a tolerogenic pathway with release of the anti-inflammatory cytokine interleukin (IL)-10. In the text, further details are illustrated.

motifs. Finally, pretreatment with flavanols leads to decrease of PMA-stimulated intracellular oxidants, which is an early event in NF-κB triggering.

CONCLUSION

There is wealth of evidences concerning the relationship between health status and integrity of vascular and neurological functions. As extensively described in the previous sections of this review, cocoa and dark chocolate-mediated induction of NO leads to vasodilation as well as inhibition of COX-2, CRP, and atherogenesis (186, 187). In addition, NO acts in concert with BDNF in order to modulate neural progenitor cell growth and synaptic metabolism for appropriateness of cognitive functions (188–190). Quite interestingly, release of NO at the thalamus level contributes to the adequate functioning of the neurovascular unit *via* increased blood flow and volume in the context of the brain (191, 192). Furthermore, polyphenols, even including those from cocoa, exert antioxidant effects, thus increasing neurological functions also preventing age-dependent damage (193). In synthesis, by analogy to other plant-derived polyphenols, cocoa flavanols may exert beneficial effects *via* activation of eNOS, inhibition of the NADPH oxidase and ROS production, downregulation of NF-κB, and regulation of MAPK and cAMP response element-binding protein pathways (194–197). In aging, especially neurological functions become deteriorated, and NO and aging seem to be interconnected. For instance, alterations of NOS have been detected in aging brain, thus influencing memory (96, 198, 199).

Conclusively, in the light of the above considerations, cocoa and dark chocolate-based diet may be beneficial in aged people for improvement of the neuro–cardiovascular connectivity.

AUTHOR CONTRIBUTIONS

All authors equally contributed to the compilation of the present review.

ACKNOWLEDGMENTS

This is a short text to acknowledge the contributions of specific colleagues, institutions, or agencies that aided the efforts of the authors.

FUNDING

This paper was supported by "Intervento cofinanziato dal Fondo di Sviluppo e Coesione 2007-2013-APQ Ricerca Regione Puglia Programma regionale a sostegno della specializzazione intelligente e della sostenibilità sociale ed ambientale-FutureInResearch."

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Nutrition

PERSPECTIVE
published: 26 September 2017
doi: 10.3389/fnut.2017.00043

Dark Chocolate: Opportunity for an Alliance between Medical Science and the Food Industry?

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Dark chocolate (DC) was originally introduced in human nutrition as a medicinal product consumable in a liquid form. Century-long efforts of food industry transformed this hardly appealing product into a valuable modern culinary delight with clear predominance of confectionery brands of DC on the market. However, current epidemiological data as well as multiple experimental and clinical observations reveal that DC consumption may have a profound effect on cardiovascular, central nervous systems, hemostasis, and lipid metabolism. However, despite of growing body of modern scientific evidence revealing medicinal properties of cocoa-based products, DC remains more gourmet culinary item than medicinal food product. Even today there are no clear dietary recommendations on consumption of cocoa flavonoids (flavanols) for health purpose. Clinical trials with DC rarely include monitoring of plasma flavanol concentration in volunteers. Moreover, there is no standardized assay or any quantitative requirements for flavanol content in the commercial brands of DC. High flavanol content is often sacrificed during manufacturing for a better taste of DC due to bitterness of cocoa flavonoids. All these problems including subsequently arising ethical issues need to be addressed by joint efforts of food industry and medical science. Moreover, application of microencapsulation technology in DC manufacturing, as well as molecular selection of best flavanol producers may drastically change bioavailability of DC bioactive ingredients and DC production technology. Nevertheless, only strict causative approach, linking possible health effect of DC to its bioactive ingredients considered as nutraceuticals, may change the current landscape in nutritional research related to cocoa-based products and create a trustworthy path for their medicinal use.

