Eur J Nutr 38:126–132 (1999)
© Steinkopff Verlag 1999

ORIGINAL CONTRIBUTION

Giovanna Caderni
Sophie Remy
Veronique Cheynier
Guido Morozzi
Piero Dolara

Effect of complex polyphenols on colon carcinogenesis

Received: 18 January 1999
Accepted: 18 May 1999

Giovanna Caderni (✉) · P. Dolara
Department of Pharmacology
Viale Pieraccini 6
I-50139 Florence
Italy

S. Remy · V. Cheynier
Research Unit Biopolymers and Aromas
INRA-ISVV
2 Place Viala
F-34060 Montpellier cedex
France

G. Morozzi
Department of Cellular
and Molecular Biology
Via Del Giochetto
I-06100 Perugia
Italy

Abstract *Background:* Complex polyphenols and tannins from wine (WCPT) are being considered increasingly as potential cancer chemopreventive agents, since epidemiological studies suggest that populations consuming a high amount of polyphenols in the diet may have a lower incidence of some types of cancer.
Aim of the study: We studied the effect of WCPT on a series of parameters related to colon carcinogenesis in rats.
Methods: WCPT were administered to F344 rats at a dose of 14 or 57 mg/kg/d, mixed with the diet. The higher dose is about ten times the exposure to polyphenols of a moderate drinker of red wine. In rats treated with WCPT, we measured fecal bile acids and long chain fatty acids, colon mucosa cell proliferation, apoptosis and, after administration of colon carcinogens, the number and size of aberrant crypt foci (ACF) and nuclear aberrations.
Results: Colon mucosa proliferation was not varied by chronic administration (90 d) of WCPT (14 or 57 mg/kg/d). The highest dose of WCPT decreased the number of cells in the colon crypts, but did not increase apoptosis. WCPT (57 mg/kg) administered before or after the administration of azoxymethane (AOM) did not vary the number or multiplicity of ACF in the colon. The number of nuclear aberrations (NA) in colon mucosa was studied after administration of 1,2-dimethylhydrazine (DMH) and 2-amino-3-methylimidazo (4,5-f)quinoline (IQ), colon-specific carcinogens which require metabolic activation. The effect of DMH and IQ was not varied by pre-feeding WCPT (57 mg/kg) for 10 d. Similarly, the levels of total, secondary bile acids and long chain fatty acids did not varied significantly in animals fed WCPT for 90 d.
Conclusions: WCPT administration does not influence parameters related to colon carcinogenesis in the rat.

Key words Colon carcinogenesis – wine polyphenols – ACF – nuclear aberrations – bile acids

Introduction

Colon cancer is one of the principal neoplastic diseases in the western world (1, 2). It is generally agreed that the composition of the diet plays a fundamental role in the induction of this type of cancer (1, 2). In the last few years, a great deal of attention has been dedicated to the study of foods which might modify the development of colon cancer, by affecting the induction or promotion phase of the carcinogenesis process.

Among various potentially interesting food components, complex polyphenols and tannins from wine (WCPT) have been considered with interest, since epidemiological studies suggest that populations exposed to polyphenols in beverages like green tea have a lower incidence of certain cancers (2). Some experimental studies have also shown a protective effect of green tea on colon carcinogenesis (3-5). On this basis, we studied the actions of WCPT in the rat using a battery of short-term assays which are utilized to assess potential chemopreventive agents.

Diet can affect colon carcinogenesis by increasing cell proliferation (6-8), a factor of risk for carcinogenesis; diet can also alter the genotoxic effect of carcinogens on the colon mucosa cells (effect on the initiation phase); finally, it can modify the growth and progression of cells initiated by carcinogens (effect on the promotion phase).

In this paper we report the effect of WCPT on colon mucosa proliferation, on the occurrence of aberrant crypt foci (ACF), putative pre-neoplastic lesions in the colon (9) and on the number of nuclear aberrations (NA) induced by colon carcinogens (10, 11). Variations in the last two parameters describe effects on the initiation phase. We also studied the effect on the promotion phase of carcinogenesis by monitoring the multiplicity of ACF in animals chronically fed WCPT. Since bile acids, and possibly long chain fatty acids, are colon co-carcinogens and promoters (12), we also measured the variations in fecal bile acids and long chain fatty acids in animals fed WCPT.

