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ORIGINAL CONTRIBUTION

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Genotoxic effect of bile acids on human normal and tumour colon cells and protection by dietary antioxidants and butyrate

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Abstract *Background* Colorectal cancer is the second cause of death for tumour worldwide. Among the risk factors for this disease the dietary habits seem to have a pivotal role. An elevated intake of fats causes a high release in the gut lumen of bile acids that are positively correlated with colorectal cancer, since they act as detergents and proliferation promoters. Recently, it was evidenced that bile acids can also be able to induce DNA damage. *Aim of the study* In this study the genotoxicity of deoxycholic acid (DCA) and chenodeoxycholic acid CDCA) has been evaluated in human normal colonocytes derived from 60 colon biopsies and in tumour cells. The involvement of reactive oxygen species (ROS) and the oxidative DNA damage was assessed. In addition, the protective effect exerted by both two well-known antioxidants commonly present in the diet, β-carotene and α-tocopherol, and butyrate which is known to be involved in the regulation of several cellular functions, has also been tested. *Methods* The DNA damage was evaluated by the "comet assay" or single cell gel electrophoresis (SCGE) both in its conventional use and by the Endonuclease III modified method, which allow to detect the presence of oxidized pyrimidines. *Results* Bile acids (CDA and CDCA) resulted genotoxic on both normal and tumour human colon cells. The inclusion of the endonuclease III digestion step in the comet assay demonstrated that bile acids induced an oxidative DNA damage. In addition, treatment of colonocytes with bile acids in the presence of the antioxidants (β-carotene, α-tocopherol) and Na-butyrate caused a reduction of DNA damage. *Conclusion* Our results suggest that bile acids may be involved in the tumour initiation by inducing a DNA oxidative damage, and so add further evidences to the preventive properties of antioxidants present in the Mediterranean diet.

Key words bile acids – oxidative DNA damage – antioxidants – Na-butyrate – normal colonocytes – tumour cell line

Introduction

Colorectal cancer is one of the most frequent worldwide human cancers, which is influenced by both genetic and environmental factors. Among the environmental factors the dietary habit seems to play a pivotal role [35], in particular, a high fat intake is considered a risk factor since it is responsible of the increased concentration of bile acids (BA) in the colon lumen [46]. Furthermore, it has been widely recognized that BA act as potent tumour promoters through several mechanisms. In fact, secondary BA possess detergent properties which damage the plasma membrane of the colon epithelial cells leading to cell death [10, 26, 36]. The consequent compensatory proliferation may increase cell susceptibility to mutagens and successively to the neoplastic transformation [21]. In addition, secondary BA may cause the selection of apoptosis-resistant cells, which may propagate the DNA damage [3, 7]. The BA tumour promoting effects may be also due to their capacity to stimulate cycloxygenase 2 (COX-2) activity with the consequent increase of prostaglandines production, which makes the cells apoptosis-resistant and stimulates the invasiveness of malignant cells [12, 20, 48]. Bile acids are also able to promote cell proliferation by inducing proto-oncogenes, like c-fos and c-jun [44], and stimulating several signal transduction pathways by PKC activation [17, 30].

Recently, many evidences suggest that BA can damage DNA and act also as tumour initiators [2, 19, 27, 34], although a direct action of BA on DNA is unlikely since DNA-bile acid adducts have not been observed either after in vitro treatment of human cancer cell lines or in in vivo studies in rats [14]. On the other hand, some studies indicate that BA may indirectly act on DNA. For instance, it has been shown that deoxycholic acid (DCA) up-regulates the DNA damage-inducible-genes (GADD) in the human tumour colon epithelial cell lines HCT116 [39]. In addition, an increasing number of studies shows that the DNA damage induced by BA could be mediated by the oxidative stress. Venturi et al. [45] have shown, by using the modified endonuclease III comet assay, that the treatment of CaCo-2 tumour cell line with DCA increases the number of oxidized pyrimidines. In addition, DCA induced DNA damage of human colon adenocarcinoma cell line HT29 was significantly reduced by the pre-treatment with the antioxidant vitamin E [4]. Moreover, Payne et al. [28] reported that DCA is able to activate both the redox sensitive transcription factor NF-kB and the enzyme poly (ADP-ribose) polymerase (PARP), both involved in the repair of oxidative DNA damage. Little attention has been paid to the BA genotoxic potential on primary normal human colon cells. Only one study, to the best of our knowledge, demonstrated that lithocholic acid at the concentration of 40 μg/ml (ca. 240 μM) induced DNA damage on normal human cells derived from colon biopsies [32].

