Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats

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Abstract

Polyphenols from tea and other beverages such as red wine have been regarded with interest as possible chemopreventive agents against cancer. Here we report that red wine polyphenols (50 mg/kg) administered with the diet to F344 rats for 16 weeks inhibited colon carcinogenesis induced by azoxymethane (ADM, 7.4 mg/kg, total dose 74 mg/kg) or dimethylhydrazine (DMH, 30 mg/kg, total dose, 300 mg/kg). Polyphenol-treated animals had a consistently lower tumour yield compared to controls. In polyphenol-treated rats, the main bacterial strains in the faeces at sacrifice were Bacteroides, Lactobacillus and Bifidobacterium spp., whereas microorganisms predominantly identified in control-fed rats were Bacteroides, Clostridium and Propionibacterium spp. Wine polyphenols (57 mg/kg for 10 days, by gavage), administered to rats not treated with carcinogens, produced a significant decrease in the basal level of DNA oxidative damage of the colon mucosa as measured with the comet assay (average pyrimidine oxidation was reduced by 62% and purine oxidation by 57%, p < 0.05). To further explore the molecular effects of wine polyphenols we used the microarray technology to study gene expression profiles: rats were treated with 50 mg/kg wine polyphenols for 14 days, mixed in the diet. Global expression analysis of 5707 genes revealed an extensive down-regulation of genes involved in a wide range of physiological functions, such as metabolism, transport, signal transduction and intercellular signalling. By analysing metabolic pathways with the GenMAPP software program we observed that two major regulatory pathways were down-regulated in the colon mucosa of polyphenols-treated rats: inflammatory response and steroid metabolism. We also found a down-regulation of many genes regulating cell surface antigens, metabolic enzymes and cellular response to oxidative stress. In conclusion, reduction of oxidative damage, modulation of colonic flora and variation in gene expression may all concur in the modulation of intestinal function and carcinogenesis by wine polyphenols.

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Keywords: Polyphenols; Colon cancer; DNA microarrays; Oxidative damage; Gut microflora; Gene expression

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1. Introduction

Environmental factors and diet have an important effect on the colon. A diet rich in vegetable and fruit reduces the risk of cancer at various sites, especially in the colon [1]. Since polyphenols are a common constituent of several fruits and vegetables, they have been considered as possible chemopreventive compounds. Grapes and red wine are an abundant source of polyphenols and represent an important dietary component for some populations. However, few experimental studies have been published on the effects and mechanisms of action of wine polyphenols. Most of the available data describe effects in rodents in vivo. Wine polyphenols have been reported to delay neurofibromalike tumours in transgenic mice [2] and to slow down the process of chemical carcinogenesis in the colon of rats [3,4]. In one study a possible mechanism of action of wine polyphenols in vitro has been studied, i.e. the variation in p53 gene expression, albeit with negative results [5].

Given these premises, to obtain information about the mechanism of action of wine polyphenols on the intestinal mucosa we investigated the effects of polyphenols administered p.o. on putatively important factors in controlling the carcinogenesis process, i.e. on the response to oxidative stress, on gene expression profiles and on colonic flora since colonic mucosa function is deeply influenced by luminal bacterial content.

2. Materials and methods

2.1. Chemicals

Azoxymethane (AOM; CAS number 25843-45-2) was purchased from SIGMA (Milan, Italy) and 1,2-dimethylhydrazine dihydrochloride (DMH; CAS number 306-37-6) from Aldrich (Milan, Italy). Wine polyphenolic extracts used in the carcinogenesis experiments were prepared from a Cabernet Sauvignon red wine by Veronique Chéynier at the University of Montpellier, France. Wine was de-alcoholised by distillation, deposited into a vinyl-divinyl benzene column, and the phenolic pool was eluted with 90% ethanol in water, concentrated by vacuum, evaporation and atomised. Wine polyphenols contained 4.4% anthocyanins, 0.8% flavonols, 2.0% phenolic acids, 1.4% catechin, 1.0% epicatechin and 28.0% proanthocyanidin units, consisting of 18.0% epigallocatechin, 13.2% catechin, 65.0% epicatechin and 3.8% epicatechin gallate, with a mean degree of polymerisation of 6.8. All these compounds accounted for 45% of the polyphenolic powder in weight. Approximately half of wine polyphenols consisted of new phenolic species produced in the course of wine making and ageing and of derived phenolic species such as flavanol-anthocyanin adducts [4].