Methods

Preparations of complex polyphenol and tannin fractions from wine

A “wine complex polyphenol and tannin” (WCPT) powder, containing wine polymeric polyphenols (i.e., proanthocyanidins from grape and various polyphenolic reaction products formed from grape components in the course of wine making) and free from low mass phenols, was obtained by using the following procedure. Two-year old red wine made from Cabernet Sauvignon, vintage 1994 at the Arzens Cooperative winery (Arzens, Aude, France) was first de-alcoholized under vacuum, filtered to remove tartaric precipitates, and deposited onto a Relite Diaion column (Mitsubichi, Japan). After washing with water to remove sugars and organic acids, the wine ‘phenolic pool’ was recovered with 90% ethanol, concentrated under vacuum and atomized. Batches of the phenolic powder, thus, obtained (380 g) were dissolved in water and chromatographed on a Toyopearl TSK HW-50 (F) column (TosoHaas, Stuttgart, Germany). The low molecular mass phenols were eluted with a mixture of ethanol:water:trifluoroacetic acid (55:45:0.005, v:v:v), and the polymeric fraction with 60% acetone in water as described earlier (13). The acetone fractions containing the wine polymeric tannins were pooled, concentrated under vacuum and atomized, yielding the WCPT powder (0.8 g per liter of wine processed).

HPLC analysis of the WCPT sample showed that it contained trace amounts of flavanol aglycones (6 mg/g) but no free anthocyanins, flavanol monomers nor phenolic acids. Genuine proanthocyanidins represented approximately half (498 mg/g) of the material present in the WCPT powder, the other half presumably consisting of ‘derived tannins’, as expected in a two-year old red wine. Their composition, determined by thiolysis (13), was qualitatively similar to that of the wine proanthocyanidins, with the following distribution of constitutive units: 18.1% catechin, 60.7% epicatechin, 3.3% epicatechin 3-O-gallate, and 17.9% epigallocatechin, and a mean degree of polymerization (mDP) of 6.3.

Animals

Male Fischer 344 rats (100 g at the beginning of the experiment) were purchased from Nossan (Correzzana, Milan, Italy). After their arrival from the supplier, animals were quarantined for 1 week, in which they were fed a standard lab chow. The rats were then shifted to a high-fat (HF) diet whose composition is based on the AIN76 diet, modified to contain a high amount of fat (230 g/kg corn oil w/w), a low level of cellulose (20 g/kg w/w), and a low level of calcium (1.3 g/kg w/w), in order to mimic the diet typical of western human populations at high risk for colon cancer (14). Dietary components were purchased from Piccioni (Gessate, Milan, Italy).

Colon mucosal proliferation

Rats were treated s.c. with 2 injections (one week apart) of AOM (15 mg/kg, total dose 30 mg/kg) or saline. One week after the last injection with AOM, rats were shifted to the different dietary treatments: control rats were fed the HF diet for 90 d whereas treated rats were given the HF diet supplemented with WCPT (14 and 57 mg/kg). At the end of this period, colonic mucosal proliferative activity was assessed in mucosal biopsies obtained after sacrifice and measured as ^{3}H-thymidine incorporation *in vitro* and autoradiography (14).

© Steinkopff Verlag 1999

Apoptosis in colonic mucosa

A morphological evaluation of apoptosis was carried out in paraffin embedded sections stained with Feulgen-Fast Green. For each rat, twenty full longitudinal crypt sections were scored at the microscope, determining in each crypt the presence of cells with the following characteristics of apoptosis: cell shrinkage and loss of normal contact with the adjacent cells of the crypt, chromatin condensation or formation of nuclear fragments round or oval in shape (known as "apoptotic bodies"). When clusters of more than one apoptotic body were seen within the diameter of one cell, these bodies were considered as fragments of one apoptotic cell. Apoptosis was quantified as apoptotic index and expressed as number of apoptotic cells/cells scored x100.

Modification of chemically-induced colon carcinogenesis

Experiments on initiation

F 344 male rats were fed the HF diet (controls, n=15) and treated rats (n=16) were fed the same diet supplemented with WCPT (57 mg/kg/d). After 10 d the animals of both groups were treated s.c. with a single dose of AOM (20 mg/kg). The number and dimensions of ACF in both groups were evaluated 30 d after administration of the carcinogen (9, 14).

