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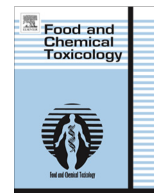

Fecal water genotoxicity in healthy free-living young Italian people

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ABSTRACT

Dietary habit affects the composition of human feces thus determining intestinal environment and exposure of colon mucosa to risk factors. Fecal water (FW) citotoxicity and genotoxicity were investigated in 33 healthy young Italian people, as well as the relationship between genotoxicity and nutrient intake or microflora composition. Two fecal samples were collected at 2 weeks apart and 3-d dietary diary was recorded for each volunteer. Cytotoxicity was measured using the Trypan Blue Dye Exclusion assay and genotoxicity using the Comet Assay (alkaline single-cell electrophoresis). Fecal bifidobacteria, total microbial count and nutrient intakes were also assessed.

High intra- and inter-variability in genotoxicity data and in bacteria counts were found. None of the FW samples were citotoxic, but 90% of FW samples were genotoxic. Seventy five percent indicated intermediate and 15% were highly genotoxic. There was a different sex-related distribution. Genotoxicity was positively correlated to the total lipid intake in females and to the bifidobacteria/total bacteria count ratio in male volunteers.

These results demonstrate that the majority of FW samples isolated from free-living Italian people show intermediate level of genotoxicity and sustain a relation between this possible non-invasive marker of colorectal cancer risk with both dietary habits and colonic ecosystem.

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1. Introduction

Cancers of colon and rectum (CRC) are the third most common type worldwide, whose mortality shows a geographical distribution even within European countries: more favorable trends were observed in most Western and Northern Europe than in Southern (particularly Spain) and in most of the Central and Eastern Countries (La Vecchia et al., 2010). These trends, otherwise reflecting the efficacy of early diagnosis programs, are likely a consequence of the differences in lifestyle habits across European people and, in particular, in dietary habits, considered one of the most important factor in colorectal cancer prevention (WCRF/AICR, 2007). Diet greatly affects the composition of feces in humans: the balance between carcinogens or oxidant and preventive or antimutagenic factors, arisen from food or formed in the colon, determines different intestinal environments and exposure of colon mucosa to risk factors for colorectal cancers (de Kok and van Maanen, 2000). Evidence suggests that intestinal microbiota highly contributes to this balance by induction of chronic inflammation following bacterial infection and/or production of toxic bacterial metabolites (Heavey and Rowland, 2004). Although the specific bacterial types associated with colorectal cancer risk have not been elucidated, it is clear that some bacterial groups (e.g., lactobacilli and bifidobacteria) have much lower enzymatic activities that can generate carcinogens than other gut microflora components such as *Clostridia* and *Enterobacteriaceae* (Azcárate-Peril et al., 2011) .

A non-invasive way to study the exposure of colon mucosa to risk factors for colorectal cancers is by means of investigating the mutagenicity of fecal sample (Bruce et al., 1977) and, more recently, the genotoxicity of the aqueous extract from stool (fecal water, FW) in colon carcinoma cells culture by single-cell gel electrophoresis (Comet Assay) (Venturi et al., 1997). This sensitive technique, enabling the detection of DNA strand breaks and alkali-labile sites in individual cells, has been successfully applied to investigate the genotoxicity of specific compounds (Venturi et al., 1997) or the genotoxicity of FW after different dietary intervention in humans (Cross et al., 2006; Glei et al., 2005; Glinghammar et al., 1997; Rieger et al., 1999). The few studies that monitored the basal

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genotoxicity of FW in healthy volunteers (Venturi et al., 1997; Woods et al., 2002) suggest that this variable, being associated to dietary habits, could be considered as a predictive biomarker for intestinal carcinogenic risk.

Since the data nowadays available are limited to the Northern European population (Sweden, English and Irish), the aim of the present study was to investigate the genotoxicity of FW in a group of healthy free-living Italian adults and its correlation with diet composition and intestinal bifidobacteria counts.

2. Materials and methods

2.1. Materials

Human Caco-2 cells were obtained from the European Collection of Animal cells Culture (UK). All cell culture reagents were purchased from Sigma Chemical Co (St. Louis, Mo) and chemicals from Merck (Darmstad, Germany).

2.2. Volunteers

Healthy female and male volunteers were recruited from college student population by advertisement of the study. Strict exclusion criteria were followed: individuals who were pregnant, taking medication (no antibiotic use for at least the last month before the study), on caloric restriction, special dietary treatments or with a daily intake of pre and probiotics products or supplements and subjects with any gastrointestinal disorder were excluded. Thirty-three subjects were selected, 16 females and 17 males, age 22 ± 4 (range 19–41), BMI 21 ± 3 (range 18–27). The study was approved by Ethical Committee of University of Milan and volunteers gave their written informed consent.

2.3. Study design

Experimental protocol has been shown in Fig. 1: each volunteer collected one sample of stool at the enrollment and a second sample after 2 weeks. Volunteers were instructed to collect the first stool passed in the morning in disposable bedpans and to keep the sample refrigerated. Aliquots of stool were immediately divided for microbiological assessment and for preparation of fecal water and stored at −80 °C. Before the second collection, volunteers filled a 3 days food records (two working days and one weekend day) to assess their food consumption. Food diaries were processed by dietitians to calculate the average amounts of energy and nutrient intakes by using commercially available software (MetaDieta, Me.Te.Da, Italy) (Welch et al., 2001). Where corresponding food items could not be found in the software, the item's energy and nutrient contents were derived from published food tables. Instructions on how to complete of the diet records were given in detail to the subjects by an expert dietitian.