2.2. Animals

We used male F344 rats (Nossan, Correzzana, Milan, Italy). The animals were housed in plastic cages at a temperature of 22 °C, with a 12:12 h light-dark cycle, according to the European Union Regulations. The experimental protocols were approved by a local Ethical Committee and by the Commission for Animal Experimentation of the Ministry of Health, Rome, Italy. We used 22 rats in the polyphenols-treated groups; the controls were 22 in the AOM group and 24 in the DMH group. The different experimental approaches are summarised in Table 1.

2.3. Diets

Dietary components were purchased from Piccioni (Gessate, Milan, Italy). Animals were fed a high-fat diet whose composition is based on the AIN76 diet, modified to contain a high amount of fat (230 g/kg corn oil w/w) and low level of cellulose (20 g/kg w/w) to mimic the diet associated with a high risk of colon cancer in human populations, as discussed in detail in previous publications [3,4].

Polyphenols were administered as a powder homogenised with food in the chronic carcinogenesis experiment; in the short-term experiment for the study of oxidative damage, given the high individual variability of this measurements, polyphenols were administered by gavage to have an exact estimate of the individual dosage. Since wine polyphenols are water-soluble and have a limited gastrointestinal adsorption, we do not expect that this variation in administration protocol could influence considerably their bioavailability.

2.4. Experimental carcinogenesis

In the experiments with AOM, rats were then treated with 10 weekly s.c. injections of AOM (7.4 mg/kg, total
Table 1

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One week after the last AOM or DMH injection, rats were randomly allocated to two different treatments: the control group was fed the high fat diet alone and the treated group was fed the same diet supplemented with red wine polyphenols, at a dose of 50 mg/kg, which was in the range of a moderate human red wine consumption [3].

One week after the last AOM or DMH injection, rats were randomly allocated to two different treatments: the control group was fed the high fat diet alone and the treated group was fed the same diet supplemented with red wine polyphenols, at a dose of 50 mg/kg, which was in the range of a moderate human red wine consumption [3].

The treatment was continued for 16 weeks. At sacrifice all the organs were macroscopically examined for the presence of tumours and classified by a pathologist using standard histological procedures, as described in detail in previous publications [3,4].

2.5. Effect on the colonic flora

In the carcinogenesis experiments using DMH after 15 weeks of feeding freshly voided faecal samples from individual rats (they spontaneously defecate after gentle human handling) were pooled in a sample, representing the faecal content of the rats treated with polyphenols or fed the control diet only; the individual samples were taken immediately after evacuation from each rat and put into sterile containers, then closed in hermetically sealed plastic bags in which an anaerobic environment was created (AnaeroGen bags, Unipath Ltd., Basingstoke, Hampshire, England, UK). The samples were conserved in a container at a constant temperature of around 4°C and brought to the laboratory in Camerino within 4 h after collection, maintaining the anaerobiosis and refrigeration temperature.
Once in the laboratory, they were divided into aliquots and immediately transferred into an anaerobic cabinet (Concept 400, Ruskinn Technology Ltd., Leeds, West Yorkshire, UK). Faecal sample pools derived from the faeces of control and polyphenol-treated rats were diluted 1:10 with a reducing solution, homogenized using a Stomacher Lab Blender. Then a series of consecutive dilutions up to $10^{-10}$ were prepared which were seeded onto selective and enriched media. Each analysis was run in triplicate. For bacterial counts and identification, 5% Columbia blood agar (bioMérieux, Marcy-l’Etoile, France) was used to count total anaerobic and aerobic bacteria. The selective media used were: Beerens’ agar [6] for Bifidobacterium counts; Rogosa agar (OXOID, Unipath Ltd., Basingstoke, UK) for Lactobacillus; MacConkey agar for enterobacteria, Chapman agar (bioMérieux) for Staphylococcus and Brain Heart Infusion agar (OXOID) supplemented with vancomycin (0.75 mg/ml) and kanamycin (10 mg/ml) (bioMérieux) for Bacteroides. The bacteria were identified through various study parameters, such as Gram stain, colony morphology and biochemical tests using enzymatic kits such as the Rapid ID 32 A (bioMérieux).