Experiments on promotion

F344 male rats were fed a HF diet for 7 d and were then administered AOM (15 mg/kg) s.c. twice with a week interval. One week after the last injection with AOM, the rats were shifted to the different dietary treatments: control rats (n=14) were fed the HF diet for 90 d whereas treated rats were given the HF diet supplemented with WCPT (14 and 57 mg/kg). The number and dimension of ACF in both groups were evaluated in the animals 90 d after administration of the carcinogen (9). Rats were sacrificed 90 d after the carcinogen administration, and the number and dimension of ACF were evaluated in both groups (9).

Induction of nuclear aberrations (NA) by colon carcinogens

F344 male rats were treated by gavage with saline or with WCPT for 10 d and were given the indicated dose of carcinogen 24 h before sacrifice. All carcinogens were administered by gavage and dissolved in water except 2-amino-3-methylimidazo (4,5-f)quinoline (IQ) which was administered in water:ethanol (60:40) to make it soluble. All controls in the IQ group were administered the same volume of water:ethanol. NA were scored in coded samples on histological sections of colon mucosal crypt after coloring with Feulgen-fast green (10) using conventional microscopy.

Bile acid and long chain fatty acid analyses in fecal samples

Bile acid and long chain fatty acid were analysed by gaschromatography mass spectrometry in the faeces of rats treated with AOM and fed WCPT for 90 d at two dose levels (14 and 57 mg/kg), according to a previously described method (15). We used the freshly collected fecal droppings of the animals of the promotion experiment on ACF described above. After collection the fecal samples were frozen at -80 °C and stored until analysis.

Statistical analysis

Data obtained from individual rats in the different dietary groups were analyzed with one-way ANOVA by calculating the contrasts between means with the Least Significant Difference method (LSD) using the Statgraphics Statistical Package (Statistical Graphic Corporation, Rockville, MD, USA). Differences were considered statistically significant when the probability level P was <0.05.

Results

Colon mucosal proliferation

For these experiments we used two levels of WCPT. The highest level used in the experiments (57 mg/kg) was equivalent to a dosage inbibed by a human of 70 kg injesting polyphenolic material of 5 l of red wine (about 0.8 g/ l). The results are shown in Fig. 1. After 90 d of feeding, a relatively long treatment period for a rodent, WCPT did not significantly modify mucosa proliferative activity, as expressed by the labelling index (Fig. 1, panel a). The distribution of labelled cells along the crypt was not varied either (data not shown). These parameters are all connected with variations of colon cancer risk (6, 8). We only observed a small, although significant, reduction in the number of cells/crypt with the highest dose of WCPT (Fig. 1, panel b), an effect of unknown biological significance in terms of cancer risk. This last result could be explained by an increased apoptosis in the intestinal mucosa, a factor which has been interpreted as protective in terms of cancer risk (16), leading damaged cells towards programmed cell death. However, as shown by Fig. 2, the level of apoptosis in the colon mucosa did not differ from baseline after feeding WCPT for 90 d.

Carcinogen-induced colon cancer pre-neoplastic lesions

We then studied the effects of WCPT on the initiation of carcinogen-induced colon cancer lesions such as ACF. WCPT did not influence the number of ACF or ACF multiplicity (mean number of aberrant crypts in ACF, ex-

