Cocoa rich diet prevents azoxymethane-induced colonic preneoplastic lesions in rats by restraining oxidative stress and cell proliferation and inducing apoptosis.

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Abbreviations used: AOM, azoxymethane; **ACF,** aberrant crypt foci; **CRC**, colorectal cancer; **ERK/MAPK,** extracellular regulated kinase/mitogen-activated protein kinase; **GSH,** glutathione; **GPx,** glutathione peroxidase; **GR,** glutathione reductase; **GST,** glutathione S-transferase; **MDA,** malondialdehyde; **PCNA**, proliferating cell nuclear antigen; **PI3K/PKB,** phosphatidylinositol 3-kinases/protein kinase B; **TUNEL,** terminal deoxynucleotidyl transferase dUTP nick end labeling.

Abstract

Cocoa is a rich source of bioactive compounds with potential chemopreventive ability but up to date its effectiveness in animal models of colon carcinogenesis has not been addressed. Herein, we investigated the *in vivo* effect of a cocoa-rich diet in the prevention of azoxymethane (AOM)-induced colon cancer and the mechanisms involved. Our results showed that cocoa feeding significantly reduced AOM-induced colonic aberrant crypt foci formation and crypt multiplicity. Oxidative imbalance in colon tissues seems to be prevented by cocoa as indicated by reduced oxidation markers levels and increased enzymatic and non-enzymatic endogenous defences. Cocoa-rich diet also exhibited antiproliferative effects by decreasing the levels of extracellular regulated kinases, protein kinase B and cyclin D1 together with pro-apoptotic effects evidenced by reduced Bcl-x_L levels and increased Bax levels and caspase-3 activity. Our findings provide the first *in vivo* evidence that a cocoa-rich diet may inhibit the early stage of colon carcinogenesis probably by preventing oxidative stress and cell proliferation and by inducing apoptosis.

Numerous epidemiological studies suggest that diets rich in natural antioxidants are associated with reduced risk of certain cancers, notably colorectal cancer (CRC) [1]. Cancer is a multistage process conventionally defined by three stages: initiation, promotion, and progression. Along this process, oxidative stress has the potential to affect a large array of carcinogenic pathways involved in proliferation of initiated cells and enhanced malignant transformation [2]. Therefore, the suppression of oxidative stress by natural antioxidant compounds seems to be an effective approach in preventing the initiation and progression of CRC.

Cocoa and its phenolic compounds, the flavanols epicatechin, catechin and procyanidins, have recently attracted a great deal of interest because of their potential ability to act as highly effective chemopreventive agents. As antioxidants, cocoa flavanols have been shown to protect cell constituents, limiting the risk factor for cancer and other chronic diseases [3,4]. In addition, they can display other chemopreventive properties which may be independent of conventional antioxidant activities [5]. In particular, recent *in vitro* studies have demonstrated that cocoa phenolic compounds can regulate several signal transduction pathways [6,7], activate redox-sensitive transcription factors [8] and modulate expression of specific genes involved in cell survival and cell death [9]. Moreover, as compared to other flavonoid containing foods, cocoa products exhibit a high concentration of procyanidins that are poorly absorbed in the intestine and consequently its beneficial effects would be more focused on the gastrointestinal tract where they may have an important local function neutralising oxidants. Despite these evidences, the efficacy of cocoa against CRC initiation and development *in vivo* remains largely unexamined. Therefore, in the present study we have used the well-defined azoxymethane (AOM)-induced colon cancer model in rats to investigate the effectiveness of a cocoa enriched diet in preventing the early phase of colon carcinogenesis and the mechanisms of action involved.

Natural Forastero cocoa powder (a kind gift from Nutrexpa, Barcelona, Spain) was used for this study. A detailed description of this cocoa is given elsewhere [10]. Diets were prepared from an AIN-93G formulation (Panlab S.L., Barcelona, Spain). The 12% cocoa diet was produced by adding 120 g/Kg cocoa to AIN-93G. This powdered supplement was formulated to provide 1 g of polyphenols per kg of diet. A similar cocoa supplementation protected liver cells against oxidative stress by activating the antioxidant defence system [11]. The composition of the diets is given in Supporting Information, Table 1. Rats were fed with control or cocoa-enriched diets during eight weeks and injected with saline or AOM (20 mg/Kg bw) during the second and third week (see experimental protocol in Figure 1 in Supporting Information). At the end of the experiment, colon samples were evaluated for aberrant crypt foci (ACF) formation, and markers of oxidative stress, proliferation and apoptosis (See Material and Methods in Supporting Information). All animals had a steady body weight gain during the treatment and the administration of AOM did not affect the growth of the rats (Table 2 in Supporting Information). Interestingly, the body weight of animals fed with cocoa diet was slightly but significantly reduced as compared to control groups (about 10%). As described elsewhere [12,13], this effect seems to be attributed to the cocoa polyphenolic fraction and its ability to reduce fat adipose tissue. Animals were treated according to the Institutional Care Instructions (Bioethical Commission from Consejo Superior de Investigaciones Cientificas, CSIC).