2.4. Microflora analysis

Bifidobacteria quantification, as well as total bacteria enumeration was accomplished using Real-Time PCR amplification on all the samples for the subjects enrolled. Bacterial DNA was isolated from faeces using the FastDNA® SPIN Kit and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA). Prior to analyzes, to avoid inhibitory effects during the amplification process, DNA has been diluted tenfold with molecular biology grade water for bifidobacteria quantification, whereas for total bacteria quantification, DNA has been diluted at 1:100 concentration with the same diluent. Real-Time PCR was carried out using Eppendorf Mastercycler ep Realplex^{4} Instrument (Hamburg, Germany). For bifidobacteria amplifications, the protocol documented by Malinen et al. (2005) was followed, whereas what described by Wise and Siragusa (2007) was used as protocol for total bacteria investigation. Bacterial quantity, after amplification, has been expressed as colony forming units (CFU)/g of wet faeces. This result has been obtained by multiplying the instrumental data, representing the number of copies of 16S rDNA, with the appropriate conversion factor, different for bifidobacteria and for total eubacteria, calculated on the basis of DNA dilution and the average number of 16S rDNA copies estimated for bacterial cells. Results of fecal flora are reported as a mean of the two stool collections.

2.5. Fecal water preparation

Fecal water (FW) was obtained, from frozen fecal samples, after defrosting for one night at 4 °C and homogenization in stomacher bags (3 cycles of 120 sec), followed by centrifugation at 35.000*g* for 2 h at 20 °C, and supernatants were carefully decanted and stored at −20 °C (Klinder et al., 2007). For citotoxicity and genotoxicity evaluation, FW samples were rapidly defrosted and filtered through a 0.45 μm filter (Whatman, Clifton, NJ); filtered solutions were used to assess FW toxicity.

2.6. Fecal water citotoxicity and genotoxicity

A suspension of Caco-2 cells, after being cultured 10 days as monolayer, were incubated with FW (50 μL + 450 μL cell suspension) or saline (negative control) or H_2O_2 50 μM (positive control) for 30 min at 37 °C (Venturi et al., 1997). Every FW recovered from stool samples was analyzed in triplicate and controls (negative and positive) were included in each batch. After the incubation, an aliquot of this cell suspension was used to assess citotoxicity of FW, by measuring cell viability, with Trypan Blue exclusion test (expressed as percentage of viable cells). Another aliquot of cell suspension was centrifuged (100*g*, for 3 min), re-suspended in 1% low-melting point agarose, and spread on microscope slide previously covered with 1% normal-melting point agarose layer. Embedded cells were lysed, DNA was allowed to unwind in electrophoresis buffer and then electrophoresis was performed. After this step, slides were washed with neutralization buffer, stained with ethidium bromide and analyzed using a fluorescence microscope (BX60 Olympus, Japan) equipped with Image-Pro Plus software (Immagini & Computer, Bareggio. Milano, Italy). Fifty images were analyzed for each slide and tail moment registered: DNA damage was expressed as percentage of DNA in the tail (Tice et al., 2000). For each subject, genotoxicity of FW was analyzed separately in the two fecal samples delivered to the laboratory; FW genotoxicity data are then reported as a mean of the two collected samples.

2.7. Statistics

ANOVA was used to assess the influence of sex on nutrient intakes, DNA damage and fecal bacteria counts. Pearson's correlation coefficient was calculated to assess association between genotoxicity of FW and nutrient intakes or fecal bacteria counts. All analyzes were carried out with Statistica software (StatSoft Inc., Tulsa, Okla) and a *p* value < 0.05 indicated a significant difference. Considering the significant influence of sex on nutrient intake, ($p < 0.01$, as summary of all effect; intakes expressed as g/day), and the influence of sex on intestinal transit time, data of male and female volunteers were analyzed separately.

3. Results

3.1. Diet

Mean nutrient intakes from the 3-d registration in males and females are reported in Table 1. Diet in our volunteers resulted quite balanced and is consistent with the last Italian food consumption survey (Sette et al., 2011). In the present study, protein intakes were 14.2% and 14.8% of daily energy; fat 28.9% and 27.7%; saturated fatty acids (SFA) 10.6% and 11.2%; carbohydrates 53.4% and 49.6% in females and males respectively and cholesterol intakes were below the limit of 300 mg/day. The recommended goal of 30 g/day of fiber intake was not reached in our volunteers, while

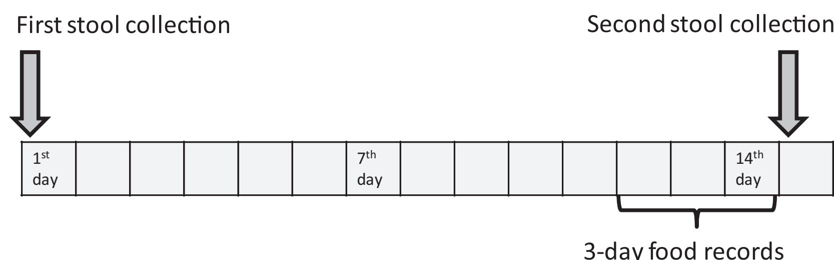

Fig. 1. Schematic diagram indicating experimental protocol.

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