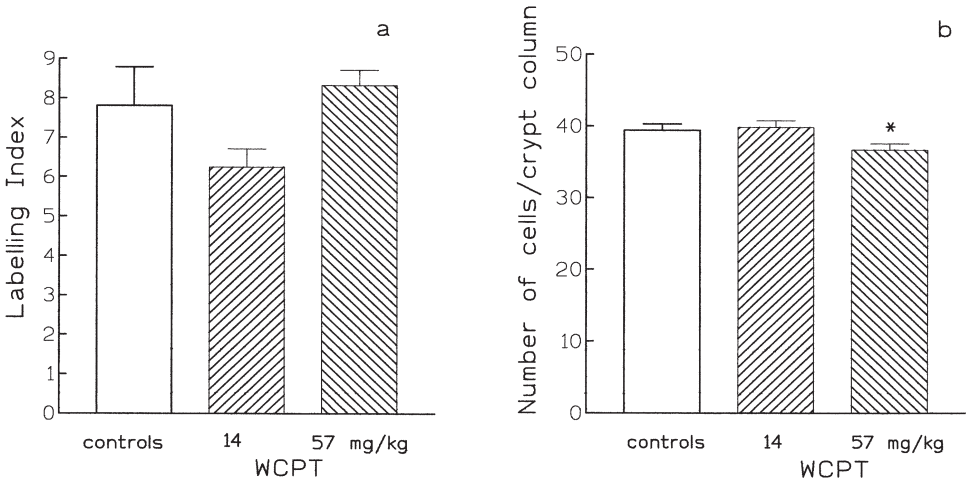
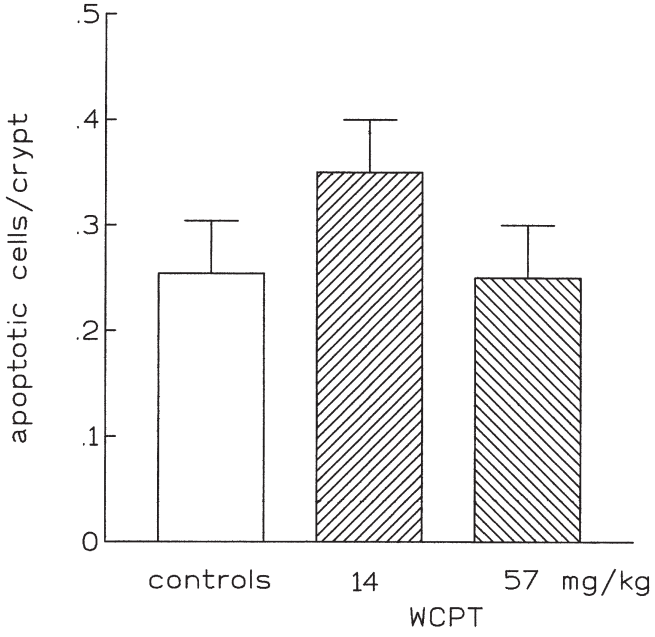


Fig. 1 Mucosal proliferative activity expressed as labelling index (a) or number of cells/crypt column (b) in AOM-treated rats fed HF diet (controls) or HF diet supplemented with WCPT (14 mg/kg or 57 mg/kg) for 90 d. Values are means + S.E.* P<0.05 when compared to controls.

pressed as AC/ ACF), when given 10 d before the carcinogen (Fig. 3) or in the promotion phase or when given for 90 d after carcinogen administration (Fig. 4).

On the basis of these results, WCPT did not seem to affect colon carcinogenesis induced by AOM. However, WCPT may have effects on the activation and deactivation of colon carcinogens, which might not be evident using AOM, a compound requiring two oxidation steps to be transformed into the ultimate carcinogen. To resolve this problem, we studied the effect of 1,2-dimethylhydrazine (DMH), a precursor of AOM, which is transformed into AOM by oxidation catalysed by monoxygenases and 2-amino-3-methylimidazo(4,5-f)quinoline (IQ), which requires oxidation, acetylation, and conjugation with sulphuric acid in order to produce genotoxic derivatives; we also used 4-aminobiphenyl (4AB), an aromatic amine that requires hydroxylation and acetylation to become genotoxic.

Fig. 2 Number of apoptotic cells/crypt in AOM-treated rats fed HF diet (controls) or HF diet supplemented with WCPT (14 mg/kg or 57 mg/kg) for 90 d. Values are means + S.E.



The modulating effect of WCPT on the initiation phase of carcinogenesis with the above carcinogens was studied utilizing the "nuclear aberration" (NA) assay, in which the effect of carcinogens on the colon is measured by scoring the number of acute morphological toxic effects on the colon mucosa cells, 24 h after the administration of a carcinogen. This test has been demonstrated to be good indicator of carcinogenic potency of chemicals on the colon (11). The results of this experiment indicate that DMH and IQ induced an increase of NA over controls; however, the administration of WCPT did not vary the induction of NA by these two carcinogens (Fig. 5 and 6, respectively). On the contrary, 4-ABP did not induce NA in our experiment (data not shown), a result in disagreement with previous data (11). Negative results with this compound have also been reported by other authors (10). For brevity, this last set of data on 4-ABP will not be reported.

Bile acids and long chain fatty acids

We also studied the effect of WCPT on the levels of fecal bile acids and long chain fatty acids in the animals treated chronically with WCPT. The results, shown in Fig. 7, did not show any statistically significant variation in the total fecal bile acid profile, although there was a reduction at the highest dose. The levels of total long chain fatty acids were also reduced by WCPT, but the differences were not significant. Analysis of the individual bile acids revealed that beta and omega-muricholic acid were lower than control levels at the highest dose of WCPT (0.54±0.08 vs.