Keywords: dark chocolate, cocoa flavanols, cardiovascular effects, biotechnology

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Nutritional Immunology, a section of the journal Frontiers in Nutrition

Received: *18 April 2017*
Accepted: *06 September 2017*
Published: *26 September 2017*

Citation:
Petyaev IM and Bashmakov YK (2017) Dark Chocolate: Opportunity for an Alliance between Medical Science and the Food Industry? Front. Nutr. 4:43. doi: 10.3389/fnut.2017.00043

INTRODUCTION

Dark chocolate (DC) was introduced into the human diet in South America at least 3,000 years ago and was brought to Europe by Christopher Columbus (1). The first specimens of the cocoa tree were transported to Spain by the end of the sixteenth century and were classified as *Theobroma cacao* by Carl Linnaeus in 1753 (2–4). In Pre-Columbian cultures, cocoa products were believed to be of divine origin and were consumed exclusively in beverage form as a remedy for fatigue, indigestion, and gastrointestinal disorders (5, 6). European dietary culture developed on Christian traditions was somewhat suspicious and xenophobic toward foreign dietary innovations, opposing the wide introduction into the human diet of coffee, tea, and cocoa beverages during the

later Medieval and Renaissance periods (3). Nevertheless, the medicinal properties of cocoa as an expectorant, diuretic, anti-depressive, weight gaining stimulant, and aphrodisiac were proclaimed in the sixteenth and seventeenth centuries (7, 8). As a result, multiple modifications of cocoa bean processing (drying, heating, and pulverization) and preparation (addition of sugar and spices) took place in small pharmacies and food shops in order to improve the bitter taste of cocoa beverages (8). As reported in 1662, British soldiers stationed in Jamaica consumed a solidified cocoa bean paste containing sugar, anise, vanilla, cinnamon, and almonds, marking the birth of DC (3). Altogether, these modifications drastically changed the taste of cocoa-based products transforming them from a medicinal product to the culinary delight.

Despite centuries of research into the health benefits of DC, most of the studies conducted in the past hardly meet the requirements of modern medical science and appear inconclusive. However, stricter guidelines for clinical trials, advances in medical statistics, and the enormous progress in molecular medicine have very recently shaped a solid scientific background to the medicinal use of DC. There are multiple questions and challenges relating to the habitual consumption of DC by individuals with risk of cardiovascular disease (CVD) and CVD patients. Many of these can be successfully addressed and resolved by the joint efforts of modern medical science and the food industry.

THE DARKER THE BETTER

Cocoa solids are intermediates of chocolate manufacturing, forming after cocoa butter extraction from the cocoa beans. Cocoa solids, called otherwise cocoa powder, confer a dark color to DC (9). In recent times the food industry produces three types of chocolate: (a) DC, prepared mostly from cocoa bean solids (up to 80% of total weight) with the addition of cocoa butter; (b) milk chocolate, derived from high-fat milk with additions of sugar and low amounts of cocoa bean solids (<10% of total weight); and (c) white chocolate, based on cocoa butter, milk, and sugar with no cocoa solids (9, 10). Most of the health benefits are attributable to the consumption of DC, while milk and white chocolate reportedly have no considerable beneficial impact on health (9–11). The overwhelming majority of food science reports have been focused on improvement of taste, texture, appearance, and shelf life of cocoa-based products, whereas the physico-chemical characteristics of chocolate predetermining its health benefits remained largely unknown until the end of last century. However, emerging pieces of evidence related to the health benefits of cocoa products, in particular the effects of DC on cardiovascular health, paved the way for studies focused on identifying the DC bioactive compounds.

Cocoa beans contain more than 300 identifiable chemical compounds (12). Many other substances appear in the DC matrix during fermentation, roasting, and processing of cocoa beans (13, 14). Scrupulous identification of the biologically active ingredients over the last decade has revealed that there are at least three groups of substances in cocoa beans with potential health effects. These are flavonoids (epicatechins and procyanids), theobromine/caffeine, and minerals—magnesium, iron, and zinc (**Figure 1**). Some other as yet unidentified compounds may contribute to the health benefits of DC (9, 15).

Although theobromine, caffeine, and minerals have distinct and independent effects on the cardiovascular system as discussed elsewhere, there is a general consensus in modern nutritional science that flavonoids (13, 15, 16) are the major group of bioactive compounds mediating the effects of DC in CVD. Since flavonoids are found predominantly in cocoa solids, cocoa-enriched DC is widely assumed to have a higher bioefficacy due to higher flavonoid content and, therefore, higher antioxidant activity (13, 15, 16). As a result, most of the current interventional studies are performed using DC containing up to 80% cocoa solids.