In vitro sensitivity of the main strains tested was also checked determining conventional MIC on selective growth media.

2.6. Oxidative damage in the colonic mucosa

Animals on a high fat diet were given a polyphenol solution in water by gavage (daily, 57 mg/kg for 10 days); controls were gavaged with water. Animals were then sacrificed, their colons were removed and colonic mucosa cells were isolated using the procedure described by Pool-Zobel et al. [7]. We used the comet assay to assess oxidative damage, as previously described [8]. After completion of the lysis step, two bacterial repair enzymes were used which introduce breaks at pyrimidine or purine oxidation sites respectively, for analysing oxidised DNA bases. The slides were incubated at 37 °C for 45 min with 50 μl of either endonuclease III for pyrimidine or formamidopyrimidine glycosylase (fapy) for purine oxidation detection (both kindly provided by Dr. A.R. Collins, University of Oslo, Norway). The specific pyrimidine or purine oxidative damage (endonuclease III- or fapy-sensitive sites) was calculated as the difference between the damage detected in the respective enzyme-treated slides and their corresponding controls (buffer-incubated slides).

Microscopic analysis was carried out using a Labophot-2 microscope (Nikon, Japan) provided with epifluorescence (excitation wavelength 546 nm; barrier 580 nm). Each experimental point was run in duplicate and the images of 50 randomly chosen nuclei per slide were captured and analysed using a custom-made imaging software coupled with a CCD camera (model C5985, Hamamatsu, Japan). The amount of DNA fluorescence migrating in the tail was measured in each nucleus and the nuclei classified into five categories from 0 to 4 with increasing tail migration. Data were expressed in arbitrary units (AU), obtained by multiplying the percentage of each comet type per slide by its category number. The mean values of each duplicate slide were further averaged to calculate single values per animal, and the average of these gave the mean values for each treatment group.

2.7. Microarrays analysis

Five male F344 rats were administered the high fat diet or the same diet with 50 mg/kg wine polyphenols for 2 weeks. At sacrifice, colon mucosa was scraped using a glass slide and stored individually at −80 °C in RNAlater (Qiagen, Milan, Italy). Using mucosal scrapings the vast majority of the cells collected are constituted of enterocytes. The use of mucosa scrapings allows the collection of relatively pure enterocytes and avoids the difficulty of mixed signals from different cells types, as usually present in mucosal biopsies (connective and vascular cells besides mucosal cells).

Total RNA was extracted using the RNeasy Midi kit (Qiagen, Milan, Italy). Using mucosal scraping the vast majority of the cells collected are constituted of enterocytes. The use of mucosa scrapings allows the collection of relatively pure enterocytes and avoids the difficulty of mixed signals from different cells types, as usually present in mucosal biopsies (connective and vascular cells besides mucosal cells).

The rat oligonucleotide array was constructed using the Rat Genome Oligo Set Version 1.1™ (Operon Technologies, CA, USA) composed of 5677 optimized oligonucleotides (70mers) each representing one rat gene, plus 24 controls (total=5707). The lyophilized oligonucleotides were resuspended in 3× SSC and printed using the Omnigrid 100 microarrayer (Genomic Solutions, Ann Arbor, MI, USA) on poly-L-lysine glass slides (Erie Scientific Company...
Portsmouth, NH, USA). After printing, microarrays were post-processed following DeRisi’s laboratory procedure (http://derisilab.ucsf.edu/).

We used the indirect labelling method described by De Risi (http://derisilab.ucsf.edu/). We performed six comparisons in duplicate: each treated rat was compared to one randomly selected control rat and the treated-pool was hybridised with the control-pool. The hybridisation was performed at 63 °C for 14–18 h.