© Steinkopff Verlag 1999

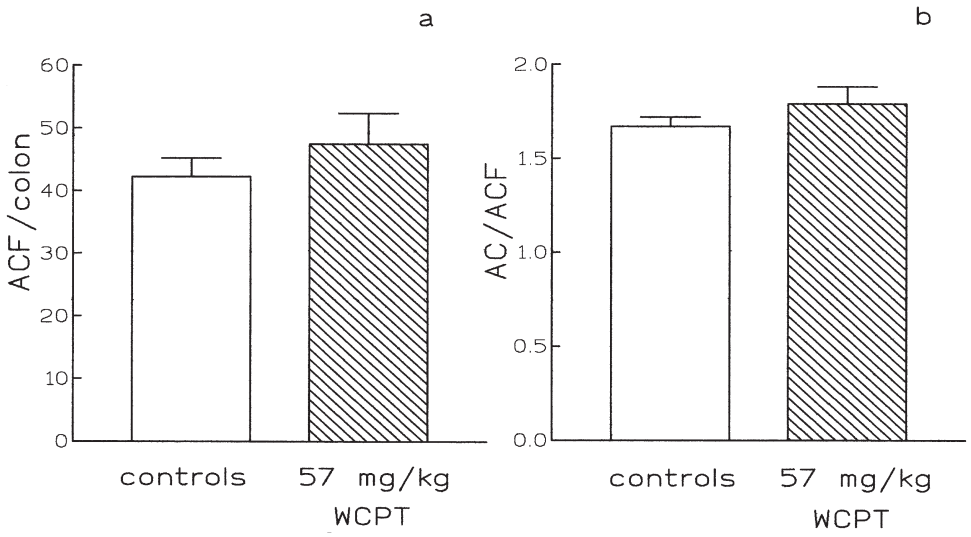


Fig. 3 Number of ACF/ colon (panel a) and multiplicity of ACF (AC/ACF, panel b) in control rats and in animals fed WCPT (57 mg/kg) 10 d before the administration of AOM. Data are means + S.E.

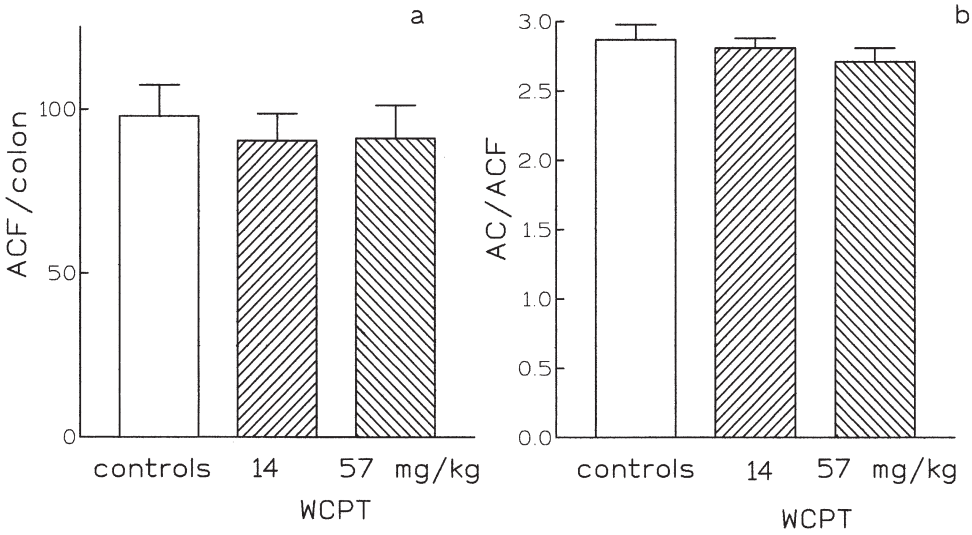


Fig. 4 Number of ACF/ colon (panel a) and multiplicity of ACF (AC/ACF, panel b) in control rats and in animals treated with WCPT (14 and 57 mg/kg) for 90 d after the administration of AOM (30 mg/kg). (n=14, in each group). The lag time after AOM was longer in these experiments than that reported in Figure 3. Data are means + S.E.

