Review Article

Cocoa: antioxidant and immunomodulator

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Cocoa, a product consumed since 600 BC, is now a subject of increasing interest because of its antioxidant properties, which are mainly attributed to the content of flavonoids such as (−)-epicatechin, catechin and procyanidins. Moreover, recent findings suggest a regulatory effect of cocoa on the immune cells implicated in innate and acquired immunity. Cocoa exerts regulatory activity on the secretion of inflammatory mediators from macrophages and other leucocytes in vitro. In addition, emerging data from in vivo studies support an immunomodulating effect. Long-term cocoa intake in rats affects both intestinal and systemic immune function. Studies in this line suggest that high-dose cocoa intake in young rats favours the T helper 1 (Th1) response and increases intestinal γδ T lymphocyte count, whereas the antibody-secreting response decreases. The mechanisms involved in this activity are uncertain; nonetheless, because redox-sensitive pathways control immune cell function, the action of cocoa flavonoids on modulating cell signalling and gene expression deserves investigation.

Cocoa: Antioxidants: Immunomodulation: Flavonoids

Cocoa, a product derived from the beans of the Theobroma cacao plant, has been consumed since 600 BC, first by ancient civilisations, such as the Mayans and Aztecs. Cocoa consumption in Europe dates from the 16th century when Hernán Cortés introduced it to the Iberian Peninsula; from there its use spread rapidly to Western Europe. Cocoa powder is a rich source of fibre (26–40 %), proteins (15–20 %), carbohydrates (about 15 %) and lipids (10–24 %; most, 10–12 %), and it contains minerals (for example, Ca, Mg, K) and vitamins (A, E, B and folic acid) (Table 1).

Bioavailability of cocoa flavonoids

The biological effects of flavonoids depend on the bioavailability of the compound. The various manners and rates in which flavonoids are absorbed have been recently reviewed. Flavan-3-ols show no changes after 40 min in the human stomach, indicating that flavonols and procyanidins are stable in the harsh environment of the digestive system. Gut flavonoid absorption basically depends on the chemical structure of the individual type. Monomeric flavonoids and certain dimeric and trimeric procyanidins are absorbed in the small intestine and are rapidly detected in the plasma, whereas large procyanidins are less efficiently absorbed, but may have an important local function in the gut, neutralising oxidants and carcinogenic compounds.

Abbreviations: PBMC, peripheral blood mononuclear cells; TCR, T cell receptor; Th, T helper.
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Cocoa has a potent antioxidant capacity as compared with other products, a quality related to its flavonoid content. Moreover, flavonoids can also be metabolised by colon microflora to phenolic acids, which are then absorbed.

The influence of the food matrix on flavonoid absorption has been an issue of discussion in the past few years, particularly after the publication of a study reporting decreased absorption of cocoa flavonoids owing to an interaction with milk proteins, which are present in most cocoa-derived products. Conversely, other studies in human subjects found no significant effects of milk on epicatechin absorption and the total amount of metabolites excreted after ingestion of a cocoa beverage. Nonetheless, differences in the excreted metabolite profile have recently been described, suggesting that milk components have an effect on cocoa flavonoid metabolism. Polysaccharides also seem to enhance flavonoid absorption, although the mechanism causing this effect remains uncertain.

Data on the distribution of flavonoid metabolites in tissues after cocoa intake are limited, even in experimental studies. Absorbed flavonoids are widely distributed and can be detected in many organs, including lymphoid tissues, at a concentration of nmol/g tissue (for reviews, see Manach et al. and de Boer et al.). Levels of flavan-3-ol metabolites have been found in rat liver and kidney over a 24 h period following flavonoid ingestion. In a study in rats and pigs, multiple tissues were found to accumulate quercetin metabolites, with high levels in rat lung and little accumulation in brain, spleen and white fat.

**Cocoa as an antioxidant**

Cocoa has a potent antioxidant capacity as compared with other products, a quality related to its flavonoid content.
the enhancement of antioxidant capacity was greatest 1–2 h after cocoa administration and gradually decreased thereafter to reach baseline levels at about 6 h post-ingestion. Plasma antioxidant capacity was not enhanced in blood collected more than 6 h after cocoa intake\(^{(48–50)}\), probably because of the short plasma half-life of flavonoids and their uptake in cells.