Administration of the colon specific carcinogen AOM to rodents induces the development of aberrant crypt foci (ACF), preneoplastic lesions that may progress into cancer later on [14]. Therefore, we first investigated the efficacy of cocoa enriched diet on inhibiting AOM-induced ACF formation. We found that all the rats injected with AOM developed aberrant crypts; however, the cocoa enriched diet significantly reduced the AOM-induced ACF formation (Figure 2 in Supporting Information). More importantly, the number

of ACF with a crypt multiplicity of four or more, that have been suggested to represent a higher risk for malignant tumour progression [14], was largely reduced by cocoa feeding (Figure 1A).

Experimental studies with rodents have demonstrated that AOM can induce ACF formation in colon by causing oxidative stress leading to DNA damage and mutations in cancer related genes [15-17]. Due to the reported antioxidant effects of cocoa and their flavonoids, oxidative stress biomarkers in the distal colon of rats were evaluated. As shown in Figure 1B, the levels of carbonyl groups (marker of protein oxidation) and malondialdehyde (MDA), a lipid peroxidation end product, were significantly higher in the distal colon of control AOM-treated rats as compared to the untreated control group whereas both increases were prevented in the AOM-treated rats fed with cocoa. These results suggest that the antioxidant properties of cocoa could prevent the colonic oxidative damage induced by AOM. Supporting this, different natural antioxidant compounds have been found to prevent the generation of reactive oxygen species (ROS) and thereby inhibit AOM-induced colon carcinogenesis in animal models [18].

AOM-induced oxidative stress lowered the endogenous antioxidant/detoxificant defence capacity in colon tissues of control rats, as evidenced by the significantly decrease in glutathione (GSH) levels and in the activity of its related enzymes glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST) (Figure 1C and 1D). Nevertheless, the levels of enzymatic and non-enzymatic defences were preserved in the colon of cocoa-fed animals. Since GSH and its related enzymes participate in the detoxification of xenobiotics, carcinogens, free radicals and peroxides [19], we can suggest that cocoa could prevent AOM-induced ACF formation by reinforcing the endogenous defence capacity in colon tissues to counteract carcinogen-induced toxicity. These results are

consistent with recent reports indicating that promotion of antioxidant enzyme activity has a chemopreventive effect on CRC [15,20].

Besides inducing oxidative damage and genomic instability, ROS can specifically activate certain redox-sensitive signalling pathways and contribute to CRC initiation/promotion through the regulation of cellular proliferation and survival [21]. The phosphatidylinositol 3-kinases/protein kinase B (PI3K/AKT) and the extracellular regulated kinase/mitogen-activated protein kinase (ERK/MAPK) are of the most important pathways activated in response to oxidative stress that promote carcinogenesis via target proteins involved in cell survival and cell cycle progression [2]. In this line, a recent *in vitro* study has showed that flavonoids such as luteolin and quercetin have anti-proliferative and pro-apoptotic effects in human CRC cells through the regulation of the ERK/MAPK and the PI3K pathways [22]. In the present work, the proliferating cell nuclear antigen (PCNA) assay was used to quantify the colonocyte proliferation. Microscopic examination of colonic tissue sections clearly showed an increased level of PCNA-positive cells in the colonic mucosa of AOM-treated rats fed with control diet and a higher percentage of proliferating cells per crypt column (Figure 2A). Indeed, the AOM-treated group fed with control diet exhibited an increase in the phosphorylation of ERKs and AKT together with an increased expression of the proliferative marker cyclin D1 (Figures 2B and C). Conversely, 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 signalling pathways involved in proliferation and thereby the progression of preneoplasic lesions in the colonic epithelial cells.

