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ORIGINAL CONTRIBUTION

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Effect of polyphenolic extracts from red wine and 4-OH-coumaric acid on 1,2-dimethylhydrazine-induced colon carcinogenesis in rats

Summary *Background* Total polyphenolic extracts from red wine protect against azoxymethane (AOM)-induced colon carcinogenesis in rats. Since red wine contains more than 200 different polyphenolic compounds, it is still unclear which substances are responsible for this effect. *Aims of the study* We investigated the effect of high molecular weight polyphenols (HMWP), low molecular weight polyphenols (LMWP) and total polyphenolic extracts from red wine (WE) on colon carcinogenesis. We also tested the effect of 4-OH-coumaric acid, a potent phenolic antioxidant present in wine and fruit. *Methods* F344 rats were treated weekly with 1,2-dimethylhydrazine (DMH) (30 mg/kg b. w. subcutaneously x 10 times). One week after the final DMH injection rats were divided into five groups and fed: a) a high fat (HF) diet containing 23 % corn oil (w/w), as control or the same basal diet supplemented with b) 0.11 % (w/w) WE; c) 0.027 % (w/w) HMWP d) 0.083 % (w/w) LMWP or e) 0.1 % (w/w) 4-OH-coumaric acid. The dietary treatments continued until sacrifice, 16 weeks after the last DMH injection. *Results* WE treated rats had significantly fewer ($p < 0.05$) colorectal adenomas than controls, while rats in other treatment groups did not differ significantly from controls (colorectal adenomas/rat were: 2.2 ± 0.3; 1.4 ± 0.2; 2.9 ± 0.5; 2.6 ± 0.4; 2.3 ± 0.3; in controls, WE, HMWP, LMWP and 4-OH-coumaric acid groups, respectively; means ± SE). The mean number of colorectal carcinomas per rat was similar among all experimental groups. Proliferative activity in the normal colon mucosa did not vary among experimental groups. *Conclusions* Total polyphenolic extracts (WE) from red wine, but neither the HMWP nor the LMWP, have some inhibitory effect on the process of colon carcinogenesis by DMH reducing the number of adenomas.

Key words red wine – polyphenols – 1,2-dimethylhydrazine – 4-OH-coumaric acid – colon carcinogenesis – cell proliferation

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Abbreviations

DMH	1,2-dimethylhydrazine
WE	total polyphenolic wine extract
HMWP	high molecular weight polyphenols
LMWP	low molecular weight polyphenols
4-OH-CA	4-OH-coumaric acid

Introduction

Diet is related to the development of colon cancer [1] and polyphenols have been proposed as one of the agents responsible for a lower risk of colon cancer associated with the consumption of vegetables [2]. Red wine is an additional source of polyphenols and polyphenolic powders obtained from red wine have de-

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layed tumor onset in transgenic mice spontaneously developing neurofibroma-like tumors [3]. Moreover, consumption of polyphenols from wine could account for the lower risk of rectal cancer among wine drinkers, compared to consumers of beer and spirits [4]. Polyphenols may exert anticancer activity through their antioxidant properties [5], inhibiting cancer cell growth [6], inducing apoptosis [7] or by other unknown mechanisms.

Recently, we showed that total polyphenolic extracts from red wine (WE) inhibited colon carcinogenesis in azoxymethane (AOM)-induced rats [8]. However, red wine contains a wide range of different phenolics [9] and a protective effect has not been assigned to a specific fraction. Although experimental studies have shown some anticancer activity for some phenols present in red wine, like catechin [10, 11], resveratrol, quercetin and gallic acid [11] and high molecular weight polyphenols, like proanthocyanidins [12], it is not yet clear which compounds present in red wine are endowed with protective activity.

On this basis, in addition to total polyphenolic extracts from red wine (WE), we studied the effects of low (LMWP) and high (HMWP) molecular weight polyphenols. The development of colonic tumors was initiated in F344 rats using 1,2-dimethylhydrazine (DMH).

In the same experiment, since oxidative damage might be related to carcinogenesis [13], we tested the effect of 4-OH-coumaric acid, a compound found as tartaric ester in wine [9], a strong antioxidant capable of reducing basal oxidative DNA damage in rat colonic mucosa also *in vivo* [14].

Methods

Chemicals

1,2-dimethylhydrazine dihydrochloride (DMH) and 4-OH-coumaric were purchased from Aldrich (Milan, Italy). Dietary components were obtained from Piccioni (Gessate, Milan, Italy).