A cocoa-enriched diet increases the antioxidant capacity of cell tissues to varying degrees, with the activity in thymus > spleen > liver\(^{(50)}\), an effect that can be attributed to differing levels of flavonoid accumulation\(^{(26)}\). Cocoa boosts catalase and superoxide dismutase activity in rat thymus, but not in spleen and liver\(^{(50)}\). Although the exact mechanism remains to be established, enhancement of superoxide dismutase and catalase activity by cocoa could be due to direct neutralisation of enzyme substrates (\(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\), respectively) or to up-regulation of antioxidant enzyme
expression. Cocoa phenols could have an important role in these actions, since they are potent antioxidants able to induce enzymes such as superoxide dismutase and glutathione peroxidase(51).

Cocoa also improves antioxidant defences in experimentally induced oxidative stress. For example, the long-term intake of a cocoa-enriched diet containing 0.3 % polyphenols reduced lipid peroxidation in the plasma and liver of hypercholesterolaemic rats(52). The consumption of 100 g chocolate containing 0.2 % polyphenols for 2 weeks counteracted oxidative stress in soccer players, as was shown by reductions in plasma malondialdehyde and α-tocopherol increases(53).

A recent review of the impact of cocoa on the cardiovascular system has compiled all the interventional studies in human subjects published over the last 10 years. In these trials, cocoa intake was seen to reduce the risk of CVD by a combination of several effects, including improvements in antioxidant status (shown by decreases in oxidative stress biomarkers, such as thiobarbituric acid-reactive species and LDL oxidation), vasodilation, and inhibition of platelet activation and aggregation(54). Other reviews have examined the beneficial effects of dietary flavonoids on health(55–58).

**Cocoa as an immunomodulator**

Cocoa has exhibited promising regulatory effects on immune cells involved in innate and acquired immunity.

**In vitro effects of cocoa on immune cells**

*In vitro* studies have demonstrated the regulatory effects of cocoa on the secretion of inflammatory mediators from macrophages and other leucocytes. Ono *et al.*(59) and Ramiro *et al.*(60) found that flavonoid-rich cocoa extract decreases the secretion of TNF-α, monocyte chemoattractant protein-1 (MCP-1) and NO by lipopolysaccharide-stimulated macrophages (Table 2). The *in vitro* anti-inflammatory behaviour of cocoa differs from that of individual flavonoids. The influence of cocoa on macrophages is much stronger when cocoa is added before lipopolysaccharide stimulation, whereas when macrophages are treated with epicatechin alone, a stronger effect is achieved with addition after stimulation (Table 2)(60). This can be attributed to partial oxidation of epicatechin by reactive oxygen species produced during the pre-stimulation period, and to the effect of cocoa procyanidins, which can be hydrolysed along the pre-stimulation period to monomeric and dimeric compounds that are taken up by macrophages(61). The inhibitory effect of whole cocoa extract on MCP-1 secretion is higher than that of epicatechin, but lower than that of isoquercitrin (Table 2)(60). These findings illustrate the differing potency of the flavonoids for modulating macrophage function, and suggest possible synergism or an influence of the wide spectrum of compounds present in whole cocoa, such as other polyphenols and methylxanthines(7).

A recent study by Kenny *et al.*(62) investigated the effect of two different fractions of purified cocoa flavonoids on lipopolysaccharide-stimulated human peripheral blood mononuclear cells (PBMC). The short-chain flavonol fraction (including monomers to pentamers), and particularly the long-chain fraction (including hexamers to decamers), enhanced the secretion of TNF-α, monocyte chemoattractant protein-1 (MCP-1) and NO by lipopolysaccharide-stimulated macrophages (Table 2). The inhibitory effect of whole cocoa extract on MCP-1 secretion is higher than that of epicatechin when it is added before stimulus.
of TNF-α, IL-1, IL-6 and IL-10 from stimulated human PBMC (Table 2). The differences between these results and those described by Ono et al. (59) and Ramiro et al. (60) may be attributable to differences in the compounds tested and the cells used, or to the experimental design. Kenny et al. (62) tested purified cocoa flavonoid fractions, whereas Ramiro et al. (60) used whole cocoa extract, which contained flavonoids and other immunomodulatory compounds. Moreover, the study by Kenny et al. (62) was performed on PBMC including several immune cell types that can interact, whereas Ramiro et al. (60) used a macrophage cell line, which may not have had the same susceptibility to cocoa compounds. As mentioned above, flavonoids can act as antioxidants or oxidants, depending on certain conditions (for example, a high flavonoid concentration induces an oxidant environment); therefore, it is reasonable to suggest that oligomeric flavonoids could induce an oxidant state in PBMC that would lead to cell activation and production of inflammatory mediators. These results do not reflect what occurs in vivo, however, when tissue cells are under the effect of flavonoid metabolites and/or different concentrations of these compounds.