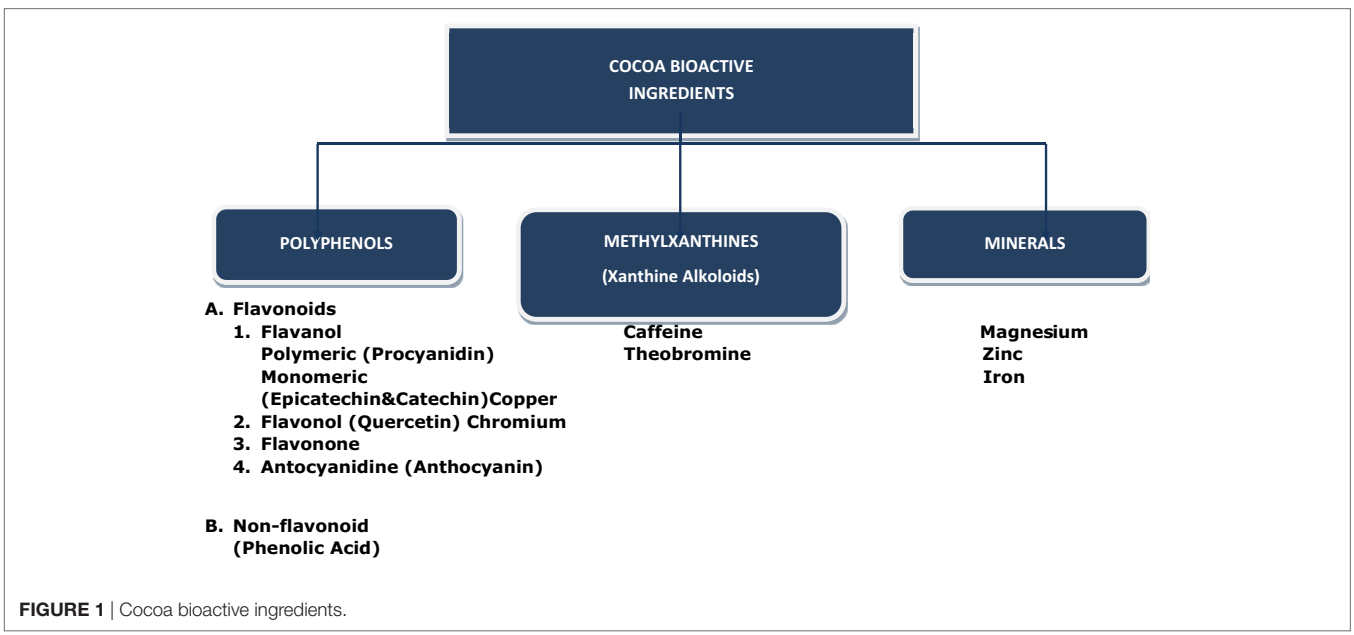

FIGURE 1 | Cocoa bioactive ingredients.

COCOA FLAVONOIDS: THEIR ACTION AND BIOAVAILABILTY

Cocoa flavonoids belong to a large class of dietary polyphenols present in fruits and vegetables. Flavonoids comprise about 12–18% by dry weight of the cocoa beans (17). Cocoa flavonoids confer a bitter taste to cocoa beans, making them revolting to human taste in unprocessed form. However, during manufacturing, the amount of polyphenols in cocoa beans may become significantly reduced thereby affecting the antioxidant properties of the final cocoa products (18). In their chemical nature, cocoa flavonoids are flavan-3-ols, this is why they are often referred to as flavanols. Flavanols are further categorized depending on their structure as catechin, epicatechin (monomers), and proanthocyanidin oligomers (19, 20). Proanthocyanidins make up >50% of the total flavonoid content in cocoa beans while catechins and anthocyanins comprise about 37 and 4%, respectively (20).

