Chapter 30

Antioxidative stress actions of cocoa in colonic cancer: Revisited

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List of abbreviations

ACF

aberrant crypt foci

AKT

protein kinase B

AOM

azoxymethane

AP-1

activator protein-1

ARE

antioxidant response element

CAT

catalase

CDK

cyclin-dependent kinase

COX

cyclooxygenase

CRC

colorectal cancer

DOC

deoxycholic

EC

(−)-epicatechin

ERK

extracellular regulated kinase

GPx

glutathione peroxidase

GR

glutathione reductase

GSH

glutathione

GST

glutathione-*S*-transferase

HO-1

heme oxygenase-1

IL

interleukin

iNOS

inducible nitric oxide synthase

JNK

c-Jun N-terminal kinase

Keap1

Kelch-like ECH associating protein-1

LDH

lactate dehydrogenase

MAPK

mitogen-activated protein kinase

NF-κB

nuclear factor kappa B

Nrf2

nuclear-factor-E2-related factor 2

PB2

procyanidin B2

PGE2

prostaglandins E2

PI3K

PI-3-kinase, phosphatidylinositol-3-kinase

PPAR

poly-(ADP-ribose) polymerase

ROS

reactive oxygen species

SOD

superoxide dismutase

t-BOOH

tert-butyl hydroperoxide

TNFα

tumor necrosis factor α

γ-GCS

gamma-glutamyl cysteine synthase

Introduction

Colorectal cancer (CRC) is one of the major causes of cancer-related mortality in the world.[1] Indeed, CRC is the third most common cancer in the world, ranked after lung and breast cancer, and it is the second most common cause of cancer death.[2] In 2018, 1.8 million cases of CRC have been detected and found to be responsible for 862,000 deaths.[2] Environmental factors, including dietary and lifestyle, play a crucial role in their etiology even though it is also attributable to inherited and acquired genetic alterations.[3] Cancer is a multistage process conventionally defined by the initiation, promotion, and progression stages. In particular, development of CRC typically follows several consecutive steps from normal epithelial cells via aberrant crypts and progressive adenoma stages to carcinomas in situ and then metastasis. Along this process, oxidative stress has the potential to affect a large array of carcinogenic pathways involved in the proliferation of initiated cells and enhanced malignant transformation.[4] In fact, the gastrointestinal tract, especially the colon, is constantly exposed to reactive oxygen species (ROS), generated during normal cellular metabolism and pathological processes.[5] ROS overproduction may provoke structure and function damages in colonic cells and induce somatic mutations and neoplastic transformation.[4] Because of this, the suppression of oxidative stress by natural antioxidant compounds has gained interest as an effective approach in CRC prevention. Chemoprevention, defined as the use of natural or synthetic compounds to prevent, block, or reverse the development of cancers seems to be an attractive option in this field and the possible impact of several nutritional agents with antioxidant and anti-inflammatory properties has been intensively studied in recent years.[6]

Accordingly, cocoa and their natural flavonoid compounds have shown a potential ability to act as a highly effective antioxidant and chemopreventive agents.[7] Flavanols are polyphenolic compounds extensively found in vegetables, fruits, and plant-derived beverages that present a potent antioxidant activity.[8] Cocoa, the dried and fermented seeds derived from *Theobroma cacao*, has the highest flavanol content of all foods on a per-weight basis and is a significant contributor to the total dietary intake of flavonoids.[9] Actually, for many individuals, cocoa products constitute a larger proportion of the diet than foodstuffs containing bioactive

compounds with similar properties such as green tea, wine, or soybeans.[10] Cocoa flavanols are powerful antioxidant agents acting directly as ROS scavengers, metal ions chelators, and free radical reaction terminators and indirectly by stimulating phase II detoxifying and antioxidant defense enzymes.[11] Additionally, polyphenolic compounds can exhibit other anticarcinogenic properties independently of their conventional antioxidant activity.[12] Based on these findings, cocoa polyphenols could be considered as promising candidates for colon cancer chemoprevention.

Nevertheless, health effects derived from cocoa flavonoids depend on their bioavailability (absorption, distribution, metabolism, and elimination), a factor which is also influenced by their chemical structure.[13] In this regard, cocoa contains high amounts of flavanols (−)-epicatechin (EC), (+)-catechin and their dimers procyanidins B2 (PB2) and B1 (Fig. 1), although other polyphenols such as quercetin, isoquercitrin (quercetin 3-*O*-glucoside), quercetin 3-*O*-arabinose, hyperoside (quercetin 3-*O*-galactoside), naringenin, luteolin, and apigenin have also been found in minor quantities.[14] Interestingly, as compared to other flavonoid-containing foodstuffs, cocoa products exhibit a high concentration of procyanidins that are poorly absorbed in the intestine and consequently their beneficial effects would be restricted to the gastrointestinal tract where they may have an important antioxidant and anticarcinogen (anticarcinogenic)etic local function.[15]