Modulation of apoptosis provides an additional protective mechanism against intestinal neoplasia. Therefore, the apoptotic process in colonic samples from rats by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining was further

investigated. The representative photographs for TUNEL positive cells clearly showed a pro-apoptotic effect of dietary cocoa in the colonic tissue of AOM-treated rats (Figure 3A). Besides, the majority of the apoptotic cells were localized on the luminal surface, implicating that cocoa feeding could ensure the reestablishment of homeostasis in the colonic mucosa by removal of damaged cells. To better characterize the mechanisms involved, the colonic expression of pro-apoptotic (Bax) and anti-apoptotic (Bcl-x_L) members of the Bcl-2 protein family as well as the activity of caspase-3 was also examined. As shown in Figure 3B and C, AOM-treatment in control rats decreased Bax expression along with a concomitant increase in Bcl-x_L proteins. However, the cocoa enriched diet was able to increase Bax expression and decrease Bcl-x_L proteins levels. Similarly, the activity of caspase-3 was significantly increased in the AOM group that was fed with the cocoa diet (Figure 3D). These findings indicate that cocoa enriched diet also induces apoptosis in colonic tissues and that the mitochondrial pathway participates in this process of programmed cell death. These data agree with recent results illustrating the apoptotic effect as a major mechanism for chemoprevention of different polyphenolic plant constituents [18,23].

Altogether, here we have shown for the first time that a cocoa enriched diet may suppress the early phase of chemically induced colon carcinogenesis probably by preventing oxidative stress and the subsequent activation of redox-sensitive signalling pathways involved in cell proliferation and pre-neoplastic condition. Interestingly, cocoa diet was also able to induce apoptosis as another complementary mechanism of chemoprevention during the progression of carcinogenesis. Taken together, our data provide evidence that consumption of cocoa would offer a natural therapeutic approach to improve individual health status including potential efficient cancer prevention with minimal toxicity.

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The authors have declared no conflict of interest.

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Legends to Figures

Figure 1.- Number of crypt multiplicity of ACF (A), MDA and Carbonyl levels (B), enzyme activity of GPx, GR and GST (C) and GSH levels (D) in colon tissues from rats injected with saline or AOM and fed with control or cocoa enriched diet. The data represent mean ± SD value from 10-12 rats in each group. Results of crypt multiplicity were statistically analyzed with Student's t test (*, p<0,05; **, p<0,01; compared to the AOM control group). In the rest of comparisons, one-way ANOVA was used. Means without a common letter differ, $P< 0.05$.

Figure 2.- Representative photographs for immunohistochemical staining of PCNA positive cells (400X magnification) and proliferative index (A) in colon tissues from rats injected with saline or AOM and fed control or cocoa enriched diet. Representative Western blot analyses (B) and percentage levels of total and phospho-ERKs and -AKT and cyclin D1 (C) in colon tissues from the indicated groups. Bars represent mean ± SD value from at least 10 rats in each group and for three independent experiments with two samples each in the Western blot analysis. Means without a common letter differ, $P< 0.05$.

Figure 3.- Representative photographs for immunohistochemical staining of TUNEL positive cells (400X magnification) and apoptotic index (A) in colon tissues from rats injected with saline or AOM and fed with control or cocoa enriched diet. Representative Western blot analyses (B), percentage levels of Bax and Bcl-x_L (C) and caspase-3 activity (D) in colon tissues from the indicated groups. Bars represent mean ± SD value from at least 10 rats in each group and for three independent experiments with two samples each in the Western blot analysis. Means without a common letter differ, $P< 0.05$.