The molecular mechanism (**Figure 2**) behind the action of cocoa flavanols is primarily connected to their effect on the nitric oxide-mediated pathway, resulting in nitric oxide production *via* $Ca(2^+)$-independent eNOS activation/phosphorylation (21). Moreover, cocoa flavanols decrease degradation of nitric oxide and increase availability of L-arginin as a NO donor (21, 22). This universal mechanism is believed to cover most of the

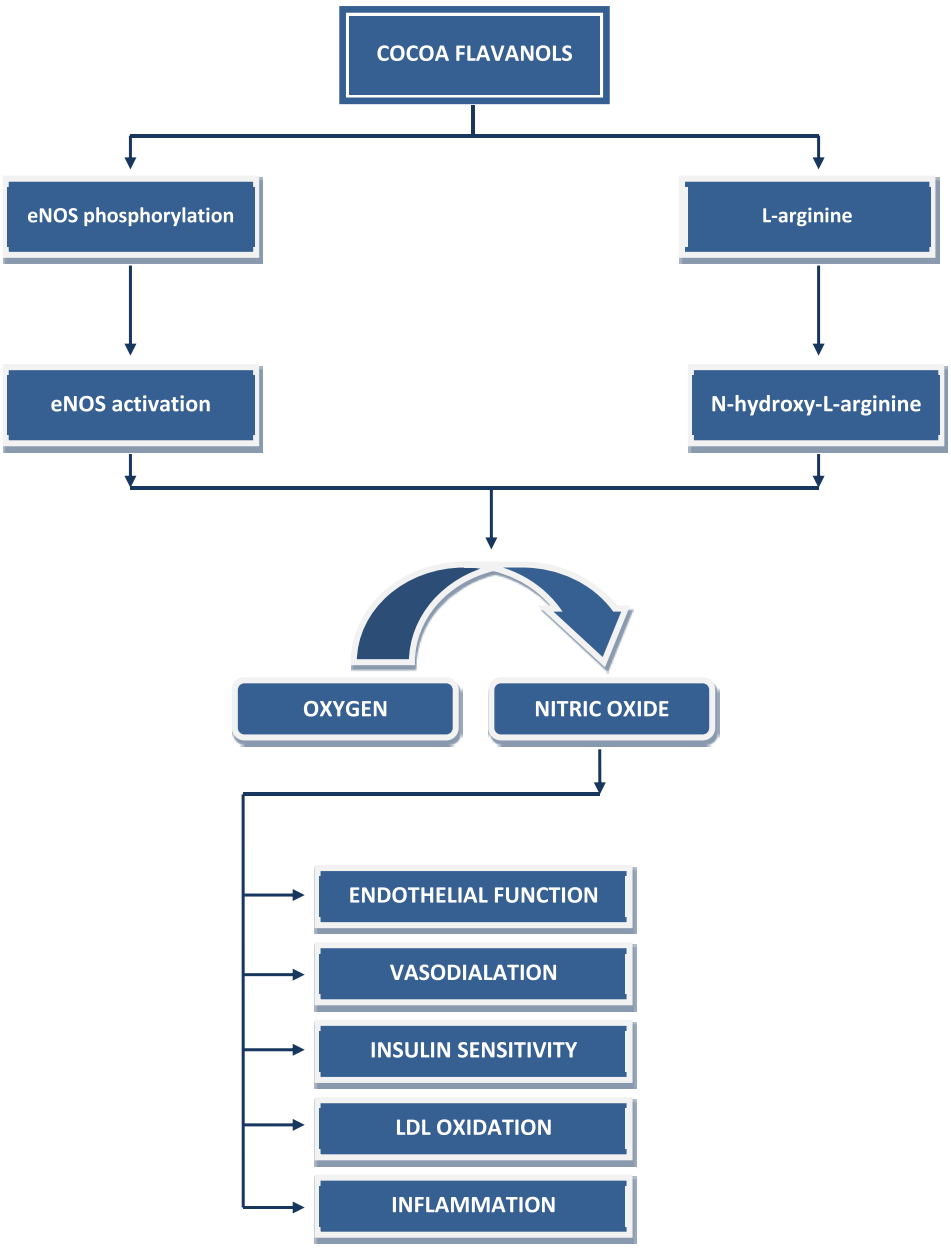

FIGURE 2 | Cocoa flavanols and nitric oxide pathway.

physiological effects of chocolate flavanols on the cardiovascular and nervous system (23).