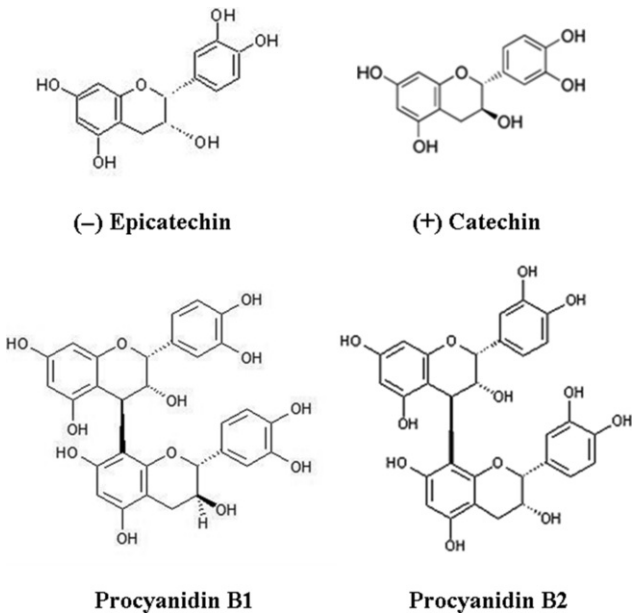

Fig. 1 Main flavonoids present in cocoa. Chemical structures of (−)-epicatechin and (+)-catechin and their respective dimmers procyanidins B2 and B1.

alt-text: Fig. 1

In general, the evidence for chemoprevention by any bioactive substance is achieved from a combination of epidemiological, animal, and basic mechanistic studies. In view of that, the mode of action of cocoa and their flavonols has been recently investigated, especially in cell culture systems. However, it remains to be demonstrated whether these mechanisms are involved in cancer prevention in humans. In this chapter, we reviewed the different in vitro studies that have identified the potential targets and mechanisms whereby cocoa and their polyphenolic compounds could interfere with colonic cancer cells. Afterward, we showed the potential antioxidant and chemopreventive activity of cocoa in an animal model of colon cancer. Finally, some evidence from human studies are also illustrated.

Chemopreventive mechanism of cocoa polyphenols in cultured colon cancer cells

In the recent past years, cocoa and their polyphenolic compounds have been widely studied for their actions against colon cancer cells and related molecular mechanisms (Table 1). All these studies have shown that the pathways responsible for the potential chemopreventive activity of cocoa and its flavonoids are mainly related to their antioxidant and anti-inflammatory properties and their ability to inhibit proliferation and to induce apoptotic cell death (Fig. 2).

Table 1 Effects of cocoa and cocoa polyphenols on colonic cancer cultured cells[a].

alt-text: Table 1

	Polyphenol	Result	Reference
Antioxidant	Cocoa Hexamer procyanidins Procyanidin B2 Epicatechin Catechin	↓ Acrylamide-induced GSH depletion, ↓ ROS generation, ↑ γ-GCS, ↑ GST ↓ DOC-induced cytotoxicity, ↓ oxidant generation, ↓ NADPH oxidase, ↓ Ca^{2+} ↓ Acrylamide-induced GSH depletion, ↓ ROS generation, ↑ γ-GCS, ↑ GST ↓ GPx, GST, GR, and Nrf2 translocation, ↓ *t*-BOOH-induced ROS production and LDH = GPx, GST and GR, and Nrf2 translocation, ↓ *t*-BOOH-induced ROS production and LDH ↓ Lipid peroxidation, ↓ ROS formation, ↑ GPx, ↑ GR, ↑ Nrf2, ↑ HO-1	[16] [17, 18] [16] [19, 20] [19] [21]
Cell cycle	Polymer procyanidins Epicatechin	G2/M arrest, ↓ ornithine decarboxylase, ↓ S-adenosylmethionine decarboxylase S arrest	[22] [23]
Apoptosis	Hexamer procyanidins Procyanidin B2 Epicatechin	↓ DOC-induced caspase-3, ↓ PPAR cleavage ↑ Bad, ↑ caspase-9, ↑ caspase-3, ↓ cytochrome *c* ↓ *t*-BOOH-induced caspase-3 ↓ *t*-BOOH-induced caspase-3	[17] [24] [19] [19]
Proliferation/survival	Cocoa Hexamer procyanidins Procyanidin B2 Epicatechin	↓ Acrylamide-induced p-JNK ↓ DOC-induced AKT, ERK, p38, and AP-1 ↓ p-AKT, ↓ p-p85-PI3K, ↓ p-GSK3 ↑ ERK, ↑ p38 = ↑ proliferation, = ↑ p-AKT, = ↑ p-ERK = proliferation, = p-AKT, = p-ERK	[16] [17] [24] [20] [25] [25]
Antiinflammatory	Cacao Hexamer procyanidins	↓ PGE2, ↓ IL-lβ, = IL-8, = NF-κB, ↑ COX-1 ↓ TNF-induced IL-S. COX-2. Inos, and NF-κB activation ↓ TNF-induced NF-κB activation and iNOS ↓ TNF-induced IL-8, improve membrane integrity	[26] [27] [28] [29]

[a] The arrow indicate an increase (↑) or decrease (↓) in the levels or activity of the different analyzed parameters. In certain cases, opposing results have been obtained since the studies were earned out in different colonic cell types and/or the final effects may depend on the dose and time of treatment with the phenolic compound.