In the present study, the genotoxic potential of BA on human normal epithelial colon cells has been investigated by the comet assay to further support the hypothesis that BA may act as initiators in colon carcinogenesis. The attention has been focused on chenodeoxycholate (CDCA) and deoxycholate (DCA), representatives of the primary and the secondary BA, respectively. The effect of two natural antioxidants, β-carotene and α-tocopherol, on BA-induced DNA damage has also been investigated. Furthermore, the protective effect of butyrate on BA-induced DNA damage has been evaluated on the basis of reported finding that short chain fatty acids, normally present in the gut lumen, protect colon cells from oxidative DNA damage [37]. Finally, the effects observed in normal human colonocytes have been compared with those found in the human tumour cell line HT29.

Materials and methods

Subjects and isolation of human colonocytes

Human colon cells, obtained from normal, endoscopically and histologically evaluated sigmoid colon mucosa of 60 healthy subjects, have been used. Each subject, informed about the aim of the present investigation, gave written consent. The study was approved by the Regional Ethical Committee and all procedures complied with ethical standards.

The colonocytes were prepared as previously described [31]. Briefly, four biopsies from each donor were pooled, minced and digested with 3 ml of a solution of proteinase K/collagenase (6 mg proteinase K + 3 mg collagenase in Hank's balanced salt solution) for 35 min at 37°C. The digestion was stopped by adding DMEM [Dulbecco's Modified Eagles Medium, supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin] to yield a final volume of 7 ml. The resulting cells were washed (1,700 rpm for 25 min) and suspended in the same medium. The cell number and viability were determined by the trypan blue exclusion method, and the concentration adjusted to 0.5×10^6 cells/ml for the different treatments.

Cancer cell line HT29

Human colon carcinoma cell line HT29 was grown at 37°C in a humidified incubator (5% CO_2 95% air) for

3–4 days, to reach the appropriate concentration, in McCoy's medium supplemented with 10% of FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Before each experiment the cells were detached by a trypsin-EDTA solution (Gibco BRL, Paisley, Great Britain), washed with the same medium, counted and adjusted to the desired cellular density for the different treatments.

Cell treatment

Two different experimental designs were carried out: the first to quantify the DNA damage induced by BA and the second to investigate the protective effect of butyrate, β-carotene and α-tocopherol, pre- or co-incubating the cells with BA and protective compounds. In the first design, 200 μl of normal colonocytes or HT29 cell suspension (0.5×10^5 cells/ml) were dispensed in eppendorf tubes and incubated with DCA or CDCA in the concentration range 0–800 μM for 30 min at 37°C in a humidified incubator (5% CO_2 , 95% air). After incubation cells were collected by centrifugation (1,400 rpm for 7 min) and used for the subsequent assays.

In the experiments carried-out preincubating cells with protective compounds, 200 μl of normal colonocytes (0.1×10^5 cells) were dispensed in eppendorf tubes, pre-incubated with Na-butyrate (6.25 mM) or β-carotene (3 μM) for 15 min, washed with DMEM and then treated with BA (DCA or CDCA 600 μM) for 30 min at 37°C in a humidified incubator (5% CO_2, 95% air). The tumour colon cells HT29 were plated at 2.5×10^5/ml on six well flat bottom plates and grown for 72 h and then the medium was removed and replaced with fresh medium containing Na-butyrate (6.25 mM) for 15 min. Subsequently, the Na-butyrate was discarded and the DCA or CDCA (600 μM) was added and manteined in contact for 30 min before to detach the cells with trypsin/EDTA for the genotoxicity and citotoxicity evaluations.

In the co-incubation experiments, 200 μl of the human colonocytes (0.1×10^5 cells) were dispensed in eppendorf tubes and co-incubated for 30 min with the BA 600 μM and β-carotene 3 μM or α-tocopherol 40 μM at 37°C in a humidified incubator (5% CO_2, 95% air). The effect of co-incubation of DCA or CDCA 600 μM with Na-butyrate at different concentration (1.56, 3.125, 6.25 and 12.50 mM) was tested as above reported both in normal colonocytes and in HT29 cells.