Wine extracts preparation

Wine polyphenolic extracts were prepared in 1998 from a 1997 red wine made by classical wine making procedures, from *V. vinifera*, var. *Syrah*, in Nîmes, France. The process used to prepare the wine polyphenolic extracts was described by Caderni and coworkers [15], except that the dealcoholization process was omitted and elution was performed with methanol. The methanol effluent was then concentrated and atomized. Further fractionation of this extract, to obtain low molecular weight polyphenols (LMWP) and high molecular weight polyphenols (HMWP), was performed as described previously [16].

The analysis of phenolic composition of wine and extracts was performed as described by Fulcrand and coworkers [16]. Wine proanthocyanidin composition was determined by thiolysis of the acetonic fraction obtained after a fractionation performed on Toyopearl TSK gel HW-50(F) as described earlier [17]. The resulting solution was then analyzed by HPLC. Identification of flavanol monomers and of the corresponding benzylthioethers derivatives was based on standard retention times established earlier [17, 18].

Wine extracts composition

The total wine polyphenolic extract (WE) was composed of 75 % (w/w) of LMWP and 25 % (w/w) of HMWP. WE contained 5.1 % (w/w) anthocyanins, 0.3 % flavanols, 2.3 % phenolic acids and 26 % proanthocyanidin units, consisting of 18 % epigallocatechin, 10.4 % catechin, 66.9 % epicatechin and 4.8 % epicatechin gallate, with a mean degree of polymerization of 8.5. Together, these compounds accounted for about 33 % by weight of the WE. The WE also contained 1.8 % polysaccharides and 2.4 % proteins, 4.4 % potassium (present mostly as tartrate), 15 % of organic acids and 0.8 % glycerol. Derived phenols formed during the course of wine-making and ageing may account for at least a part of the remainder.

LMWP was mainly composed of monomers and some oligomeric compounds, for example proanthocyanidin dimers and derived oligomeric compounds [17], while HMWP was similar to WE but devoid of polyphenol monomers. Neither LMWP nor HMWP contained water soluble compounds (salts, organic acids, polysaccharides, proteins) which were still present in WE. The polyphenolic composition of WE used in this experiment was similar to that used in previous work [8].

Animals

Male F344 rats were obtained from Nossan, Correzzana, Milan, Italy, housed in plastic cages with wire tops, maintained at a temperature of 22 °C, with a 12-h light-dark cycle. Animal care followed the European Union Regulations on the Care and Use of Laboratory Animals [20]. The experimental protocol was approved by a local Ethical Committee for Animal Experimentation, Florence, Italy and by the Commission for Animal Experimentation of the Ministry of Health, Rome, Italy. Following their arrival from the supplier, animals (n = 108) were quarantined for 1 week, during which they were fed standard lab chow (Teklad Global diet 18 % protein, Har-

lan, Correzzana, Italy). They were then transferred to a HF diet, the composition of which was based on the AIN76 diet, modified to contain a high level of fat (23 % corn oil w/w), a low level of cellulose (2 % w/w) to mimic the high risk of colon cancer in human populations consuming high fat diets [8]. Rats then received 10 injections (s. c.) of DMH (30 mg/kg body weight; dissolved in sterile saline and buffered with sodium hydroxide at neutral pH) at 1 week intervals (total dose 300 mg/kg body weight). One week after the final DMH injection, rats were randomly allocated to five experimental groups: a) Controls (n = 24), with HF diet alone; b) the WE group (n = 22), fed the HF diet supplemented with 0.11 % (w/w) WE; c) the HMWP group (n = 20), fed HF diet supplemented with 0.027 % (w/w) HMWP; d) the LMWP group (n = 20), fed HF diet supplemented with 0.083 % (w/w) LMWP; and e) the 4-OH-coumaric acid group (n = 22), fed HF diet supplemented with 0.1 % (w/w) 4-OH-coumaric acid, a dose previously used to assess the effect of potential chemopreventive agents [21].

The dosage of WE was similar to that used in a previous experiment [8]. The dietary levels of HMWP and LMWP were derived from the proportions of HMWP and LMWP present in WE. Diets were freshly prepared every two weeks, by mixing each of the red wine extracts or 4-OH-coumaric acid with the remainder of the components, and were stored at –20 °C. Rats were allowed food and water ad libitum and small stocks of diet were kept at room temperature to replenish feeders on alternate days.

Histopathological evaluation of the tumors

At sacrifice, all organs were macroscopically examined for the presence of tumors or other pathological lesions. Tissues with abnormal morphology were fixed in 10 % buffered formalin and embedded in paraffin blocks. Histological sections stained with hematoxylin and eosin were used to confirm the presence and type of tumors by histopathological examination, which was performed by a pathologist unaware of the experimental codes. Suspected macroscopic lesions were measured with callipers [8]. Tumors were evaluated on the basis of the histotype, grading and pattern of growth; adenomas were classified as tubular, tubulovillous and villous according to Morson et al. [22].