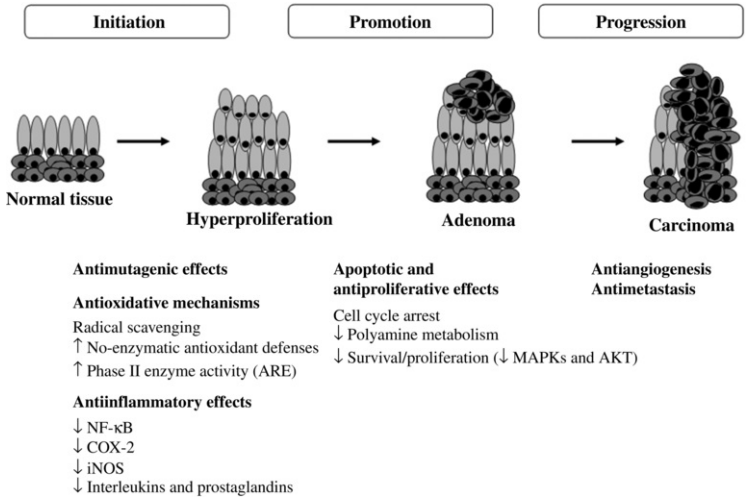

Fig. 2 Mechanisms involved in the potential chemopreventive effects of cocoa and its flavonoids against colorectal cancer. The arrows indicate an increase (↑) or decrease (↓) in the levels or activity of the different analyzed parameters.

alt-text: Fig. 2

Antioxidant effects

Aerobic organisms cannot avoid free radical and reactive oxygen species (ROS) generation. Overproduction of ROS may lead to the formation of highly reactive oxidation products, activation of carcinogens, and formation of oxidized DNA bases and DNA strand breaks. These alterations might cause mistakes during DNA replication and genetic alterations, increase transformation frequencies, modulate transcription of redox-regulated proteins, ultimately leading to enhanced cell proliferation and tumor promotion/progression.[12] In a physiological situation, cells maintain the balance between generation and neutralization of ROS through the enzymatic and nonenzymatic defenses, such as glutathione (GSH), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), nitric-oxide synthase, lipooxygenase, xanthine oxidase, etc. However, when the cellular balance is altered and cellular defenses overwhelmed, cells can be damaged as mentioned above.

Cocoa and its flavonoids exert strong antioxidant effects. Thus, cocoa possesses a potent antioxidant capacity as compared with other foods or products, such as teas and red wine, and this property has been related to its flavonoid content.[30] Interestingly, the antioxidant properties of cocoa and its flavonoids are partly based on their structural characteristics, including the hydroxylation of the basic flavan-ring system, especially 3′,4′-dihydroxylation of the B-ring (catechol structure), the oligomer chain length, and the stereochemical features of the molecule.[31] These structural characteristics of flavanols represent the molecular basis for their hydrogen- donating (radical-scavenging) properties and their metal-chelating antioxidant properties. In addition, cocoa and its flavonoids can prevent the DNA damage caused by free radicals or carcinogenic agents acting through the modulation of enzymes related to oxidative stress (CAT, GR, GPx, SOD, etc.) and the alteration of the procarcinogenic metabolism by inhibiting phase-I drug-metabolizing enzymes (cytochrome P450) or activating phase II conjugating-enzymes (glucuronidation, sulfation, acetylation, methylation, and conjugation).

Protective effects

Prevention of ROS generation and the preservation of the cellular antioxidant defenses seem to represent an important mechanism of the chemoprevention of natural polyphenols.[12] In this line, intestinal Caco-2 cells pretreated with a cocoa phenolic extract or with the pure cocoa flavanols (−)-epicatechin (EC) and procyanidin B2 (PB2) at physiological concentrations (i.e., 10 μg/mL for cocoa phenolic extract and 10 μM for EC and PB2, respectively) for 20 h counteracted acrylamide-induced cytotoxicity (5 mM for 24 h) by inhibiting GSH consumption and ROS generation.[16] Both cocoa phenolic extract and PB2 almost completely blocked the decrease of GSH induced by acrylamide and totally abrogated the subsequently increased ROS generation, whereas these effects were only partially restored with EC. This result suggests that the minor effect exerted by EC could be partially ascribed to the fact that EC mainly acted as a scavenger of free radicals. However, similar to what was reported for other polyphenols and antioxidants,[12] PB2 and a cocoa phenolic extract could protect cell constituents not only by neutralizing several types of radicals but also by upregulating antioxidant defenses as well as by interacting with signaling pathways involved in cell survival. Thus, PB2 and the cocoa phenolic extract increased the levels of gamma-glutamyl cysteine synthase and glutathione-*S*-transferase (GST) in the mentioned experimental conditions Caco-2 cells.[16]

Pure PB2 and EC (1–10 μM) decreased ROS production but did not affect GSH content in Caco-2 cells, and PB2 (1–10 μM), evoked a substantial increase in GPx, GR, and GST activity after 20 h of incubation.[19] Thus, pretreatment of Caco2 cells with EC and PB2 for 20 h before the oxidative insult induced by the potent prooxidant *tert*-butylhydroperoxide (*t*-BOOH at 400 μM) attenuated or blunted ROS production, respectively. In addition, EC and PB2 protected cells from necrosis, as lactate dehydrogenase (LDH) leakage decreased after 1 and up to 6 h of incubation with the prooxidant, respectively.[19] All together suggests that at least two mechanisms could be involved in the protection of Caco2 cells afforded by flavanols: (1) the inherent antioxidant capacity to quench ROS and (2) the improvement of the endogenous antioxidant defenses.