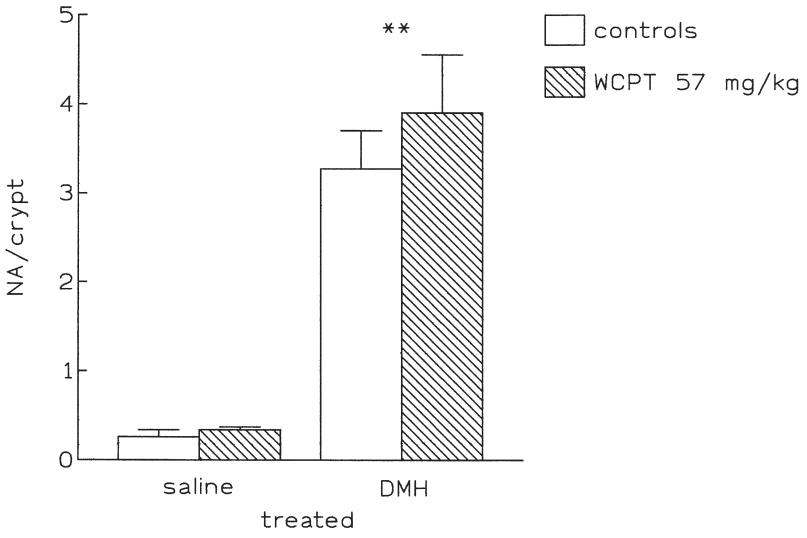


Fig. 5 Nuclear aberrations (NA) in colon mucosa of control rats and in animals fed HF diet supplemented with WCPT (57 mg/kg) for 10 d before the administration of DMH. NA were scored 24 h after carcinogen administration and are expressed as mean number of NA/ crypt + S.E.
**= p.01 relative to the saline-treated rats.

0.25± 0.05 and 0.86±0.14 vs. 0.36±0.1, respectively), but that of hyodeoxycholic acid was higher (0.11±0.04 vs. 0.28±0.06). These variations are probably of modest biological significance, since the total amount of secondary bile acids, which are supposed to act as co-carcinogens and promoters at the level of the colon (12, 17), was not changed significantly.

Discussion

In our experiments on F344 rats, the administration of WCPT, before or after the administration of AOM, did not affect a series of short-term and intermediate assays correlated to colon carcinogenesis.

We administered doses which are higher than ordinary human exposure to WCPT (of the order of 5 mg/kg for a moderate drinker), since they had no toxic effect on the animals and such high doses could be considered for human use if some chemopreventive effect were to be found – obviously not administered as wine – which would contain intoxicating amounts of ethanol.

Of all the parameters analysed relative to cell proliferation (number of cells/crypt, labelling index, distribution of the proliferative activity along the crypt), we observed a significant decrease only in the number of cells/crypt in rats treated with the highest dose of WCPT. The significance of the variations in this parameter in terms of colon cancer risk is not clear. In fact, some authors consider a low number of cells/crypt to be a protective factor, some others the reverse (18, 19). All the other parameters were not varied compared to controls. To have some important biological meaning this effect should be dose-dependent. The observed difference probably derives only from biological fluctuation. WCPT, therefore, do not decrease cell proliferation in the colon, a factor which has been interpreted as an indicator of chemoprevention (8).

We also controlled for the number of apoptotic cells/crypt in the rats treated with carcinogens and WCPT. It has been suggested (16) that increased apoptosis might protect against colon cancer by eliminating mutated or damaged cells in the mucosa. However, such an