Detection of DNA damage (comet assay)

200 μl (0.1×10^5 cells) of cell suspension were used for the comet assay ("single cell gel electrophoresis assay" SCGE) carried out according to a previously described method [41]. Cell suspensions were centrifuged at 1,200 rpm for 5 min. The supernatant was discarded and the pellet was mixed with 75 μl of low melting agarose (Gibco BRL, Paisley, Great Britain) 0.7% in PBS and distributed onto conventional microscopic slides pre-coated with normal melting agarose (Gibco BRL, Paisley, Great Britain) 0.5% in PBS and dried at 50°C. After the agarose was solidified (4°C for 10 min) a second layer of low melting agarose was applied. Successively, the slides were immersed for 1 h at 4°C in the lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl pH 10, containing freshly added 1% Triton X100 and 10% DMSO) and then placed into a horizontal electrophoresis apparatus filled with freshly made solution (1 mM Na_2EDTA, 300 mM NaOH). Following 20 min of pre-incubation (unwinding of DNA), electrophoresis was run for 20 min at a fixed voltage of 25 V and 300 mA adjusted by increasing or lowering the electrophoresis buffer volume in the tank. Then, the slides were washed three times with the neutralization buffer (0.4 M Tris–HCl, pH 7.5), stained with 50 μl ethidium bromide (20 μg/ml), and kept in a moisture chamber in the dark at 4°C until analysis. All the above steps were performed under red light environment to avoid additional DNA damage.

An Endonuclease III enzyme (New England Biolabs, Celbio) modified comet assay was also carried-out in order to evaluate the presence of oxidative DNA damage [6]. In this context H_2O_2 15 μM was introduced as positive control and cell treatment was performed at 4°C for 5 min. The enzyme (1 μl) was reconstituted with 1 ml reaction buffer [PBS (Na_2HPO_4 10 mM, NaH_2PO_4 10 mM, KCl 2.7 mM, NaCl 120 mM, pH 8.00) + bovine serum albumine 100×]. After cellular lysis the slides were washed three times with PBS for 5 min and then 55 μl of Endonuclease III (50 μl, 1 U/ml) were added to the cells. Control with enzyme reaction buffer alone was included and all slides were incubated at 37°C for 45 min.

Cells were analyzed 24 h after staining at 400× magnification, using a fluorescence microscope (Axiolab, Carl Zeiss, Jena, Germany) equipped with a 50-W mercury lamp. Microscopic images show circular shapes (undamaged DNA) and "comet" like shapes, in which the DNA migrated from the head to form a tail (damaged DNA). For each slide, one hundred images were scored by a computerized image analysis system (Comet assay II, Perceptive Instruments, Haverhill, UK) able to calculate the "tail moment", the parameter considered more directly related to the DNA damage. The tail moment is defined as the product of DNA content in the tail and the mean distance of migration in the tail. The 75th percentile (upper quartile, level of damage that 75% of the cells

Table 1 Correlation coefficients between subject age and DNA damage

Variable	DNA damage								
	Basal			DCA induced[a]			CDCA induced[a]		
	n	*r*	*P*	*n*	*r*	*P*	*n*	*r*	*P*
Age (all subjects)	60	0.261	<0.05	44	0.316	<0.05	30	0.145	NS
Age (no smokers)	50	0.322	<0.05	36	0.319	<0.05	26	0.189	NS

n number of subject, *r* correlation coefficient, *P* significant coefficient
[a]Normal colonocytes were treated with 600 μM of DCA and CDCA for 30 min

do not exceed) value for tail moment was calculated from 100 cells per slide and provided a single value that gave an appropriate representation of the distribution of the data for statistical analysis.

Detection of apoptotic and necrotic cells

To assess that the DNA damaged cells were neither apoptotic nor necrotic, a fluorescent microscopy method was applied as previously described [11]. Briefly 200 μl of cells were centrifuged (1,400 rpm) and the pellets were resuspended in 15 μl of complete DMEM medium containing the DNA binding dyes Hoechst 33342 (HO 342, 20 μg/ml in PBS) and Propidium Iodide (PI, 10 μg/ml in PBS). After 10 min of incubation at room temperature, the cells were examined by using a fluorescent microscope (Zeiss, R.G., equipped with a 50-W mercury lamp) with ultraviolet excitation at 340–380 nm. For each of the experimental conditions, three slides were prepared and 100 cells were counted in each slide.