Effects on phase I and II enzymes

Enzymes of the phase I of drug metabolism (cytochromes P450) transform xenobiotics by adding functional groups which render these compounds more water-soluble. Phase I functionalization may be required to efficiently detoxify carcinogens.[12] Phase II enzymes such as GST and sulfotransferases conjugate transformed phase I metabolites and xenobiotics to endogenous ligands like GSH, glucuronic, acetic, or sulfuric acid and enhance excretion and detoxification in form of these conjugates.[12] Therefore, reduction of elevated phase I enzyme activities to physiological levels and enhanced excretion of carcinogens via upregulation of phase II enzymes are considered a strategy in chemoprevention. In addition, the transcription factor NF-E2-related factor-2 (Nrf2) and the Kelch-like ECH-associated protein 1 (Keap1) are considered as chemopreventive targets because both proteins participate in the regulation of the antioxidant response element (ARE). Thus, the modification of the protein Keap1 can lead to the accumulation of Nrf2 in the nucleus and the subsequent ARE activation.[12]

Cocoa and its phenolic compounds also exert their protective effect toward oxidative stress through the modulation of phase I and II enzyme activities and Nrf2. Accordingly, catechin (100 μM) increased the expression of Nrf2 and heme oxygenase-1 in a time-dependent manner in intestinal Int-407 cells.[21] PB2 (1–10 μM) alone also evoked a dose-dependent increase in GPx, GR, and GST after incubating Caco-2 cells for 20 h, which could be related to an improved cell response to an oxidative challenge.[19] Hence, cells treated with 10 μM PB2 for 20 h, and then submitted to oxidative stress induced by *t*-BOOH (400 μM, for 1 or 6 h, respectively) showed a reduced ROS production, restricted activation of caspase 3 and higher viability than cells plainly submitted to the stressor.[19]

Furthermore, PB2 (10 μM, for 20 h) showed a protective effect against the oxidative injury induced by 400 μM *t*-BOOH in Caco-2 cells through the upregulation of the expression and activity of GST P1 via a mechanism that involved extracellular regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) activation and Nrf2 translocation.[20] Thus, PB2 treatment increased the protein levels of Nrf2 in the nucleus at 3 h, peaked at 6 h and

continued elevated up to 20 h of treatment. Accordingly, this procyanidin significantly enhanced the mRNA levels and activity of GST P1 at 4–20 h of incubation, which was accompanied by an increment in the levels of protein expression at 8 and 20 h.[20]

Similarly, cocoa procyanidins protected Caco-2 cells from the loss of integrity induced by a lipophilic oxidant.[17, 18] Interestingly, a hexameric procyanidin fraction isolated from cocoa (2.5–20 μM) interacted with the Caco-2 cell membranes preferentially at the water-lipid interface without affecting their integrity after 30 min of incubation. Moreover, the hexameric procyanidin fraction inhibited the deoxycholic (DOC)-induced cytotoxicity and partly prevented the oxidant generation following NADPH oxidase inhibition, as well as DOC-triggered increase in cellular calcium.[17, 18] The limited effects on LDH release observed after 6 h of incubation for lower molecular weight procyanidins, i.e., monomer-tetramer, stress the relevance of the membrane-related effects of larger procyanidins. These differential actions have to be explained as a compromise between the incorporation of the compounds into the cells that decreases as procyanidin oligomerization increases and the adsorption to the cell surface that increases as procyanidins oligomerization increases.

Effects on apoptosis and proliferation

Apoptosis and proliferation in cells are also modulated by ROS generation.[12] Indeed, suppression of cell proliferation, as well as induction of differentiation and apoptosis are important approaches in cancer chemoprevention.

Cell cycle

Deregulated cell cycle and resistance to apoptosis are hallmarks of cancer.[12] Cell cycle control is a highly regulated process that involves the modulation of different cell cycle regulatory proteins, such as cyclins, cyclin-dependent kinases (CDKs), CDK inhibitors, etc.[12] ROS generation could induce an alteration of cell cycle-specific proteins that can affect and/or block the continuous proliferation of cancer cells.

Procyanidin-enriched extracts and procyanidins inhibited Caco-2 cell growth.[22] After 48 h of incubation, procyanidins extracts with a flavanol and procyanidin content of 501 mg/g, caused only 25% growth inhibition, whereas the procyanidin-enriched extracts (flavanol and procyanidin content: 941 mg/g) induced a 75% growth inhibition. On the contrary, cocoa powder samples, which consisted of a flavanol and procyanidin content of 141 mg/g, showed no growth inhibitory effects in Caco-2 cells. Moreover, 50 μg/mL procyanidin-enriched extracts blocked the cell cycle at G2/M phase, without inducing apoptosis, and decreased the polyamine metabolism by inhibiting the ornithine decarboxylase and *S*-adenosylmethionine decarboxylase activities, which has partly been related to the accumulation of cells at the G2/M phase.[22] Cocoa procyanidin hexamers (2.5–50 μM for 24–72 h) also decreased cell viability in Caco-2, HCT15, HT29, HCT116, SW480, and LoVo cells in a dose-dependent manner, showing a more prominent effect than EC or cocoa procyanidin hexamers at the same concentrations.[24] Thus, cocoa procyanidin hexamers (10–30 μM, 72 h) arrested the cell cycle of Caco-2 in G2/M and induced apoptosis.[24] Interestingly, hexamers did not affect the cell viability of differentiated intestinal Caco-2 cells, which highlight the different effect of the same compound depending on the proliferating state of the colon cell, being this aspect crucial in CRC prevention.[24] Similarly, incubation of LoVo cancer cells with 690 and 1380 μg/mL EC for 24 h induced S phase arrest in the cell cycle progression but it did not induce apoptosis.[23] Importantly, lower concentrations of EC seemed to promote a slight proliferation of LoVo cells.