In general, bioavailability of dietary flavonoids is fairly limited due to their short half-life, hydrophobicity, and susceptibility to oxidation (24). The absorption rate of cocoa flavanols is greatly influenced by interaction with the food matrix and co-ingested constituents (24, 25). Theobromine and epicatechins can be efficiently absorbed in the small intestine, whereas proanthocyanidins and oligomeric procyanidins are known to have a limited rate of intestinal absorption and become absorbable only in the colon after metabolic transformations by intestinal microbiota (24–26). Absorption rate of cocoa polyphenols is highly dependent on polymerization rate and unabsorbed portion of polyphenols undergo fecal elimination (26). A lipid environment promotes bioavailability of cocoa flavanols in the intestinal lumen due to possible micellization of polyphenols (27). Epicatechins can be detected in the plasma of volunteers 2 h after DC consumption and have a relatively high clearance rate comprising 2–4 h (28). Therefore, postprandial assessment of bioaccessible fraction of polyphenols in blood seems to be more valuable than information revealing total phenolic content in cocoa-based products.

DC AND CARIDOVASCULAR HEALTH

The first considerable epidemiological evidence suggesting a possible relationship between DC consumption and cardiovascular health came in 1992 from the Dutch Zutphen Elderly Study published in The Lancet. The study reported an inverse association between dietary flavonoid consumption, hypertension, and cardiovascular death rates in a large cohort of elderly volunteers (29). Extremely interesting information has been obtained from mortality pattern analysis in the Kuna Indians of Panama. Widespread consumption of cocoa flavanols consumed as a beverage in the Kuna Indians provides an intake of up to 900 mg/day of cocoa flavanols and coexists with a significantly reduced rate of CVD and diabetes-related mortality (30). Lower values for the systemic blood pressure over the lifetime of the Kuna Indians were reported by others (31) and providing another piece of evidence supporting epidemiological evidence for the Panama study. The subset of epidemiological research was strengthened by the IDEFICS study which reported that consumption of DC in childhood appears to affect clustered CVD risk factors in European populations (32). The inverse association between DC consumption and coronary heart disease was also reported in the general population of the USA (29, 30). However, some other results suggest that the inverse association may be much stronger for stroke than myocardial infarction (33, 34).

Interventional studies provide further evidence supporting the link between cardiovascular health and DC consumption. Recently performed meta-analysis (42 acute or short-term studies, 1,297 participants) indicates that regardless of the daily amount, regular consumption of cocoa-based products significantly improves flow-mediated dilation (FMD) and reduces systemic blood pressure (35). Although the blood pressure reducing effect of DC seems to be modest and rarely exceeds 3–4 mmHg, the degree of statistical significance and magnitude of changes in blood pressure reflect the amount of cocoa flavanol ingested and the duration of intervention (36, 37). However, the seemingly small impact of cocoa flavonoids on blood pressure could be translated into enormous public health benefits. In the best case scenario assessed by the Markov model (38), regular consumption of DC by individuals with metabolic syndrome may have a significant impact of cardiovascular health, reducing probability of cardiovascular events by 85 per 10,000 population. Despite some legitimate concerns regarding best case scenario epidemiological modeling, these results encourage further research.

Moreover, there is a growing number of smaller interventional studies suggesting that DC consumption may improve endothelial function, decrease arterial stiffness index and aortic pulse wave velocity, reduce platelet adhesion, and improve inflammatory parameters and brachial artery FMD in CVD patients (39–43). It is very encouraging that most of the clinical observations can be reproduced to some extent in experimental settings (10, 12, 26).