Figure 1

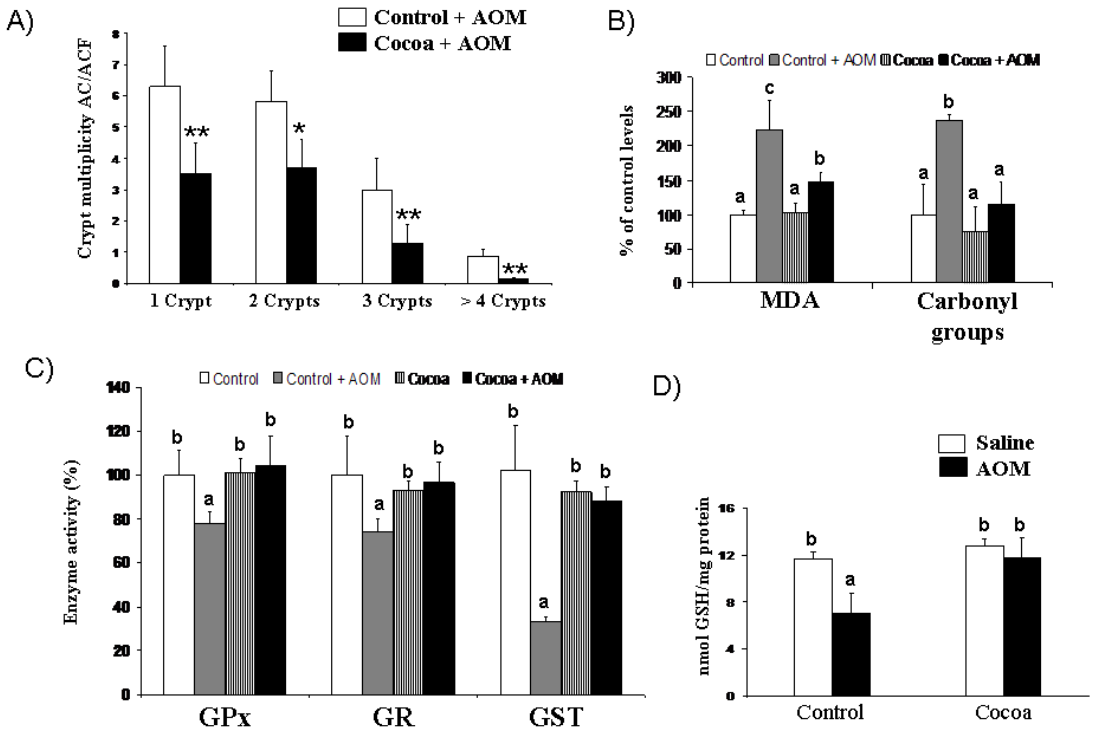

A)
Control + AOM
Cocoa + AOM
Crypt multiplicity AC/ACF
1 Crypt
2 Crypts
3 Crypts
> 4 Crypts
B)
Control
Control + AOM
Cocoa
Cocoa + AOM
% of control levels
MDA
Carbonyl groups
C)
Enzyme activity (%)
GPx
GR
GST
D)
Saline
AOM
nmol GSH/mg protein
Control
Cocoa