Apoptosis

ROS generation has been described as a critical upstream activator of the development of apoptosis.[12] At molecular level, it has been widely reported the existence of two mechanisms for the activation of the programmed cell death: (1) the extrinsic pathway, which is mediated by death receptors, and (2) the intrinsic mechanism (mitochondria-mediated), that is, regulated by pro- and antiapoptotic proteins of the Bcl-2 family. Both cascades converge in a common executor mechanism involving DNA endonucleases that activate proteases (caspases) and lead to cellular death.[12]

Treatment of cells with natural antioxidants prevents the cytotoxicity induced by oxidative stress inducers through the ability of these compounds to restrain the increase in ROS levels and the subsequent activation of caspase-3 which leads to apoptosis induction.[12] Consistent with the above, after 20 h of incubation EC or PB2 (10 μM) effectively reduced the apoptotic effects induced by *t*-BOOH (400 μM for 4 h) in Caco-2 cells.[19] Similarly, pretreatment with 10 μM hexameric procyanidins for 30 min also delayed the DOC-induced Caco-2 cell apoptosis, as restrained caspase-3 activation, given that poly-(ADP-ribose) polymerase cleavage was observed only after 6 h incubation.[17] Nevertheless, in Caco-2 cells cocoa procyanidin hexamers (20 μM, 24 h) induced apoptosis, as increased mitochondrial Bad, caspase-3 and -9 activities, and decreased mitochondrial cytochrome *c* levels.[24]

Proliferation/survival

Phosphatidyl-inositol-3-kinase (PI3K)/protein kinase B (AKT), growth factor receptors/Ras/mitogen-activated protein kinases, and nuclear factor kappa B (NF-κB), which also importantly contribute to the inflammatory process (see below), constitute the most important signaling pathways regulating cell proliferation and survival.[12]

Cocoa phenolic compounds can interact with signaling proteins and modulate their activity. In this line, pretreatment with 10 μM hexameric procyanidins (30 min) prevented oncogenic events initiated by DOC through the interaction with Caco-2 cell membranes and inhibited the DOC-promoted activation of AKT, ERK, and p38, as well as the downstream transcription factor activator protein-1 (AP-1).[17] Conversely, cocoa procyanidin hexamers (10–40 μM for 24 h) inhibited the PI3K/AKT pathway, as well as GSK-3 levels in Caco-2, which was associated with the repression of proliferative/survival routes and the induction of apoptosis.[24] Interestingly, PB2 and EC (10–50 μM) did not have

an obvious effect on Caco-2 and SW480 colon carcinoma cells after 24 h of incubation. However, PB2 promoted cell growth in SW480 cells by increasing p-AKT and p-ERK levels[25] and activated Nrf2 translocation and increased both GST P1 protein and activity, via ERK and p38 pathways, which were also essential routes for the cytoprotective effect exerted by the flavanol in Caco-2 cells.[20] This different response depending on the distinct chemical structure of the compound and the different degree of cell differentiation highlights the importance of an integrated approach to study the biological effects of phytochemicals.[25]

Antiinflammatory effects

The manifestation of oxidative stress by infections, immune diseases, and chronic inflammation has been associated with carcinogenesis.[12] Thus, chronic inflammation is a risk for colorectal cancer,[12] and most inflammation-associated colorectal cancers are characterized by the activation of the transcription factor NF-κB and inflammatory mediators such as tumor necrosis factor α (TNFα), cyclooxygenase-(COX)-2, etc., being all these proteins also related to cell proliferation, antiapoptotic activity, angiogenesis, and metastasis.[12]

The cocoa extract inhibited the inflammatory mediator prostaglandins E2 (PGE2) in human intestinal Caco-2 cells.[26] Thus, cells incubated with a polyphenolic extract of cocoa (equivalent to 50 μM of gallic acid) for 4 h and stimulated with interleukin-(IL)-1β for 24 or 48 h showed a decrease in PGE2 synthesis, whereas IL-8 secretion and NF-κB activity remained at high levels.[26] Surprisingly, in the absence of pro-inflammatory stimulus, the cocoa polyphenolic extract induced a basal PGE2 synthesis in Caco-2 cells after 24 h of incubation. This effect has been associated with induction of COX-1, which seems to be implicated in maintaining the mucosal integrity.[26]

More recently, pretreatment with a cocoa phenolic extract, at a physiological concentration (10 μg/mL) for 20 h, reduced the increase in inflammatory markers such as IL-8 secretion, COX-2, and inducible nitric oxide synthase (iNOS) expression induced by the pro-inflammatory agent TNFα (40 ng/mL for 24 h) in Caco-2 cells.[27] In this work, cocoa phenolic extract selectively decreased both phosphorylated levels of c-Jun N-terminal kinase and nuclear translocation of NK-κB induced by TNFα, indicating that this pathway could be an important mechanism contributing to the reduction of intestinal inflammation.