The effect of cocoa flavonoids on adaptive immunity has been investigated using lymphocyte cultures. In phorbol-myristate acetate-stimulated lymphocytes, cocoa extract reduces lymphocyte proliferation by down-regulating IL-2 secretion and IL-2 receptor expression (Table 3) (63–65). In keeping with the results obtained in macrophages, the inhibitory effect of whole cocoa extract on lymphocytes is higher than that of epicatechin (65). In addition, cocoa procyanidins decrease IL-2 in phytohaemagglutinin-stimulated PBMC (Table 3) (64), demonstrating that flavonoids are the main compounds responsible for the regulation of lymphocyte activation. Moreover, it has been reported that epicatechin, catechin and dimeric procyanidins, the flavonoid forms usually found in plasma, reduce IL-2 secretion by phorbol-myristate acetate-stimulated Jurkat T cells (61). Although the exact mechanism is still unclear, cocoa flavonoids have been shown to inhibit IL-2 at the transcriptional level (64). Moreover, given the regulatory effect of IL-2 on its receptor, IL-2 receptor down-regulation could also be a consequence of the IL-2 decrease.

Considering the effects of cocoa on cytokines attributed to effector T helper (Th)1 and Th2 cells, cocoa extract has been found to slightly increase in vitro IL-4 secretion and, therefore, Th2-response (Table 3) (65). In this setting, cocoa produces a less pronounced effect than epicatechin. The effects of cocoa flavonoids on cytokine secretion seem to be related to the degree of polymerisation: short-chain cocoa procyanidins increase IL-4 and IL-5, whereas long-chain procyanidins reduce both these Th2 cytokines (Table 3) (66,67).

The mechanism by which cocoa exerts its opposing effects on Th1/Th2 cytokines remains to be established. It is likely that cocoa differentially modulates transcription factor activation: signal transducer and activator of transcription-4 (STAT4), involved in IL-2 expression, and STAT6, the main IL-4 inducer (68). Cytokine interactions should also be taken into account; the increase in Th2 cytokines, which are known to be potent down-regulators of Th1, could contribute to IL-2 inhibition.

Cocoa procyanidins promote homeostatic levels of transforming growth factor-β (TGF-β) in PBMC. TGF-β is
enhanced in non-stimulated immune cells, whereas it is decreased in stimulated cells (Table 3)(69). Although TGF-β is generally considered a regulatory cytokine that helps to maintain an appropriate Th1/Th2 balance in certain situations (for example, in advanced CVD), it can act as a pro-inflammatory mediator through the induction of immune cell recruitment and activation(70,71).

In summary, cocoa down-regulates both macrophage and lymphocyte activation in vitro. Given their powerful antioxidant activity, flavonoids seem to be the perfect candidates for immune regulation; nonetheless, studies showing opposite effects among different cocoa flavonoid fractions suggest that other compounds may contribute to cocoa’s immune effects. In any case, as the profile of flavonoids absorbed in vivo differs from that present in crude cocoa extract, the physiological relevance of these data is limited.

In vivo effects of cocoa on the immune system

A few studies have gone farther than in vitro assays: to investigate the in vivo influence of cocoa on lymphoid organs and immune cell functionality.

The effect of long-term intake (3 weeks) on rat thymus has been recently reported(50). In young rats, an isoenergetic diet containing 10 % cocoa promotes the progression of immature thymocytes (double negative T cell receptor (DN TCR) αβlow and double positive (DP) TCRαβlow cells) towards more mature T cell stages (CD4+CD8− TCRαβhigh cells)(50). Thymus cell differentiation is triggered by complex signalling cascades, most of which are sensitive to changes in the redox environment(72). As was mentioned previously, long-term cocoa intake increases the thymic antioxidant status, as has been shown by enhanced superoxide dismutase and catalase activities(50). These activities might stimulate a slight shift towards a mildly oxidising environment that would favour lymphocyte maturation. In addition, high cocoa intake may promote the differentiation of other immune cell subsets, such as B cells, T cells, myeloid cells, natural killer cells and dendritic cells(73).