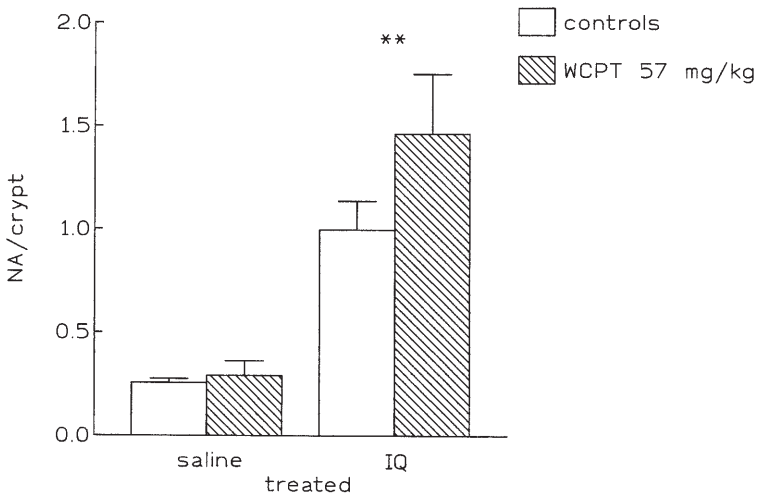


Fig. 6 Nuclear aberrations (NA) in colon mucosa after administration of IQ (250 mg/kg) to control rats and rats fed HF diet supplemented with WCPT (57 mg/kg) for 10 d. NA were scored 24 h after carcinogen administration and are expressed as a mean number of NA/ crypt +S.E. **= p<0.01 relative to the saline-treated rats.

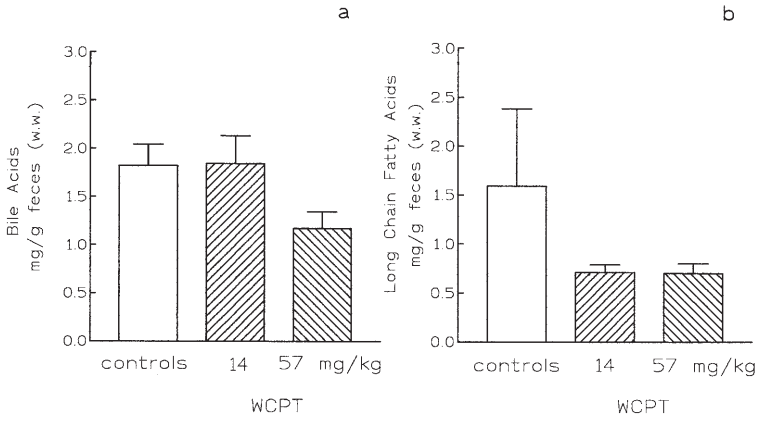


Fig. 7 Levels of fecal bile acids and long chain fatty acids in control rats and in rats treated with WCPT for 90 d. All animals had been administered AOM (30 mg/kg s.c.) 90 d before sacrifice. Values are means + S.E

© Steinkopff Verlag 1999

effect was not observed in the rats administerd AOM and fed WCPT.

We also carried out a series of assays which can predict the influence of a dietary supplement on the initiation phase (induction of ACF or NA) or in the promotion phase of colon carcinogenesis (growth of ACF). A potential chemopreventive agent should decrease the number of ACF or of NA or decrease the growth rate of ACF after carcinogen administration.

We observed none of these effects. The administration of WCPT does not modify these processes, induced with a variety of carcinogens (AOM, DMH, and IQ).

The supplementation with WCPT at the highest dose tends to decrease the total concentration of bile acids and of long chain fatty acids in the feces. However, these differences were not statistically significant. Bile acids are co-carcinogens and promoters at the level of the colon (13, 17). On the other hand, levels of secondary bile acids, supposedly the most toxic bile acids for the colon mucosa, are not modified by WCPT administration.

Polyphenolic compounds have been reported to protect against oxidative damage in different organs including the colon (3-5); therefore, it is possible that WCPT exert antioxidant effects on colonic mucosa. However, in the present set of experiments, we did not measure parameters related to oxidation damage.

Our data do not indicate that WCPT is an active chemopreventive agent using assays related to colon carcinogenesis in the rat. However, anti-tumour effects have been described in the literature using green tea extracts or red wine solids (4, 20); therefore, the possibility exists that low molecular weight phenols and flavonoids contained in crude extracts, may have cancer preventive activity. In conclusion, before excluding an effect on the colon of WCPT or crude extract from wine, long-term carcinogenesis experiments should be performed.

Acknowledgments This work was financially supported by grants from the European Community AIR (Agro Industrial Research) Program (Grant CT94/0933), European Community FAIR program (Grant No. CT95/0653), by the Ministero della Università e della Ricerca Scientifica e Tecnologica, and by the University of Florence, Italy. The authors acknowledge the skillful technical assistance of Ms. Maddalena Salvadori.