EC and procyanidins can inhibit NF-κB at different levels in the activation pathway. A decrease in cell oxidants that are involved in NF-κB activation is a potential mechanism of modulation by these compounds. Thus, incubation of Caco-2 cells for 30 min with 2.5–20 μM hexameric procyanidins, prior to treatment with 10 ng/mL TNFα for further 5–30 min inhibited the TNFα-induced NF-κB activation (inhibitor of κB phosphorylation and degradation, p50 and RelA nuclear translocation, and NF-κB–DNA binding), iNOS expression, and cell oxidant increase.[28] These effects have been suggested to occur because hexameric procyanidins can inhibit NF-κB activation by interacting with the plasma membrane of intestinal cells, and through these interactions preferentially inhibits the binding of TNFα to its receptor and the subsequent NF-κB activation.[28] In addition, in HT-29 cells pretreatment with cocoa-high-molecular-weight polymeric procyanidins fractions (≥ 7 polymerization degree, 10–25 μg/mL for 24 h) were more effective than cocoa monomers or oligomer-procyanidin rich fractions to preserve the membrane integrity and reduce the levels of IL-8 in response to pro-inflammatory conditions (5 ng/mL TNFα for 6 h).[29]

Chemopreventive mechanism of cocoa in animal models of colon cancer

Studies with colonic cell culture model have clearly demonstrated the antioxidant and chemopreventive abilities of cocoa and its flavonoids, but only experimental models for colorectal cancer could offer the opportunity to assess the contribution of this natural dietary compound to the potential prevention of CRC. To this end, carcinogen-induced rodent models have been shown to mimic many features of human non-familial colorectal cancer (nongenetic based),[32] which is the most frequent and occurs sporadically. The induction of colon tumors is achieved by the administration of carcinogens such as nitrosamines, heterocyclic amines, aromatic amines, 1,2-dimethylhydrazine and azoxymethane (AOM). In particular, administration of AOM to rodents induces the development of colonic preneoplastic lesions (aberrant crypt foci, ACF) that may progress into cancer with time.[33] ACF represent the earliest identifiable intermediate precancerous lesions during colon carcinogenesis in both laboratory animals and humans[34] and can be identified microscopically on the surface of the colon mucosa after methylene blue staining (Fig. 3).

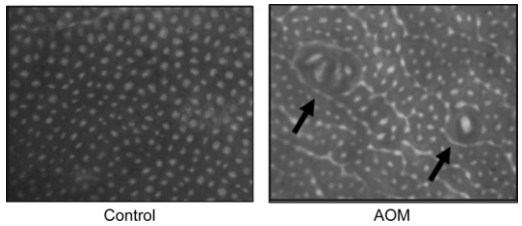

Fig. 3 Mucosal surface of colon from control and azoxymethane (AOM)-injected rats. Mucosal of colon were stained with methylene blue and observed under light microscope (40 × magnification). The presence of aberrant crypt foci (ACF) is indicated by arrows.

alt-text: Fig. 3

Contrary to the strong evidence for the antioxidant and cancer preventive activity of cocoa and their components in cultured cells, there are only a few studies in rats that have demonstrated the potential chemopreventive ability of cocoa on colon carcinogenesis.[35–37] In one of the studies, male Wistar rats were fed with a cocoa-enriched diet (12%) starting 2 weeks before the carcinogenic induction and throughout the experimental period (8 weeks).[35] As expected, all the rats that were injected with AOM developed colonic preneoplastic lesions (aberrant crypts foci, ACF). Nevertheless, the cocoa-enriched diet significantly reduced the AOM-induced ACF formation and especially those ACF with a larger number of crypts (≥ 4 crypts) which exhibit a higher tendency to progress into malignancy (Table 2). Therefore, this study showed for the first time that a cocoa-enriched diet was able to suppress the early phase of chemically induced colon carcinogenesis. More recently, two new studies using the azoxymethane/dextran sulfate sodium model in BALB/c mice have also described the antitumor effects of a 5% and 10% cocoa-rich diet on colitis-associated cancer (CAC).[36, 37]

Table 2 Effect of dietary cocoa on aberrant crypt foci (ACF) formation in azoxymethane (AOM)-treated rats.

alt-text: Table 2

	ACF formation	ACF/cm^2	Crypts multiplicity of ACF			
			1 Crypt	2 Crypts	3 Crypts	> 4 Crypts
Control	0/8	0	0	0	0	0
Cocoa	0/8	0	0	0	0	0
Control + AOM	12/12	16.1 ± 6.2^a	6.3 ± l.9^a	5.8 ± 2.8^a	3.0 ± l.4^a	0.87 ± 0.23^a
Cocoa + AOM	12/12	8.8 ± 2.5^b	3.5 ± l.0^b	3.7 ± l.5^a	1.3 ± 0.6^b	0.14 ± 0.05^b

Values are means ± SD. Means in a column without a common letter differ, $P < .05$.

The most relevant in vivo mechanisms involved in the chemopreventive effects elicited by cocoa are briefly described below. These mechanisms included the prevention of oxidative stress, cell proliferation and cell inflammation, and the ability of cocoa diet to induce cell apoptosis (Fig. 2).