Fluorescent cDNA bound to the microarray were detected with a GenePix 4000 microarray scanner (Axon Instruments, Foster City, CA, USA), using the GENEPIX 4000 software package to locate spots in the microarray. We performed a ratio-based normalisation, using the Acuity software (Axon Instruments) and a cluster analysis of the data, using the Eisen software available at http://rana.stanford.edu in order to identify gene clusters representing a possible “polyphenol signature”. All genes showing a change of two-fold or more in at least one experimental condition were included in the analysis. Cluster analysis was performed using the K-means method. Log-transformed fold changes were arbitrarily clustered into groups of genes having similar expression profiles. The number of nodes was chosen to give the largest number of fundamentally different patterns.

To identify statistically significant pathways responding to polyphenol treatment in rat colon mucosa we used the “GenMAPP” program [9]. GenMAPP is a recently reported tool for visualising gene expression data in the context of biological pathways. We imported the results of our data set into the GenMAPP program, which identifies pathways containing differentially expressed genes in treated animals versus controls.

2.8. Statistics

Comparisons of tumour outcomes were done using a Poisson regression model fitted to the data [10]. We tested the presence of over dispersion by maximum likelihood, using a negative binomial model to take multiple occurrences per rat into account.

Other comparisons of the treated and control groups were performed using standard variance analysis for continuous responses (p-level fixed at 0.05, two-sided) and multiple comparisons by taking the control group as a reference.

3. Results

3.1. Tumour induction

The number of tumours/rat (adenomas or cancers) in different locations of the colon after induction with AOM or DMH is shown in Fig. 1. Polyphenol-treated animals had a consistently lower tumour yield. However, individual variability was considerable and a statistically significant reduction of tumour number per rat was observed only for adenomas in DMH treated rats and for total colon tumours (sum of adenomas and cancers) in AOM treated rats.

3.2. Variation of the colonic flora

A similar total faecal bacterial count and ratio between anaerobic and aerobic bacteria was found after 72 h of incubation in aerobiosis and anaerobiosis, in controls and polyphenol-treated rats (data not shown). On the contrary, polyphenols had a very strong effect on the average percentage counts of the main genera of bacteria (Fig. 2). Prevailing microorganisms identified in the faeces from control rats included Bacteroides, Clostridium and Propionibacterium spp. The genera found in the faeces of polyphenol-treated rats were Bacteroides, Lactobacillus and Bifidobacterium. The percentage of Clostridium and Lactobacillus spp. were quite different between the two groups (p < 0.05). The visible increase in bifidobacteria was below statistical
2. Bacterial genera predominantly identified in the faecal content of rats induced with DMH (30 mg/kg × 10), fed a high fat-low fibre diet (controls, A) or the same diet containing 50 mg/kg polyphenols (polyphenol treated, B) after 15 weeks of feeding following the last carcinogen injection. Values are expressed as means of three replicates ± S.D. The reported values are percentages calculated on the total number of anaerobic microorganisms. *p < 0.05 compared to the control group (A) with unpaired Student’s t-test.

significance. The tested strains proved sensitive to wine polyphenols in vitro only at the relatively high concentrations of 0.1–1 g/ml (data not shown).

3.3. Variation in oxidative damage

Treatment with wine polyphenols did not modify the basal level of single-strand breaks in DNA isolated from colon mucosal cells after 57 mg/kg wine polyphenols administration for 10 days (70 ± 6.8 AU in controls and 67.5 ± 7.1 AU in polyphenol-treated animals). Using a calibration method, originally developed by Collins et al. [11], which involves the use of X- and γ-rays, it was possible to convert the values in arbitrary units to DNA break frequency. Calculated with this method, the basal level of DNA breaks resulted to be around 1.2 per 10^8 Da of DNA in both control and treated animals. According to the same calculation, the basal frequency of endonuclease III- and fapy-sensitive sites was 0.66 and 0.49, respectively. Polyphenol administration (Fig. 3) induced a significant decrease (~62%) in the basal pyrimidine oxidation level (endonuclease III-sensitive sites, p < 0.05), and a 57% decrease in purine oxidation (Fapy-sensitive sites) as compared with the controls (p < 0.05).