The influence of cocoa intake is not only restricted to T cell maturation in the thymus, but also affects lymphocyte composition and function in other immune tissues. High cocoa intake (10 %) for 3 weeks increases the percentage of B cells and decreases the percentage of Th cells in the spleen of young rats (Fig. 3)(74). In addition, the composition of gut-associated lymphoid tissue, the first line of immune cells to oral challenge and diet(75), is also influenced by cocoa intake(76). Peyer’s patches and mesenteric lymph nodes are gut-associated lymphoid tissue compartments that show changes in lymphocyte composition in young rats fed 10 % cocoa during 3 weeks(76). In Peyer’s patches, cocoa intake reduces the TCRαβ+ T cell percentage (mainly the Th subset) and increases B and TCRγδ+ T cell percentages (Fig. 3). Similarly, in mesenteric lymph nodes, high cocoa intake decreases the Th percentage and raises the percentage of γδ T cells(76).

One of the most important findings of these studies is that a diet containing 10 % cocoa fed to rats increases γδ T cell percentages in gut-associated lymphoid tissue(76). These results are consistent with the effects of apple polyphenol intake in healthy mice(77). Intestinal γδ T lymphocytes are mainly involved in innate immunity, where they have a noteworthy role, with participation in oral tolerance, mucosal tissue repair, and immunity against viral antigens and tumour cells(78–80). In murine models of food allergy, apple polyphenols prevented the development of oral sensitisation, and this inhibition correlated with a rise in the intestinal γδ T cell population(77). Taken together, these results suggest that certain diets rich in flavonoids from cocoa or other sources may increase γδ T cell functionality. This finding could be especially important during childhood when the immune system is maturing(81).

On the other hand, a diet containing 10 % cocoa during 3 weeks seems to produce a relative reduction of Th cells in
secondary lymphoid tissues\(^{(74,76)}\). This finding appears to contrast with the impact on thymic tissue, in which cocoa intake promotes Th maturation\(^{(60)}\). These contradictory findings may result from a reduction in thymic Th mobility or an increase in maturation speed, leading to a short life of these lymphocytes. The effects of cocoa on Th cell percentage also suggest an influence on the proliferation rate of these cells. \textit{In vitro} studies have shown that cocoa inhibits Th activation\(^{(65)}\); this might explain the smaller percentage of Th cells in lymphoid organs. However, research in the proliferative response and secretion of IL-2 (the main cytokine involved in Th proliferation) in the spleen and mesenteric lymph nodes of rats fed high doses of cocoa has not shown any reduction\(^{(74,76)}\). Because the results of these studies were expressed as relative percentages, it is conceivable that the decrease in Th percentage in spleen and gut-associated lymphoid tissue may be due to an increase in the absolute number of other lymphocytes, such as B cells. Nonetheless, the ability of these cells to secrete antibodies is down-regulated in rats fed a high-cocoa diet, as was reflected by lower plasma IgG, IgM and IgA levels\(^{(74)}\) and gut secretory IgA and secretory IgM\(^{(76)}\) (Fig. 4). This effect cannot be directly related to a decrease of IgG-secreting cells\(^{(74,76)}\); instead, it could be the result of down-regulation of B cell differentiation caused by the decrease in Th2 cytokines, including IL-4\(^{(74,76)}\).

A decrease in IL-4 secretion was detected in the spleen and mesenteric lymph nodes of rats fed a 10% cocoa diet\(^{(74,76)}\). These results suggest that intake of high doses of cocoa in young rats can favour the Th1 response, in contrast to what has been seen in \textit{in vitro} studies\(^{(60)}\). The reason for these contradictory results may reside in the differing compounds that might explain the smaller percentage of Th cells in lymphoid tissues. Flavonoids present in cocoa, such as fibre and lipids, should also be taken into account in future studies. To date, evidence of cocoa’s immunoregulatory activity has been documented in experimental animal studies. It is difficult to extrapolate these results to human consumption because of the differences in metabolism. However, if flavonoid content were responsible for the immunomodulatory effects, a useful goal for the future could be the design of cocoa formulations with a higher flavonoid content, as has been recently reported\(^{(60)}\).