EFFECT ON NEUROLOGICAL AND COGNITIVE FUNCTIONS

In general, it is believed that dietary polyphenols may have a measurable and reproducible effect on neurological functions (26). However, the impact of cocoa bioactive compounds on the central nervous system (CNS) in particular remains poorly understood and requires further investigation. The ability of cocoa polyphenols to modulate nitric oxide production may represent a major mechanism explaining effects of DC on CNS. Vasodilation and subsequently increased cerebral blood flow promotes oxygen and glucose delivery to the neurons enhancing thereby their function and blood vessel formation in the hippocampus (44). The possible effects of DC on neurological functions may also originate from the antioxidant properties of cocoa polyphenols. Age-related cognitive deterioration and certain neurodegenerative disorders, including Alzheimer's and Parkinson's diseases, are closely related to the accumulation of reactive oxygen species (ROS) in the brain (45, 46). Therefore, the preventive effect of cocoa polyphenols on various molecular events initiated by ROS (inhibition of mitochondrial complex I, activation of caspase-3, and apoptosis) reported in different experiments might be explained by the anti-radical properties of bioactive components in cocoa (44, 46). Supplementation of mice with a diet containing cocoa polyphenols and theobromine has also been shown (47) to enhance cholinergic and catecholaminergic transmissions in brain and cause an increase in superoxide-dismutase activity, reversing thereby the metabolic changes associated with neurodegeneration. In mouse hippocampal sections, cocoa extracts have also been shown to reduce oligomerization of amyloid peptide β which is a keystone feature of Alzheimer's disease (48).

The effect of cocoa bioactives on signaling pathways in neurocytes may provide another rationale for linking DC to regulation of brain functions. Cocoa flavanols and methylxanthines have been shown to affect the activation cascade in

phosphatidylinositide 3-kinase/protein kinase B and target of rapamycin signaling pathways (49–51). These play a crucial role in synaptic function, neuronal growth, mechanisms of memory as well as in pathogenesis of neurodegenerative disorders (52). Anti-inflammatory action of cocoa bioactives is another feature which may contribute to the neuroprotective effect of DC (53). As reported, acute ingestion of DC may decrease concentration of adhesion molecules and 4-series leukotrienes in serum, nuclear factor κB activation in leukocytes and the expression of CD62P and CD11b on monocytes and neutrophils in volunteers (54). However, the reproducibility and magnitude of these changes needs to be further investigated. Other experimental studies suggest that cocoa flavanols may reduce cytokine production (55, 56). Suppressed cytokine production caused by DC/cocoa flavanol intake has been shown to be accompanied by inhibition of indoleamine 2,3-dioxygenase, an enzyme controlling tryptophan degradation (56). Therefore, increased availability of tryptophan for serotonin synthesis in the brain after DC intake may result in the enhancement of serotoninergic stimulation in neurons associated with improved mood and cognition (56). This pathway may establish a new molecular paradigm connecting DC consumption, mood, and cognitive function.

Clinical results relating to the effect of DC on mood and cognitive function are rather controversial. There is a certain degree of reproducibility in reports describing increased cerebral blood flow including brain areas responsible for cognition following cocoa flavanols intake (57, 58). However, it is still debatable if acute or chronic DC/cocoa flavanol intake has a measurable impact on mood and cognitive performance (59–61). The discrepancies in these results may reflect variability in the pre-existing health status of volunteers, different spectrum of flavanols used, as well as differences in the cognition assessment protocols.

CALORIC BURDEN VERSUS HEALTH BENEFITS

High sugar content and excessive caloric value of cocoa-based products lead negative perception of chocolate by dieticians (62). Indeed, some trials include ingestion up to 100 g of DC daily thereby providing up to 50 g of carbohydrates, 35 g of fat, and 600 cal (63). However, it was shown recently by the HELENA study that higher chocolate consumption is associated with lower fat deposition in European adolescents (64). In addition, cocoa polyphenols are shown to reduce biosynthesis and intestinal absorption of lipids and carbohydrates (65). Restoration of insulin sensitivity and the hypoglycemic action of DC has been reported by others (22, 66). Thus, the perception of chocolate as a nutritional factor promoting obesity is not justified at least in the case of DC.

However, high sugar content does not have to be an indispensable feature of DC. A similar perception of DC taste and sweetness can be achieved by replacing sugar with inulin, a prebiotic known for its ability to increase mineral absorption and reduce intestinal infection and colon cancer rates (67). Moreover, inulin controls the diversity of the intestinal microbiota by increasing representation of bifidobacteria (68). Therefore, an inulin-containing DC could become a valuable prebiotic product with a potential use in the management of CVD. Such an assumption becomes plausible in the wake of recent discoveries revealing the keystone role of the intestinal microbiota in CVD (69, 70).