Statistical analysis

For statistical analysis the two-tailed Student's paired and unpaired t-test was adopted. Values of $P < 0.05$ were chosen for the rejection of the null hypothesis. Data are expressed as means ± SEM.

Results

General characteristics of the subjects participating at the study

The characteristics of the 60 subjects involved in this study are as follow: the mean age was 56 years (range 30–83), 28 subjects were males (46.6%) and 10 were smokers (16.6%). The mean value of basal DNA damage of normal colonocytes, expressed as 75th percentile, was 0.53 ± 0.48 (range 0.005–3.22). The correlation between subject characteristics and both the basal and BA induced damage, was assessed by simple regression analysis. As reported in Table 1 a positive correlation was found between age and basal DNA damage, and between age and DCA-induced DNA damage. This result was independent from smoking habit because the exclusion of smokers did not modify the correlation. On the other hand, no significant correlation between gender and smoking habits with colonocytes DNA damage was found, probably in relation to the low number of smokers considered in the study.

DCA and CDCA genotoxicity and cytotoxicity in normal and tumour colon cells

Human colonocytes and colon tumour cells HT29 were treated with increasing concentrations of DCA and CDCA for 30 min. In both cell types a clear dose-dependent genotoxic effect induced by the two BA was observed (Fig. 1a, b) and the DCA induced DNA damage was slightly higher than that induced by CDCA in both kind of cells. HT29 tumour cells were more susceptible than the freshly isolated normal colonocytes. In fact, treatment of HT29 cells with DCA and CDCA at 600 μM increased DNA strand breaks respectively by 24 and 20-fold above the basal level, whereas in normal colonocytes the increase was 7.5 and 3.0-fold, respectively. Highest concentrations of BA were not tested since it is unlikely that, under physiological conditions the concentrations of these compounds in the gut lumen exceed 800 μM. In addition, it is known that high concentrations of BA are able to induce a general damage to the cells (apoptosis and/or necrosis) with the consequent overestimation of the DNA damage measured by the comet assay. For this reason the cytotoxic and apoptotic effects of BA on both colonocytes and HT29 were also evaluated under the experimental conditions used. The treatment at the highest doses of DCA and CDCA caused a low percentage of apoptotic cells and a weak cytotoxicity (viability > 75%). In any

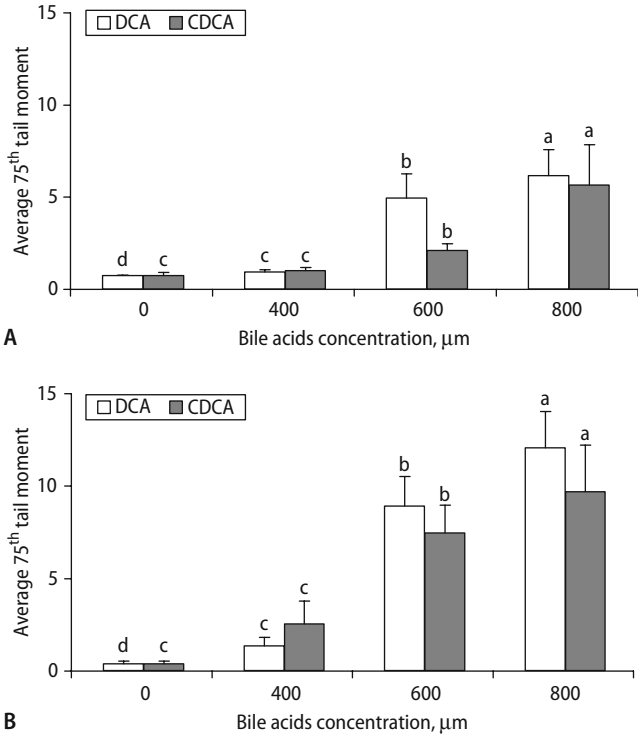

Fig. 1 Dose-dependent effect of DCA and CDCA on DNA damage in normal colonocytes (**A**) and tumour colon cell HT29 (**B**) after treatment at 37°C for 30 min. The values are average 75th tail moments ± standard error of the mean (SEM), $n = 28$ with DCA and $n = 15$ with CDCA in normal colon cells and $n = 6$ in HT29 with both BA. For each compound, value without a common letter differ, $P < 0.05$

case, the dead cells have been excluded from the DNA damage evaluation because their particular "cloud" form as reported in previous studies [15].