**Are the antioxidant properties of cocoa responsible for its immunoregulatory role?**

The exact mechanism by which cocoa modulates innate and acquired immune functions remains unclear. As was indicated above, cocoa is a rich source of flavonoid antioxidants, which might promote changes in redox-sensitive signalling pathways involved in the expression of many genes and, consequently, in several cell functions, such as the immune response.

In macrophages and lymphocytes, cocoa compounds can target transcription factors, such as NF-κB which is redox-sensitive and triggers expression of over 100 genes, many of them involved in the immune response\(^{(87)}\). NF-κB is found in the cytoplasm of non-stimulated cells bound to IκB inhibitor proteins (IκB). Upon cellular stimulation, IκB is phosphorylated by the serine-specific kinase, inhibitor of κB kinase (IKK), allowing NF-κB to translocate to the nucleus where it is reduced to initiate transcription of cellular genes\(^{(88)}\). Other redox-sensitive kinases, including mitogen extracellular signal-regulated kinase 1 (MEKK-1), protein kinase B (PKB or AKT)/phosphatidylinositol 3-kinase (PI-3-K) and c-Jun N-terminal kinase (JNK), can affect NF-κB activation. Monomeric flavonoids present in cocoa, such as epicatechin, catechin and querectin, are known to inhibit the NF-κB pathway and decrease TNF-α and NO production in stimulated macrophages\(^{(89,90)}\). Mackenzie et al.\(^{(51)}\) shed light on the regulatory role of cocoa flavonoids on the NF-κB pathway. Epicatechin, catechin and B dimeric procyanidins can act at different stages of NF-κB activation: at early stages, accumulated flavonoids in the cytosol regulate oxidant
levels and reduce IKK phosphorylation, and at later stages, flavonoids – mainly dimeric procyanidins, which penetrate the nuclei – selectively prevent NF-κB binding to its consensus sequence\(^{(61)}\). More recently, Kang et al.\(^{(91)}\) showed that cocoa procyanidins inhibit the kinase activity of mitogen extracellular signal-regulated kinase 1 (MEK1), thus attenuating activation of NF-κB and activator protein-1 (AP-1). These results support an inhibitory effect of cocoa on cytokine production by interacting with NF-κB activation. In addition, apart from the effects on NF-κB, cocoa flavonoids may also have an influence on other transcription factors involved in cytokine production, such as AP-1\(^{(91)}\) and signal transducer and activator of transcription-4 (STAT4)\(^{(92)}\).

This evidence is in keeping with the statement that the antioxidant properties of cocoa are responsible for its immunoregulatory role, but it also shows that certain cocoa flavonoids can directly interact with cell signalling and gene expression factors. Therefore, antioxidant-independent mechanisms must also be considered to better understand the effects of cocoa \textit{in vivo}. In addition, future mechanistic studies should look into the specific metabolites of cocoa that interact with cells \textit{in vivo} and determine the physiological concentrations of these metabolites after normal cocoa intake.

Conclusions

There is an increasing interest in food compounds that can promote health and reduce the risk of disease. Because of its antioxidant activity, mainly attributed to flavonoids, cocoa is currently attracting considerable attention in this regard. The health benefits of cocoa in reducing cardiovascular risk are emerging. In addition, the influence of whole cocoa and cocoa flavonoids on the immune system is gaining recognition, with the work on this subject being reviewed in the present paper. Cocoa has been shown to have an effect on innate and acquired immune function. Various \textit{in vitro} studies have attributed down-regulation of the inflammatory response to cocoa compounds. However, more \textit{in vivo} approaches investigating this anti-inflammatory effect are needed to estimate the true impact of cocoa in this respect.

Cocoa has shown regulatory effects on the acquired immune response in both \textit{in vitro} and \textit{in vivo} experiments. In rats, high cocoa intake modulates intestinal and systemic immune cell functionality. Because immune cell function is controlled by redox-sensitive pathways, flavonoids, which are potent antioxidant compounds, seem to be the best candidates as the source of cocoa’s beneficial effects. In addition, there is some evidence that certain cocoa flavonoids can directly interact with cell signalling and gene expression factors. Further research is needed to shed light on the interactions between cocoa and cell physiology, contributing thus to the body of knowledge of the effects of food compounds on health.

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References