Cocoa prevented AOM-induced oxidative stress in colon tissues

The suppressive effect of cocoa on AOM-induced preneoplastic lesions has been associated with its antioxidative properties. AOM is metabolized in the liver to a methyl-free radical who in turn generates hydroxyl radical or hydrogen peroxide capable of oxidized DNA, RNA, lipids, or protein of colonic epithelial cells.[38] As a consequence, the levels of lipid and protein peroxidation, indicative of oxidative injury, increased in the colon of animals treated with AOM.[39] However, in animals fed with 12% cocoa-diet, the increased levels of protein and lipid oxidative damage induced by AOM were strongly prevented, demonstrating that cocoa possesses a potent antioxidative effect in vivo on the stressed colonic tissue.[35] In particular, cocoa feeding was able to avoid oxidative stress by reverting to control values the diminished levels of GSH and the activities of GPx, GR, and GST provoked by the toxicant. Similar results were found by Panduragan et al.[36] in an animal model of AOM/DSS-induced colitis-associated cancer (CAC). Furthermore, they also demonstrated that cocoa upregulated the expression of Nrf2 and its downstream targets protecting colon tissues from oxidative damage during colorectal carcinogenesis. Since Nrf2 and its antioxidant enzymes participate in the detoxification of xenobiotics, carcinogens, free radicals, and peroxides,[40] it can be suggested that cocoa could prevent ACF formation by reinforcing the endogenous defense capacity in colon tissues to counteract carcinogen-induced toxicity. Consequently, the increased cellular defense in the colon of cocoa-fed animals treated with AOM seems to be an effective strategy to protect against carcinogen-induced toxicity and largely accounts for the chemoprotective activity of cocoa.

Cocoa prevented cell proliferation in AOM treated animals

Besides inducing oxidative damage and genomic instability, ROS can specifically activate certain redox-sensitive signaling pathways and contribute to CRC initiation/promotion through the regulation of cellular proliferation and survival.[41] Among these, PI3K/AKT and ERK/MAPKs are within the most important pathways activated in response to oxidative stress and play important roles in the carcinogenesis of many types of cancers including colon cancer.[4] Accordingly, AOM treatment clearly elevated the proliferative activity of the colonic mucosa and this increase was accompanied by the phosphorylation of AKT and ERKs and the over-expression of cyclin D1, a preneoplastic marker involved in cell cycle progression.[35] However, cocoa intake prevented all these processes induced by AOM, suggesting that cocoa, by its ability to restrain oxidative stress could also inhibit the consequent activation of signaling pathways involved in proliferation and thereby the progression of preneoplasia in the colonic epithelial cells. Supporting this, a recent in vitro study has shown that flavonoids such as luteolin and quercetin have antiproliferative and proapoptotic effects in human CRC cells through the regulation of the ERK/MAPK and the PI3K pathways.[42] In the same line, the expression of PCNA was increased in the colon of AOM/DSS-induced CAC mice model while cocoa was able to reduce cell proliferation through the inactivation of p-STAT3.[37]

Cocoa prevented AOM-induced inflammation in colon tissues

In recent years, considerable evidence has demonstrated that ROS are also involved in the link between chronic inflammation and cancer.[4] Redox status has an impact on the transcription factor NF-κB which regulates the expression of the pro-inflammatory enzymes COX-2 and iNOS. Both enzymes are implicated in chronic inflammation causing a microenvironment that contributes to the development of preneoplastic lesions in the colon carcinogenesis.[43] In fact, iNOS and COX-2 have been found to be increased in human CRC and AOM-induced rat colon carcinogenesis.[44] A cocoa-rich diet was able to suppress the intestinal inflammation induced by AOM through the inhibition of NF-κB signaling and the downregulation of the pro-inflammatory enzyme expressions of COX-2 and iNOS.[27] iNOS expression is frequently observed in dysplastic, but not in hyperplastic, ACF indicating that iNOS plays an important role in the early stages of tumor formation.[45] On the other hand, tumorigenic mechanisms of COX-2 include inhibition of apoptosis via increased Bcl-2 and activation of proliferation via MAPK or PI3K/AKT signaling pathways.[46] Thus, the effect of a cocoa-rich diet preventing iNOS and COX-2 expression induced by AOM seems to be related to the inhibition of ACF formation observed in the AOM group treated with cocoa. Besides, cocoa also was able to inhibit the NF-κB signaling pathways and suppress the expressions of pro-inflammatory cytokines during the early stage of colitis.[37]

Cocoa-induced apoptosis in AOM-treated animals

During the promotion/progression phase of carcinogenesis, apoptosis is the main biological event involved in the removal of the initiated/mutated colonic epithelial cells.[4] In fact, many natural dietary compounds have been shown to suppress ACF formation by increasing apoptosis.[6] Accordingly, cocoa supplementation clearly induced apoptosis in the colon tissue of AOM-treated rats[35] and the colon of AOM/DSS-induced mice.[37] Indeed, cocoa was able to modulate the expression of pro- and antiapoptotic proteins (Bax and Bcl-x_L, respectively) and to provoke caspase-3 activation, suggesting that cocoa induces apoptosis with the participation of the mitochondrial pathway. These data are in agreement with other results illustrating the apoptotic effect as the major mechanism for chemoprevention of different polyphenolic plant constituents.[12] Consequently, the proapoptotic in vivo effect of cocoa seems to be a complementary mechanism both to reduce preneoplastic lesions induced by AOM and to prevent promotion/progression of carcinogenesis, playing thus an important role in its anticarcinogenic potential.

Human studies

There is increasing evidence to support an inverse association between the intake of dietary fruit and vegetables and several cancers, including colorectal cancer.[47, 48] Although the anticarcinogenetic mechanism of these foods is unclear, the presence of dietary polyphenols, in particular flavonoids, maybe one of the reasons. Most studies carried out in cell cultures and experimental animal have supported the chemopreventive efficiency of cocoa polyphenols in colorectal cancer, but the outcome of epidemiologic studies on cocoa intake and risk of colon cancer is not conclusive.