The possible involvement of reactive oxygen species (ROS) on BA-induced DNA damage was evaluated in HT29 cell line by using the endonuclease III modified comet assay (Fig. 2). The treatment of cells with BA and H_2O_2, used as positive control, was carried out at 4°C to inhibit the endogenous DNA-repair systems. The digestion of DNA with endonuclease III increased the number of DNA strand breaks when the cells were treated with H_2O_2 15 µM. Similarly, both DCA and CDCA 600 µM caused a significant increase of oxidative damage to DNA pyrimidines. The BA treatment at 4°C in absence of Endonuclease III did not induce a significant DNA damage.

Protective effect of β-carotene and α-tocopherol on BA induced DNA damage in normal colonocytes

In order to add further evidences to the hypothesis that ROS are involved in the BA induced DNA damage in normal colonocytes the effect of two well known antioxidants, β-carotene and α-tocopherol, has been evaluated. When the colonocytes were treated with DCA and CDCA (600 µM) in the presence of β-carotene 3 µM and α-tocopherol 40 µM a total protection of DNA damage was observed (Table 2), but protection was not observed when the cells were preincubated with β-carotene and, after its removal, exposed to the BA (600 µM) (data not shown).

Effect of Na-butyrate on BA induced DNA damage in normal colonocytes and HT29 cells

The co-incubation of both cells types with DCA (Fig. 3a) and CDCA (Fig. 3b) in the presence of Na-butyrate at different concentrations significantly reduced the DNA damage. It is interesting to underline that the protective activity of Na-butyrate seems to be inversely correlated to its concentration; in fact the maximum effect was observed at the lowest concentration tested while at the highest one the effect was lost (Fig. 3). The pre-incubation of normal human colonocytes and HT29 tumour cells for 15 min with Na-butyrate at the same concentrations used above did not prevent the BA induced DNA damage. In

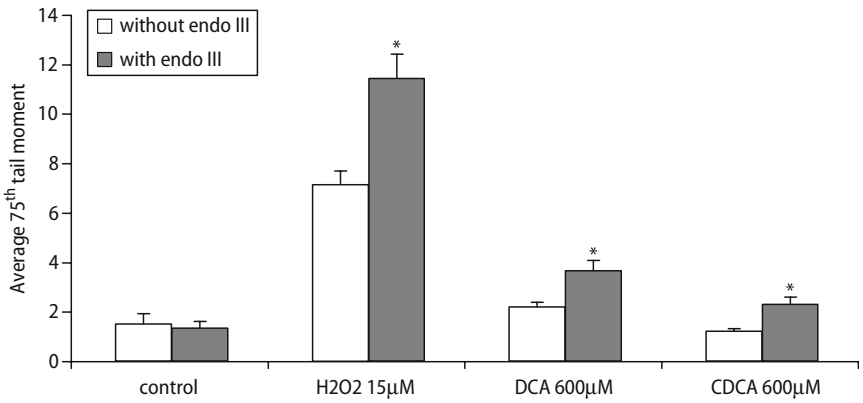

Fig. 2 Effect of Endonuclease III digestion on genotoxicity of BA to HT29 cells in the comet assay. The treatment was made to 4°C for 5 min and H_2O_2 15 µM was used as positive control. The values are average 75th tail moments ± standard error of the mean (SEM), $n = 6$. *Significantly elevated tail moments after endonuclease III treatment ($P < 0.05$)

Table 2 Effect of co-incubation with β-carotene 3 μM and α-tocopherol 40 μM* on BA induced DNA damage

Protective agent	DNA damage		
	Basal	DCA	CDCA
None	0.552 ± 0.062[b]	4.872 ± 1.299[a]	2.019 ± 0.37[a]
β-carotene 3 μM	0.806 ± 0.147[b]	0.701 ± 0.166[b]	0.735 ± 0.148[b]
α-tocopherol 40 μM	0.613 ± 0.122[b]	0.441 ± 0.081[b]	0.424 ± 0.051[b]

*Normal colonocytes were treated for 30 min with 600 μM of DCA and CDCA in the presence of the two antioxidants. The values are average 75th tail moments ± standard error of the mean (SEM), $n = 6$. Values without a common letter differ, $P < 0.05$

addition, treatment with butyrate alone at the highest concentration (12.5 mM) did not modify the basal DNA damage in both kind of cells (data not shown).

