

Cytokinesis-Block Micronucleus Cytome Assays for the Determination of Genotoxicity and Cytotoxicity of Cecal Water in Rats and Fecal Water in Humans

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Abstract

We tested the cytokinesis-block micronucleus cytome assay using the WIL2-NS human B lymphoblastoid cell line as a biomarker of genotoxicity and cytotoxicity of cecal water from rats and fecal water from humans. Cecal water was assessed in rats fed either a diet rich in fat, low in calcium and fiber, and barbecued red meat as the protein source (high colorectal cancer risk diet) or a diet high in fiber and calcium, low in fat, and casein as the protein source (low colorectal cancer risk diet) for 2 weeks. There was a significant 7.6-, 1.8-, and 4.0-fold increase in binucleated (BN) cells with micronuclei (Mn-BN), BN cells with nucleoplasmic bridges (Npb-BN), and necrotic cells ($P < 0.001$) with 1-h incubation with a 10% dilution of the cecal water isolated from rats fed the high colorectal cancer risk diet compared with rats fed the low colorectal cancer risk diet. In humans,

fecal water samples collected from feces of free-living volunteers showed that 24-h exposure to 1% dilution of fecal water produced a significant 2.6-, 6.5-, 7.5-, and 2.2-fold increase in Mn-BN, Npb-BN, BN cells with nuclear buds, and necrotic cells compared with controls ($P < 0.05$). The coefficients of variations for interindividual differences for Mn-BN, Npb-BN, BN cells with nuclear buds, and necrosis biomarkers were greater than corresponding coefficients of variations for intraindividual variation. These results indicate that the cytokinesis-block micronucleus cytome assay can be used successfully to determine the interindividual variation in genotoxicity and cytotoxicity of cecal or fecal water and to identify dietary patterns that are likely to increase carcinogenic events in the colon. (Cancer Epidemiol Biomarkers Prev 2007;16(12):2676–80)

Introduction

Epidemiologic and experimental studies suggest that dietary factors play a major role in the modulation of colorectal cancer (1). Diets high in red meat and fat are associated with an increased risk of developing colorectal cancer (2–5), whereas diets low in fat and high in fiber are in most cases protective (6–8).

As human feces represent the outcome of the digestion and fermentation processes, the assessment of the carcinogenic potential of fecal contents provides a noninvasive procedure for studying the environment in the distal colon and its potential contribution to colorectal cancer risk (9, 10). It is suggested that fecal water (aqueous phase of the feces) is more likely to exert a direct effect on the colonocytes than compounds that are bound to food residues and bacterial mass (11), indicating that genotoxic potential of fecal water may be a suitable biomarker for studying the effect of diet on colorectal cancer risk in humans. Using animal models, it is also possible to investigate cecal water genotoxicity, which may provide further information on any carcinogenic load generated at the proximal end of the colon.

Various methods have been used to measure the fecal or cecal water genotoxicity. These methods include the Comet assay or measurement of DNA adducts (12–14). The cytokinesis-block micronucleus (CBMN) assay is an alternative well-established approach to measure the cecal or fecal water genotoxicity (15, 16). This assay measures genotoxicity at the chromosomal level using three biomarkers: micronuclei (Mn; biomarker of chromosome breakage and/or loss), nucleoplasmic bridges (Npb; biomarker of DNA strand break misrepair and/or telomere end fusion), and nuclear buds (Nbud; biomarker of gene amplification and/or elimination of DNA repair complexes). The same assay can also measure frequency of apoptotic and necrotic cells and nuclear division cytotoxicity index (NDCI), a measure of cytostatic effects (15, 16). Given that the CBMN assay provides a picture of the genotoxic, cytotoxic, and cytostatic effects of a particular cellular insult, this has led to the conception of the CBMN assay as a 'cytome' assay (CBMN-Cyt), as every cell in the system is assessed for its viability, mitotic, and DNA damage status (15).

In this study, we will validate the use of the CBMN-Cyt assay in WIL2-NS cells as a biomarker of genotoxicity and cytotoxicity of cecal water from rats and fecal water from humans.

Materials and Methods

Rat Cecal Water Studies. Twenty 8-week-old male Sprague-Dawley rats (Animal Resource Centre, Murdoch

Received 5/29/07; revised 9/12/07; accepted 9/25/07.

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doi:10.1158/1055-9965.EPI-07-0488

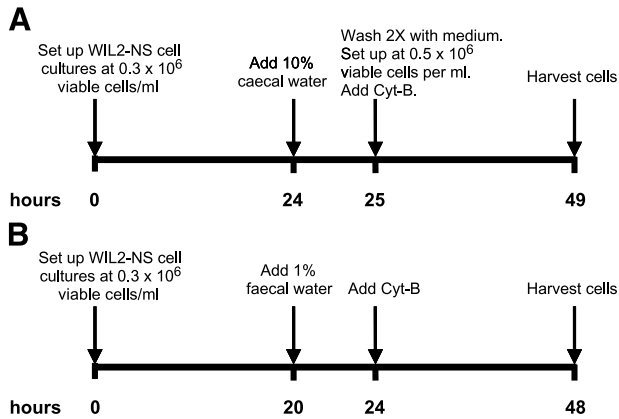


Figure 1. Schematic protocol of cecal water (A) and fecal water (B) CBMN-Cyt assay experiment.