HEPATIC BIOAVAILABILITY AS A NEW MODE OF COCOA FLAVANOL ACTION

Ingested polyphenols are known to be widely distributed among internal organs and tissues (digestive system, endothelial cells, heart, kidney, skin, and others) (71). Chocolate flavanols may directly influence some hepatic functions. In particular, catechins and proanthocyanidins are shown to affect lipid turnover in liver *via* SREBP pathway (72). Chocolate polyphenols have also distinct activity on insulin signaling and hepatic glucose production (73). Moreover, they increase hepatic ApoAI transcription and reduce oxidative stress often in dose-dependent manner in cultured hepatocytes (74, 75). These results are consistent with clinical observations revealing hypolipidemic action of cocoa polyphenols and their effect on glucose homeostasis (76). Therefore, the hepatic mode in cocoa polyphenol action becomes an emerging reality although is poorly understood and remains to be thoroughly investigated in future studies.

Several liver-targeted delivery systems have been recently developed (77). In particular, the lycosome hepatic delivery technology (78) employs a network of carotenoid receptors highly expressed on hepatocytes for targeted delivery of bioactive compounds to the liver. This microencapsulation protocol has been recently successfully applied for hepatic delivery of stilbene polyphenols (resveratrol), some hydrophobic peptides from whey protein, and simvastatin, an inhibitor of HMG-CoA reductase (79–81). Lycosome technology was also applied for DC production. As we reported (82), lycosome-formulated DC has a superior ability in the reduction of blood pressure and plasma lipids when compared with regular formulation of DC with similar cocoa flavanol content.

CONCLUSION: TRANSFORMING DC INTO NUTRACEUTICAL PRODUCT

Recently, DC has been credited with a health status in Europe (83). However, this declaration may be premature and imposes substantial obligations on both food industry and medical science to become a reality. As mentioned in the Lancet Editorial (84) almost decade ago and remains true today chocolate represents more food than medicine. Indeed, huge variability in flavanol content and cocoa processing, absence of clinically justified recommendations on cocoa flavanol/DC, as well as obvious predominance on the market of confectionary DC brands with unknown polyphenol content undermine nearest perspectives for medicinal use of DC. Currently, DC consumption remains astonishingly low even in the European countries, where flavonoid intake arising from chocolate consumption accounts for about 1/600 of daily flavonoid intake from all other sources (85).

Transformation of DC into nutraceutical product brings multiple ethical and financial challenges for manufacturers and medical science. It needs to start with agricultural practices and selection cultivars. Selection of *T. cacao* genomic variants with best flavor profile is under way (86). This approach becomes conceivable after recent genome-based categorization of *T. cacao* cultivates into 10 major clusters (87, 88). The genomic-based search for best flavanol spectrum producers using molecular biology holds enormous promise for medicinal use of DC. However, selection of cultivars suitable for medicinal use requires a precise knowledge of flavanol spectrum conferring bioefficacy for DC. Unfortunately, this information is hardly available now since most of the clinical trials do not provide scrupulous information revealing flavanol content neither in DC products nor plasma of patients before and after treatment.

Moreover, DC production technologies need to be reevaluated from the standpoint of modern food chemistry. Up to 90% of cocoa flavanols are known to be lost in the cocoa beans during post-harvest processing (88). Since taste and flavor improvement remains a major motive for innovations in chocolate manufacturing, reduced flavanol content resulting in diminished bitterness of DC often concurs with interests of chocolate manufacturers, limiting thereby bioefficacy of DC final product. However, taste and flavor should not be sacrificed for high flavanol content. As recently shown, microencapsulation of cocoa flavanols increases their bioavailability and masks sensory perception of flavonoids (25, 89). Modern microencapsulation technologies can be applied for cocoa polyphenols to enhance probiotic properties of DC (90, 91). All these challenges can be successfully addressed by joint efforts of food industry and medical science.

AUTHOR CONTRIBUTIONS

Both authors contributed equally into gathering of factual data and writing the manuscript.

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