Determination of proliferative activity in colonic mucosa

The number of mitotic cells within colonic crypts was assessed using the isolated crypt technique as described [23, 24]. Briefly, samples (n = 10 for each experimental group) of fixed colonic mucosa with no gross pathology, were hydrated, hydrolyzed in concentrated HCl, stained with Feulgen's reagent and whole crypts isolated using a low-power dissecting microscope. Crypts were mounted in acetic acid:water (45:55) under cover slips, lightly compressed and examined for mitoses by medium-power bright-field light microscopy. All nuclei in prophase, metaphase, anaphase and telophase were recorded as mitoses. Using an eyepiece graticule the spatial distribution of mitotic events along the crypt was recorded by dividing the crypt into 5 approximately equal zones. A total of 10 crypts from each sample were assessed and the means calculated.

Statistical analysis

The data for the tumor occurrence were analyzed by fitting a Poisson regression model, as previously described [25]. Data for proliferative activity were analyzed by ANOVA with Tukey's treatment.

Results

The mean body weight of the rats at the beginning of the experiment was 74 g ± 0.9 (mean ± SE, n = 108). After the last DMH injection, the mean weight of the rats was 281 g ± 1.9. One week later the animals were randomly divided into 5 groups and placed on the experimental diets for 16 weeks. No significant differences in final body weight were found between any of the treatment groups (final b. w.: 380 g ± 9; 390 ± 5; 380 ± 9; 388 ± 9 and 393 ± 9 in the controls, WE, HMWP, LMWP and 4-OH-coumaric groups, respectively, means ± SE).

Tumor induction

All rats developed intestinal tumors, the majority of which were localized in the colon and rectum. They also developed squamous papillomas of the outer ear. Animals fed WE supplemented diet had significantly less ($p < 0.05$) adenomas in the colorectum compared to those on the control diet, while all other dietary treatments had no significant effect (Fig. 1 A). In addition, the degree of dysplasia in colorectal adenomas and their dimensions also did not differ among groups (data not shown). In contrast, the number of carcinomas in the colorectum, the degree of differentiation, dimensions and invasiveness of tumors did not vary between groups (Fig. 1 B and data not shown).

The number of tumors in the small intestine did not vary significantly; however, there was a tendency towards a decrease in number in the 4-OH-treated group (number of total tumors in the small intestine: 0.87 ± 0.17; 0.86 ± 0.16; 0.95 ± 0.17; 0.8 ± 0.17; 0.45 ± 0.16

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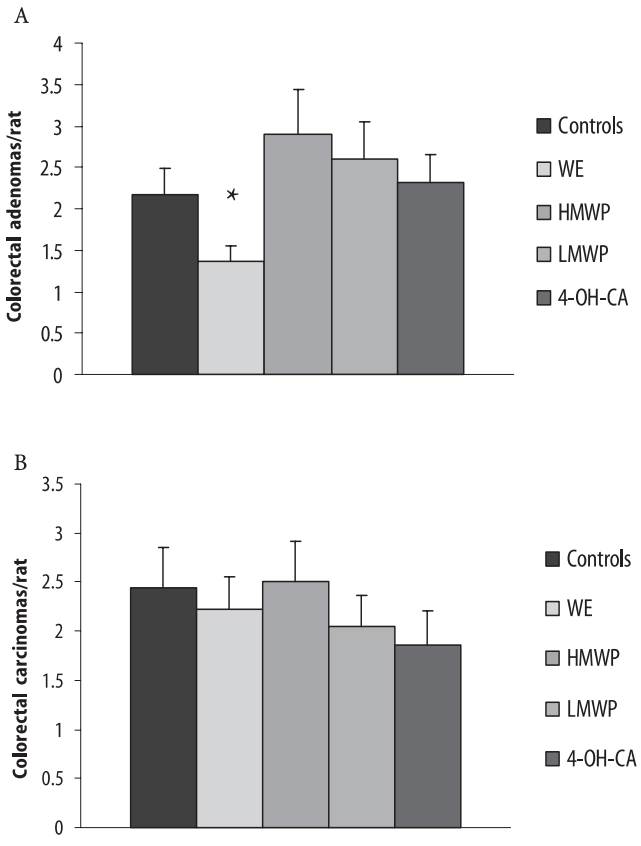

Fig. 1 **A** Effect of the different diets on the number of colorectal adenomas in rats previously treated with DMH. **B** Number of colorectal carcinomas/rat; Values are means ± SE (n = 24, 22, 20, 20, 22 in controls, WE, HMWP, LMWP and 4-OH-coumaric acid groups, respectively)

in controls, WE, HMWP, LMWP and 4-OH-coumaric acid groups, respectively; means ± SE).