3.4. Gene expression profiles

By averaging the results of the five individual rats analysed, 0.35% of the genes (20 out of 5677) were up-regulated and 6.44% were down-regulated (366) in polyphenol-treated rats. Cluster analysis of the normalised data (Fig. 4) showed that the pooled RNA from treated and control rats was only partially representative of the effects of wine polyphenols in individual rats, although many genes had similar expression patterns. Supplementary Tables 1 and 2 (provided as additional information) report the list of genes up and down-regulated (showing a change of 2.0-fold or more); the mean values reported are the averages of the fold changes in each treated rat (n = 5). For each mean value we also calculated the coefficient of variation (CV) as a measure of the inter-individual variability.

As a whole, genes down-regulated by wine polyphenols were related to intercellular signalling, signal transduction, nutrient digestion and absorption. We also found down-regulation of genes codifying cell surface antigens, metabolic enzymes and genes related to cellular response to oxidative stress (Fig. 5). By using a pathway visualisation tool, at least two regulatory pathways were found to contain clusters of differentially expressed genes: inflammatory response and steroid hormone metabolism genes.

The results on the up- and down-regulation of specific genes were not controlled individually with
Fig. 4. Gene expression patterns in the colon mucosa of five different rats (lines 1–5) and in pooled RNA samples from all treated and control rats. Gene expression data of 5707 genes from five rats and pooled RNA samples were analysed by K-means clustering: each polyphenol-treated rat was compared with each control rat (five right lanes); or pooled RNA samples, obtained by mixing equal amount of RNAs from each treated rat, were compared to the pooled RNA extracted from controls (first lane). Genes are listed in rows. Individual expression of rats or pooled RNA are in columns. The ratio of gene expression levels (treated/controls) is colour-coded: red indicates up-regulation while green indicates down-regulation and black no change in treated vs. control rats.

4. Discussion

The results of our studies indicate that treatment of F344 rats with a diet supplemented with polyphenols from red wine is able to inhibit some steps in DMH- or AOM-induced intestinal carcinogenesis, reducing the numbers of adenomas or the number of total tumours, respectively. The alcohol-free polyphenolic extract from red wine used in the present experiments contained monomeric polyphenols such as anthocyanins, monomeric flavanols, flavonols and phenolic acids, along with complex phenols and tannins with various degrees of polymerisation [3,4]. The effect on carcinogenesis was not very strong, but the decrease in tumour yield was consistent with the two different carcinogens used. It has been reported [2] that wine solids with a similar phenolic composition but containing relatively large amounts of non-phenolic compounds (such as tartaric acid and sugars) delay spontaneous tumour onset in transgenic mice.

Our results also show that treatment with wine polyphenols in carcinogen-treated rats is associated with a strong variation in the colonic flora. Although the total bacterial counts and anaerobe/aerobe ratio of microorganisms in the polyphenol-treated rats were similar to controls, Propionibacteria, Bacteroides and Clostridia were decreased and Lactobacilli and Bifidobacteria were increased in the colon content. Lactobacilli and Bifidobacteria are considered beneficial for intestinal function [12–14], whereas Clostridia have detrimental actions on colonic mucosa [15,16], thus indicating that these alterations in the composition of the colonic flora may provide possible protection from exposure to carcinogens, inflammation or increased oxidative stress. These last results are very interesting, because they document for the first time that wine polyphenols have effects on the bacterial content of the colon that mimic the action of fibres and prebiotics, compounds with known beneficial effects on colon function.

We further searched for possible cellular mechanisms of action of wine polyphenols. Using the comet assay technique, we observed that wine polyphenols can substantially reduce basal DNA oxidative damage due to pin variability, presence of “bad spots”, ratio signal/background).
in vivo in the colonic mucosa in rats not treated with carcinogens, after only 10 days of treatment. These data suggest that dietary polyphenols might have protective and therapeutic potential pathological processes with a component of oxidative damage. Although many dietary compounds have been described as having anti-oxidant activity in vitro, very few have the capability of reducing the basal level of oxidative damage of DNA in vivo, since the level of oxidation damage is tightly regulated in each tissue and species in vivo.