Discussion

In the present study, both the basal and the BA induced DNA damage in normal colonocytes isolated from 60 subjects has been measured. A positive correlation between age and both basal and BA induced DNA damage was found, probably related to the minor efficiency of human DNA repair systems with aging [9]. It was not found any correlation between DNA damage with sex and smoking habit.

The involvement of BA in colon carcinogenesis has been widely reported underlining that the different compounds have distinct biological effects [23]. In the present study, at least as regards the DNA damage, no significant differences have been observed considering CDCA, a primary BA and DCA, a secondary one. Similar effects of the two BA has been reported with regard to the reduction of cell viability through the induction of apoptosis [16] and the COX-2 gene up-regulation [20]. In this context it must be observed however that the concentration of primary BA in the colon results very low at normal physiological conditions in comparison with that of the secondary ones, but it may increase as a consequence of intestinal dismicrobism due to an excessive use of drugs or to the assumption of a diet rich in simple sugars and poor in fibers [24].

The DCA induced DNA damage in HT29 cell line confirms other reports [5, 12], but in the present study, for the first time, a genotoxic effect induced by

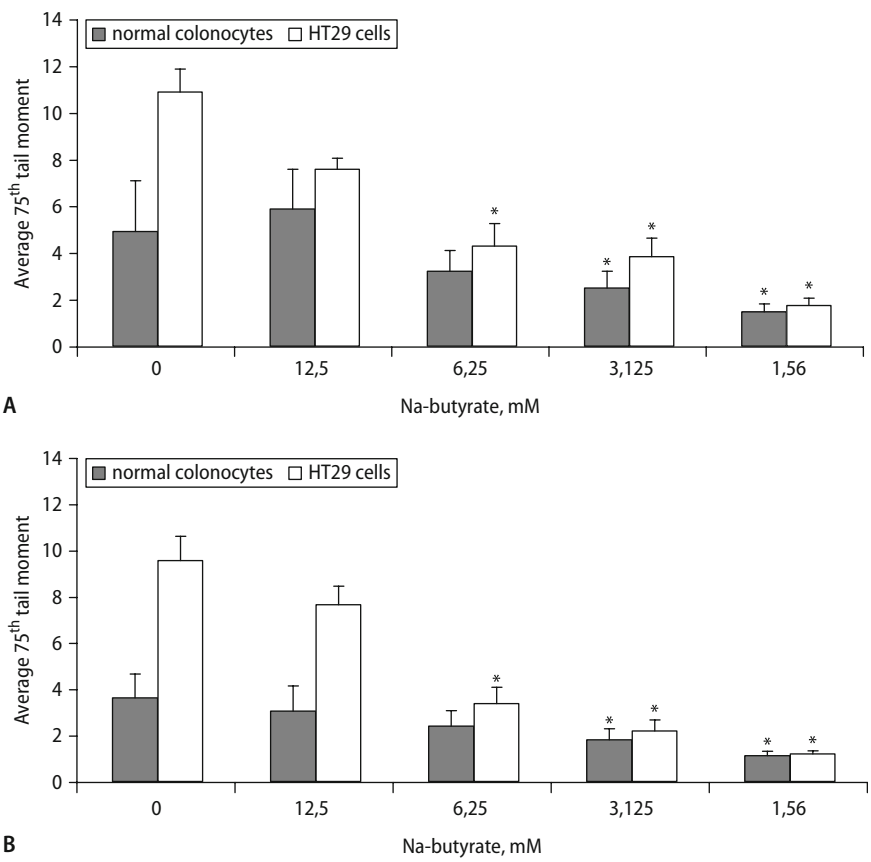

Fig. 3 Dose-dependent effect of Na-butyrate co-incubated for 30 min with DCA 600 μM (**A**) and CDCA 600 μM (**B**) on normal colonocytes and HT29 tumour cells. The values are average 75th tail moments ± standard error of the mean (SEM), $n = 4$. *Significantly lower tail moments ($P < 0.05$) versus 0

BA (DCA and CDCA) on normal colonocytes obtained from biopsies is shown. This result demonstrates that BA could be a real risk factor for colorectal cancer, involved not only in the promotion but also in the tumour initiation.