The mean number of intestinal tumors/rat (i. e. adenomas and carcinomas in the colorectum and small intestine) was also very similar in response to all dietary treatments (5.5 ± 0.5; 4.7 ± 0.4; 6.3 ± 0.8; 5.4 ± 0.5; 4.7 ± 0.5 in controls, WE, HMWP, LMWP 4-OH-coumaric acid groups, respectively; means ± SE).

Proliferative activity in normal colon mucosa

There was no significant difference between groups in the mucosal proliferative activity, expressed as number of mitoses per crypt (Table 1). However, there was a trend towards increased proliferation in all experimental groups compared to the control diet. Spatial distribution of mitoses along the crypt was also unaffected by dietary treatments (data not shown).

Table 1 Effect of the different diets on the proliferative activity in normal colonic mucosa of rats previously treated with DMH. Data are means ± SE; n = 10

Experimental groups	Mitoses/crypt
Control	3.01 ± 0.18
WE	3.65 ± 0.33
HMWP	3.98 ± 0.24
LMWP	3.71 ± 0.47
4-OH-coumaric acid	3.63 ± 0.43

Discussion

The present results show that a diet supplemented with WE suppresses the formation of colorectal adenomas, although it has no effect on the total number of carcinomas in response to DMH treatment.

This effect of WE on adenomas rather than carcinomas is consistent with our previously reported data in AOM-treated rats given WE [8]. In the present experiment, at variance with previous data with AOM [8], we did not observe a significant reduction in the total number of colorectal tumors/rat (adenomas and carcinomas).

We suggest that this discrepancy might be due to a higher yield of carcinomas in the present experiment, probably due to a more aggressive protocol used for colon tumor induction (30 mg/kg DMH x 10 times compared to 7.4 mg/kg AOM x 10 times in the previous experiment). However, since colon cancer progress from adenoma to carcinoma [26], the ability of WE to reduce the number of adenomas indicates that it has potential to inhibit the process of carcinogenesis.

Neither HMWP nor LMWP, at the concentrations present in WE, reduced the incidence of colorectal adenomas or carcinomas. Evidently a polyphenol mixture is more active than separate components. These conclusions are in accordance with previous observations, showing that HMWP do not inhibit aberrant crypt foci (ACF), putative preneoplastic lesions [15].

Ebeler and coworkers [10] have suggested that red wine extracts reduce carcinogenesis in transgenic mice due to their catechin content. Similarly, other studies have suggested that compounds present in red wine, such as resveratrol and quercetin have anticarcinogenic activity [11, 28]. Our data indicate that a protective effect of wine polyphenols is better observed administering a complex mixture.

Antioxidant activity has been suggested as a mechanism for the carcinogenesis inhibition. However, 4-OH-coumaric acid, a potent DNA antioxidant *in vivo* [14], was virtually inactive in the present experiment.

Dietary components may inhibit colon cancer reducing proliferation in the colon mucosa [27]. Indeed, Gee et al. [28] demonstrated a reduction in the incidence of

ACF in the distal colon of rats fed a diet containing 0.1 % quercetin, six weeks post DMH treatment. In that experiment the carcinogen treatment was relatively mild and the protective effect of quercetin appeared to be associated with reduced mitosis in the colonic crypts. In our experiment the effect of red wine polyphenols was not associated with a significant variation in proliferative activity.

The induction of apoptosis in colonic tumors has been suggested as a possible mechanism of chemoprevention [29]. Accordingly, we previously showed that in the tumors from rats treated with WE, apoptosis is increased compared to control rats [8]. Moreover, the same tumors had lower levels of GST-P, GST-M2, COX-2 and i-NOS mRNA [30], enzymes which modulate the colon carcinogenesis process.

In addition, gut microflora modulation has been suggested as a possible mechanism of colon cancer chemoprevention. In fact, it has been demonstrated that some colonic bacteria, such as *Lactobacilli* and *Bifidobacteria* [31, 32], might protect against colon carcinogenesis. Thus it is worth noting that preliminary results obtained in our laboratory indicate that the diet containing WE beneficially affects microflora composition, increasing *Lactobacilli*.

Whether these or additional undiscovered mechanisms are responsible for the suppressive effects of polyphenols on tumor development is not yet clear.

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