Finally, a short term experiment was performed to analyse how red wine polyphenols modulate the gene expression profile of the colon mucosa. The global expression analysis of 5707 genes in rats fed 50 mg/kg of polyphenols for 2 weeks revealed extensive gene down-regulation; these responsive genes (366) are involved in a wide range of physiological functions, such as metabolism, transport, signal transduction and intercellular signalling. This broad range of effects is not surprising, since multiple cellular processes are regulated by nutritional factors.

Transduction pathways, metabolic pathways and other functional groupings of rat genes were systematically evaluated for differential regulation using “GenMAPP”, a novel tool for visualising gene expression data in the context of biological pathways. GenMAPP converts gene expression data into charts showing significant expression changes in specific pathways. We thus discovered that genes in at least two major regulatory pathways were significantly altered after polyphenols administration: the inflammatory response pathway and the steroid hormones metabolism. We found that nine genes encoding for cell surface antigens and 41 genes related to immune and inflammatory responses were down-regulated by wine polyphenols. These effects may explain previously described anti-inflammatory properties of polyphenols [17,18] and are in agreement with our previous findings of a lower expression of iNOS and COX2 in tumours from polyphenol-treated rats [19].

GenMAPP analysis also revealed the down-regulation of a cluster of steroid metabolism genes, many of which are involved in the synthesis of estrogens (encoding for hydroxy-delta-5-steroid dehydrogenase, UDP glycosyltransferase 2, steroid 5-alpha-reductase 2 and hydroxysteroid dehydrogenase 17.

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**Fig. 5.** Diagram of the down-regulated genes after polyphenol-treatment and their function. The % indicated the % frequency of genes in a functional class relative to the total amount of genes varied by the treatment.
beta type 7 (17HSD). It has been recently reported that pomegranate polyphenols affect oestrogen biosynthesis by inhibiting aromatase and 17HSD activity, also showing an anti-proliferative effect on human breast epithelial cells [20]. Human 17HSD is widely expressed in human tissues and it has been proposed as a regulator of steroid hormone metabolism in peripheral tissues [21].

Other down-regulated genes after polyphenol administration were the insulin-like growth factor gene, which has effects on cell proliferation, differentiation, and apoptosis [22]; genes involved in the metabolism of sugars, alcohols, fatty acids and cholesterol, xenobiotic metabolism (cytochromes P450, mainly of classes 1, 2 and 11) and genes encoding for proteins involved in radical scavenging (glutathione peroxidase and superoxide dismutase 3), possibly as a result of the potent antioxidant effect of polyphenols in vivo [23].

We also found a down-regulation of eight genes involved in energy metabolism pathways, including the NADP-dependent dehydrogenase/reductase, pyruvate dehydrogenases and cytochrome-c oxidase (COX6, COX7, COX8 and COX11). Alterations in genes involved in energy metabolism and mitochondrial function would be expected to decrease electron transport activity and mitochondrial oxidative stress. It has in fact been reported that phenolic compounds such as quercetin and tea polyphenols inhibit mitochondrial ROS production [24].

Among the genes up-regulated by polyphenols were cholesterol 7a-hydroxylase (CYP7A1), the rate-limiting enzyme in the pathway of bile acid biosynthesis [25] and two triglyceride lipases. It has been suggested that CYP7A1 deficiency would reduce the conversion of cholesterol to bile acids, resulting in elevated liver cholesterol levels, down-regulated LDL receptors, and hypercholesterolemia [26,27].

In conclusion, polyphenols from red-wine inhibit the process of chemical colon carcinogenesis in rodents, modify colon microbial ecology, reduce colonic mucosa DNA oxidation and have complicated effects on gene regulation, possibly affecting the mucosal response to inflammatory and carcinogenic agents. It is not clear at present how the observed variations in gene regulation are specifically connected to protection from oxidative damage and/or inhibition of carcinogenesis.

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Appendix A. Supplementary data


References