Epidemiologic studies

The first report in support of a potential association between flavanol/catechin intake and colorectal cancer was published in 2002 with data obtained from the Iowa Women's Health Study (United States), where a cohort of 34,651 postmenopausal cancer-free women aged 55–69 years was followed from 1986 to 1998. Among several cancers studied, data suggested that catechin intake may protect against rectal cancer, furthermore, catechins derived primarily from fruits tended to be inversely associated with upper digestive tract cancer, whereas catechins derived from tea were inversely associated with rectal cancer.[49] The Kuna tribe in Panama has been widely regarded as the most significant example in support of the preventive effect of cocoa intake in cancer. The population of this tribe inhabits the San Blas district of Panama and has cocoa as their main beverage.[50] When death certificates from year 2000 to 2004 were surveyed in order to compare cause-specific death rates between mainland Panama and the San Blas islands, the rate of cardiovascular disease and different types of cancer, including colorectal, among island-dwelling Kuna was much lower than in mainland Panama. This natural protection was rapidly associated with the elevated intake of cocoa flavanols, which intake probably exceeds 900 mg/day, a figure that represents the highest flavonoid-rich diet of any world population.[50]

Just within the last 3 years, a number of epidemiological studies and meta-analysis have failed to show a protective effect of flavanols from cocoa or other sources on colon and rectal cancer. Thus, during up to 26 years of follow-up, 2519 colorectal cancer cases Nimptsch and colleagues found no support for the hypothesis that a higher habitual intake of any flavonoid subclass decreases the risk of colorectal cancer.[51] Data from the European Prospective Investigation into Cancer and Nutrition (EPIC) study, which included 476,160 men and women from 10 European countries during a mean follow-up of 14 years, found that intake of flavanols was not related to colorectal, colon, or rectal cancer risks.[52] Also in 2018, a massive meta-analysis performed by Chang and coworkers found no association between flavanol intake and risk of colorectal cancer, although they found an inverse association with other flavonoid subtypes.[47]

Considering specifically cocoa and/or chocolate flavanols, in a pioneer case–control study in Burgundy (France), chocolate was identified as a risk factor for colorectal cancer.[53] An early study in North Carolina failed to detect a significantly lower prevalence of adenomatous polyps and colorectal cancer with chocolate consumption.[54] In the same line, a French study from 2005 showed no significant association between a high chocolate dietary pattern and any stage of colorectal disease ranging from polyps to adenomas and colorectal cancer.[55] Finally, early this year Morze et al. have reported that chocolate consumption is not related to risk for colorectal cancer.[56] It is worth mentioning that any type of chocolate was included in all the above meta-analysis.

Intervention studies

There are properly no human intervention studies attempting to show a correlation between cocoa intake and cancer prevention, but a few human intervention trials indicate that cocoa favorably affects intermediary factors in cancer progression.[7] In this regard, several recent studies have focused on the modulation of antioxidant and anti-inflammatory status by consumption of cocoa products. In a study by Spadafranca et al.[57] dark chocolate consumption significantly improved DNA resistance to oxidative stress. In this study, healthy subjects were assigned to a daily intake of 45 g of dark chocolate or white chocolate for 14 days and oxidative damage to mononuclear blood cells DNA was reduced in the dark chocolate group 2 h after consumption; 22 h later the effect disappeared. Similarly, cocoa consumption reduced NF-κB activation in peripheral blood mononuclear cells in healthy voluntaries,[58] but biomarkers of inflammation, including IL-6, were unaffected in patients at high risk of cardiovascular disease consuming cocoa powder.[59]

Summary points

- Cocoa and its main phenolic compounds regulate cellular redox status and multiple signaling pathways associated with cell proliferation, differentiation, apoptosis, and inflammation.
- Animal studies have established that cocoa and its main phenolic components might prevent and/or slow down the initiation-promotion of colon cancer.
- In humans, interventional studies have described favorable changes in antioxidant biomarkers.
- Daily intake of small quantities of cocoa or chocolate, which provide flavanols and procyanidins, in combination with a standard dietary consumption of flavonoids, would constitute a natural approach to prevent colon cancer with insignificant toxicity.
- Carefulness is obligatory to extrapolate the in vivo cellular results to in vivo animal colon cancer models and, even more notably, to humans.
- Cocoa molecular mechanisms of action remain unclear and further investigations are deserved.
- More well-designed epidemiological and intervention studies are needed to demonstrate the potential colorectal cancer preventive activities of cocoa in humans.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Abstract

Colorectal cancer is the third most common cancer in the world and is one of the major causes of cancer-related mortality in the world. Cancer is defined as a multistage process and divided into the followin stages: initiation, promotion, and progression. In this disease, oxidative stress is involved in a large array of carcinogenic pathways associated with the proliferation of initiated cells and cellular malignancy. Therefore the suppression of oxidative stress by natural antioxidant compounds has gained interest as an effective approach in colorectal cancer prevention. Accordingly, cocoa and its phenolic components have demonstrated t exert antioxidant activity and to be able to interfere with multiple carcinogenic signaling pathways. In this chapter, we reviewed the different in vitro and in vivo studies that have identified potential targets an mechanisms whereby cocoa and their flavonoid compounds could interfere with colonic cancer cells. In addition, recent evidence from human studies is also illustrated.

Keywords: Cocoa flavonoids; Colorectal cancer; Antioxidant defenses; Apoptosis; Proliferation; Inflammation; Signaling pathways

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