References

1. Potter JD, Slattery ML, Bostick RM, Gapstur SM (1993) Colon Cancer: A review of the epidemiology. Epidemiol Rev 15:499–545
2. World Cancer Research Fund and American Institute for Cancer Research (1997) Food Nutrition and the prevention of cancer:a global perspective. BANTA, Menasha, WI, USA
3. Yamane T, Hagiwara N, Tateishi M, Akachi S, Kim M, Okuzumi J, Kitao Y, Inagake M, Kuwata K, Takahashi T (1991) Inhibition of azoxymethane-induced carcinogenesis in rat by green tea polyphenol fraction. Jpn J Cancer Res 82:1336–1339
4. Narisawa T, Fukaura Y (1993) A very low dose of green tea polyphenols in drinking water prevents N-methyl-N-nitrosourea-induced colon carcinogenesis in F344 rats. Jpn J Cancer Res 84:1007–1009
5. Yamane T, Nakatani H, Kikuoka N, Matsumoto H, Iwata Y, Kitao Y, Oya K, Takahashi T (1995) Inhibitory effects and toxicity of green tea polyphenols for gastrointestinal carcinogenesis. Cancer 77:1662–1667
6 Risio M, Lipkin M, Newmark H, Yang K, Rossini FP, Steele VE, Boone CW, Keloff GJ (1996) Apoptosis, cell replication, and Western – style diet – induced tumorigenesis in mouse colon. Cancer Res 56:4910–4916
7. Stamp D, Zhang XM, Medline A, Bruce WR, Archer MC (1993) Sucrose enhancement of the early steps of colon carcinogenesis in mice. Carcinogenesis 14:777–779
8. Lipkin M (1988) Biomarkers of increased susceptibility to gastrointestinal cancer: new applications to studies of cancer prevention in human subjects. Cancer Res 48:235–245
9. Bird RP (1987) Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. Cancer Lett 37:147–151
10. Wargovich M, Golberg MT, Newmark HL, Bruce WR (1983) Nuclear aberrations as a short-term test for genotoxicity to the colon: evaluation of nineteen agents in mice. JNCI 71:133–137.
11. MacLellan EA, Bird RP (1988) Specificity study to evaluate induction of aberrant crypts in murine colons. Cancer Res 48:6183–6186
12. Reddy BS, Wynder EL (1977) Metabolic epidemiology of colon cancer. Fecal bile acids and neutral sterols in colon cancer patients and patients with adenomatous polyps. Cancer 39:2533–2539
13. Souquet JM, Cheynier V, Brossaud F, Moutounet M (1996) Polymeric proanthocyanidins from grape skins. Phytochemistry 43:509–512
14. Caderni G, Luceri C, Lancioni L, Tessitore L, Dolara P (1998) Slow-release pellets of sodium butyrate increase apoptosis in the colon of rats treated with azoxymethane, without affecting aberrant crypt foci and colonic proliferation. Nutr Cancer 30:175–181
15. Luceri C, Caderni G, Lancioni L, Aiolli S, Dolara P, Mastrandrea V, Scardazza F, Morozzi G. (1996) Effects of repeated boluses of sucrose on proliferation and on aom-induced aberrant crypt foci in rat colon. Nutr Cancer 25:187–196
16. Samaha, HS, Kelloff, GJ, Steele, V, Rao, CV, Reddy, BS (1997) Modulation of apoptosis by sulindac, curcumin, phenylethyl-3-methylcaffeate, and 6-phenylhexyl isothiocyanate: Apoptotic index as a biomarker in colon cancer chemoprevention and promotion. Cancer Res 57:1301–1305
17. Bull AW, Marnett LS, Dame EJ Nigro ND (1983) Stimulation of deoxythymidine incorporation in the colon of rats treated intrarectally with bile acids and fats. Carcinogenesis 4:207–210
18. Newmark HL, Lipkin M, Maheshwari N (1990) Colonic hyperplasia and hyperproliferation induced by a nutritional stress diet with four components of western style diet. JNCI 82:491–496
19. Lipkin M, Uehara K, Winawer S, Sanchez A, Bauer C, Phillips R, Lynch HT, Blattner WA, Fraumeni JF (1985) Seventh-day adventist vegetarians have a quiescent proliferative activity in colonic mucosa. Cancer Lett 26:139–144
20. Clifford AJ, Ebeler SE, Ebeler JD, Bills ND Hinrichs SH, Teissedre PL, Waterhouse AL (1996) Delayed tumor onset in transgenic mice fed an amino acid-based diet supplemented with red wine solids. Am J Clin Nutr 64: 748–756