Figure 2

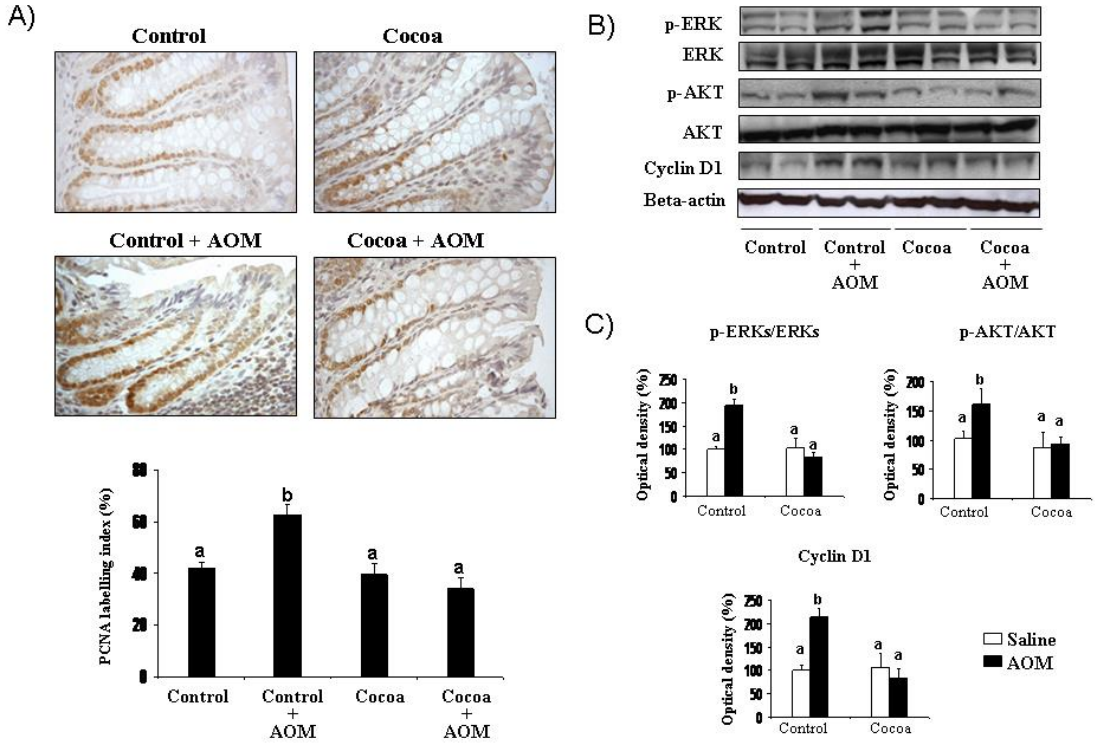
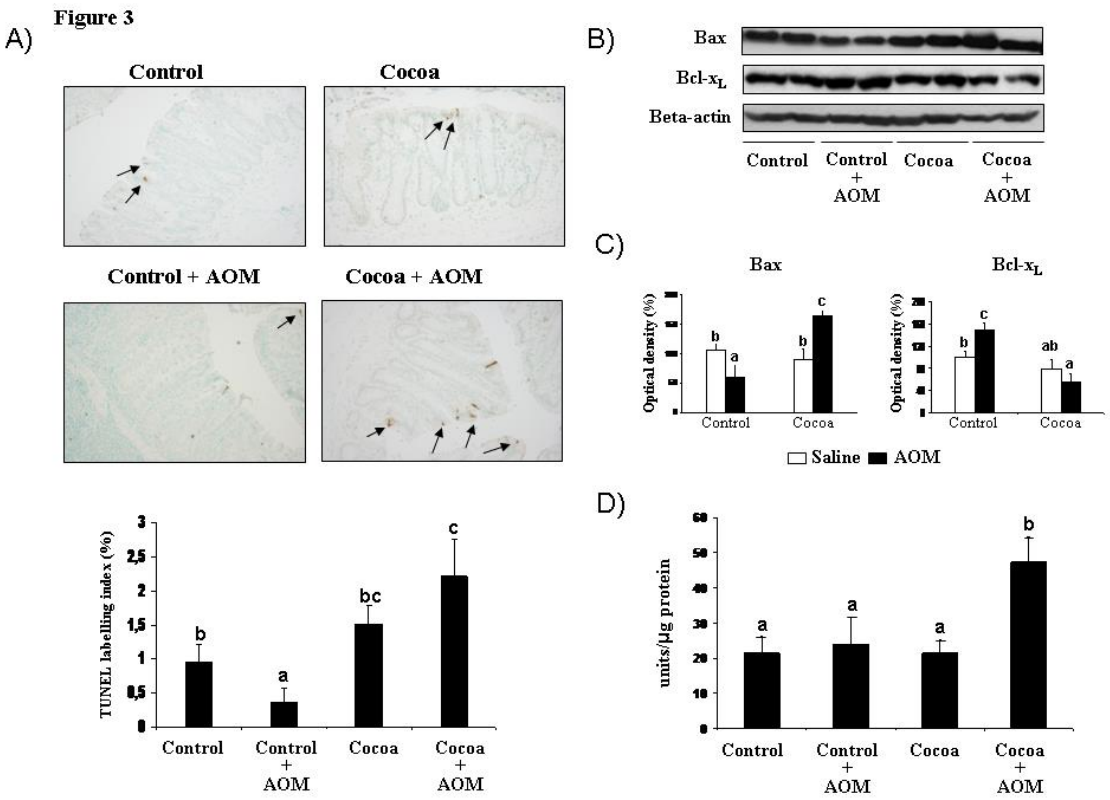

A)
Control
Cocoa
Control + AOM
Cocoa + AOM
PCNA labelling index (%)
a
b
a
a
Control
Control + AOM
Cocoa
Cocoa + AOM
B)
p-ERK
ERK
p-AKT
AKT
Cyclin D1
Beta-actin
Control
Control + AOM
Cocoa
Cocoa + AOM
C)
p-ERKs/ERKs
p-AKT/AKT
Optical density (%)
Control
Cocoa
Cyclin D1
Saline
AOM

Figure 3


A)
Control
Cocoa
Control + AOM
Cocoa + AOM
TUNEL labelling index (%)
b
a
bc
c
Control
Control + AOM
Cocoa
Cocoa + AOM
B)
Bax
Bcl-x$_L$
Beta-actin
Control
Control + AOM
Cocoa
Cocoa + AOM
C)
Bax
Bcl-x$_L$
Optical density (%)
Control
Cocoa
Saline
AOM
D)
units/µg protein