The mechanisms by which BA induce DNA damage are, up to now, not well known. The results of the present investigation obtained with the use of endonuclease III modified comet assay at 4°C suggest that the DNA damage could be mediated by ROS production. In fact, a significant increase, even low, in BA derived DNA damage has been observed in agreement with Venturi et al. [45] finding, that reported an increase in the DNA damage induced by DCA 300 μM on $CaCo_2$ cell line by using the same modified comet assay. The lack of BA ability to induce DNA damage at 4°C in the absence of endonuclease III, seems to demonstrate that BA are not directly responsible for the damage but rather that BA are able to induce cellular processes active only at physiological temperature.

Data from the literature suggest that DCA can induce ROS production by several pathways; for instance by its detergent effect on membrane bound enzymes, such as phospholipase A2 (PLA2) with the consequent activation of cyclo-oxygenase (COX) and lipo-oxygenase (LOX) [48], by damaging mitochondria and by activating the redox-sensitive transcription factor, NF-kB, resulting in increased levels of nitric oxide synthase 2 [29].

The reduction of BA induced DNA damage resulting from the co-incubation of human normal colon cells with CDCA and DCA and with the strong antioxidants compounds, β-carotene and α-tocopherol, further support the hypothesis that ROS are involved in BA induced DNA damage. Similar results have been obtained by Booth et al. [4] co-incubating HT29 tumour cells with litocholic acid and α-tocopherol 50 μM for 60 min. Since the antioxidant mechanism of both β-carotene and α-tocopherol is related to the interruption of the chain of events involved in lipid peroxidation [4] it can be hypothesized that the BA induced DNA damage is mediated, at least in part, by peroxides produced at the level of the membrane. It is well known that BA possess a detergent action on plasma membrane and activate phospholypase A2 with the consequent production of arachidonic acid [48], which can be responsible for the enhancement of lipid peroxidation [42].

In contrast to what observed in co-incubation experiments, the pre-incubation of normal colonocytes with BA and β-carotene did not show any appreciable protection in disagreement with the finding of Lowe et al. [22] who showed a reduction of DNA damage produced by xanthine/xanthine oxidase reaction in HT29 cell line pre-incubated for 2 h with β-carotene 1–3 μM. Apart the different cellular system considered, the differences found could be related probably to the short pre-incubation time (30 min) employed in the present study to avoid that normal colon cells undergo apoptosis, which occurs when the exposure time is lengthened [13].

The protective effect of Na-butyrate was investigated in normal colonocytes and HT29 cell line, considering that this short chain fatty acid has opposite effects on normal and tumour colon cells, at least, with regard to cellular proliferation and apoptosis [40]. Despite the fact that in a previous work [37] the protective effect of pre-incubation with Na-butyrate on oxidative DNA damage induced by H_2O_2 was observed, in this investigation no protection was observed when the oxidative DNA damage was induced by BA. As reported above also in this case the short preincubation time could have a fundamental role. The protection resulted evident when the cells were co-incubated with Na-butyrate and BA and the effect was more evident at the lowest butyrate concentration tested (1.56–3.125 mM). A similar peculiar dose-dependent effect has been also reported by Janson et al. [18]. It must be observed that these last concentrations are comparable with those really present in the gut at least until 3.125 mM [8, 47]. This finding add a further evidence of the ability of butyrate in interfering with some negative effects evoked by BA, such as: the increase of mucosal DNA and protein content in vivo in rats [43], the hyperproliferation of human colonic epithelium [1] and the reduction of the spontaneous apoptosis in AA/C1 adenoma cells [25].

About the possible butyrate protective mechanism, Pool-Zobel et al. [33] reported that the treatment of normal colonocytes, HT29 and LT97 tumour cells lines with butyrate up-regulates the glutatione S-trasferase genes expression, a phase II enzyme involved in the cellular protection from products of oxidative stress. In addition, it is known that butyrate is a good inhibitor of histone deacetylase and it has been shown that inhibition of this enzyme, by increasing the acetylation of the transcription factor Sp1, prevents oxidative stress-induced death in neuronal cells [38]. It cannot be excluded however that the reduction of DNA damage observed only in the co-incubation experiments could be due to an interaction between the two reagents (BA and Na-butyrate) even if experimental data are, at the moment, not available.

In conclusion, these findings and in particular the results obtained on normal colonocytes, demonstrate that BA may act not only as promoters but also as initiators of the multistage process of carcinogenesis. Moreover, the observation that butyrate and antioxi-

dants (β-carotene and α-tocopherol) may protect cells from BA-induced genotoxicity add further support to the hypothesis that foods rich in fibres and antioxidants may be protective against the processes involved in the carcinogenesis.

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