University, Perth, Western Australia, Australia) were housed in stainless steel wire bottom cages at $23 \pm 2^\circ\text{C}$ with a 12-h light/12-h dark cycle. Rats were randomly divided into two dietary treatment groups with *ad libitum* access to experimental powdered diets and distilled water for a period of 2 weeks. Body weights were recorded weekly.

Experimental diets were modified forms of the AIN-76 formulation (17). Fat was based on sunflower seed oil, fiber was supplied as α -cellulose, and the protein was either barbecued beef or casein. The high colorectal cancer risk (HR) diet contained high fat (20%), low fiber (2%), low calcium (0.1%), and barbecued beef as the protein source equivalent to 17% of total diet. The low colorectal cancer risk (LR) diet contained low fat (5%), high fiber (5%), high calcium (0.5%), and casein as the protein source equivalent to 17% of total diet. Ethics approval was obtained from Commonwealth Scientific and Industrial Research Organisation Human Nutrition Animal Ethics Committee.

Cecal contents were collected from anesthetized rats after 2 weeks on their respective diets. Samples were centrifuged at $40,000 \times g$ for 2 h at 4°C and the supernatant was removed, filtered (0.2 μm filter; Millipore), and stored at -20°C until required.

WIL2-NS cells (American Type Culture Collection number CRL8155; obtained from the laboratories of Prof. A.A. Morley, Flinders Medical Centre, Bedford Park, South Australia, Australia) were cultured and maintained in RPMI 1640 supplemented with 5% fetal bovine serum, 2% antibiotic solution (5,000 units/mL penicillin/5 mg/mL streptomycin; all from Trace Biosciences), and 2 mmol/L L-glutamine (Sigma). The cells were cultured in plastic T-75 flasks at 37°C in a humidified atmosphere containing 5% CO_2 . The experimental protocol is shown in Fig. 1A. One thousand binucleated (BN) cells were scored for frequency of Mn-BN, Npb-BN, and Nbud-BN. Five hundred cells were scored for rate of necrosis and NDCI. These biomarkers were scored using established criteria (16). Apoptotic cells were not scored as these are virtually absent in the WIL2-NS cultures due to p53 deficiency (18). NDCI was calculated as follows: $\text{NDCI} = [\text{nec} + \text{M1} + 2(\text{M2}) + 3(\text{M3}) + 4(\text{M4})] / \text{T}$, where nec = necrotic cells; M1 to M4 = number of viable cells with one to four nuclei; and T = total cells (viable and nonviable). All

assays were done in duplicate. The WIL2-NS cell line has been validated for *in vitro* MN assay genotoxicity testing due to a low background MN frequency and excellent cellular morphology (19, 20).

Human Fecal Water Studies. Fecal samples were collected from six subjects on one occasion, when convenient within the week. One of these subjects also provided one fecal sample per week for 6 weeks. Samples were stored at -20°C until required. Volunteers were not required to modify their habitual diet during the period of fecal sample collection because only baseline data were collected in this study. Ethics approval was obtained from Commonwealth Scientific and Industrial Research Organisation Human Nutrition Ethics Committee.

Fecal samples were thawed at room temperature for 3 h, homogenized, and centrifuged at 40,000 rpm at 4°C for 50 min. The supernatant was collected and stored at -20°C until required. Fecal water was thawed at room temperature on the day of the assay. Fecal water was diluted to 10%, 1%, 0.1%, and 0.01% in RPMI 1640 and sterile filtered to determine the concentration that produced a sufficiently large genotoxic effect to be measured without excessive cell death and cytostasis. Fecal water from all six individuals was tested at the various concentrations but only 1% fecal water was tested for the six separate samples from the one individual.

WIL2-NS cells were cultured and maintained in RPMI 1640 containing 5% fetal bovine serum, 1% antibiotic solution, and 1% L-glutamine at 37°C in a humidified atmosphere with 5% CO_2 . The experimental protocol is shown in Fig. 1B. Slides were scored for the CBMN-Cyt biomarkers using established scoring criteria as described above (21).

Statistical Analysis. In the rat studies, differences in Mn-BN and Nbud-BN frequency between dietary groups were analyzed using the nonparametric Mann-Whitney *U* test because results were not normally distributed; results for Npb-BN, which were normally distributed, were analyzed using Student's *t* test. In the human study, dose-response effects were analyzed by repeated measures ANOVA and Bonferroni multiple comparison test was used to determine significance of difference between treatments. Differences at $P < 0.05$ were considered significant. All calculations were done using GraphPad Prism version 4.00 software.

Results

Rat Cecal Water Study. All rats gained weight normally during intervention. There was no significant difference in final body weights between diets (370 ± 7 g and 383 ± 11 g for the LR and HR diets, respectively).

Figure 2 shows a 7.6-fold increase in Mn-BN ($P = 0.0002$) and a 2.8-fold increase in Npb-BN ($P = 0.0003$) in the HR diet group compared with the LR group. Differences between diets for Nbud-BN did not achieve statistical significance despite a 0.5-fold increase in the HR group. There was a 2.7-fold increase in necrotic cells ($P = 0.0006$) in the HR diet group compared with the LR treatment and a significant 33% reduction in the NDCI ($P = 0.0004$) in the HR group.

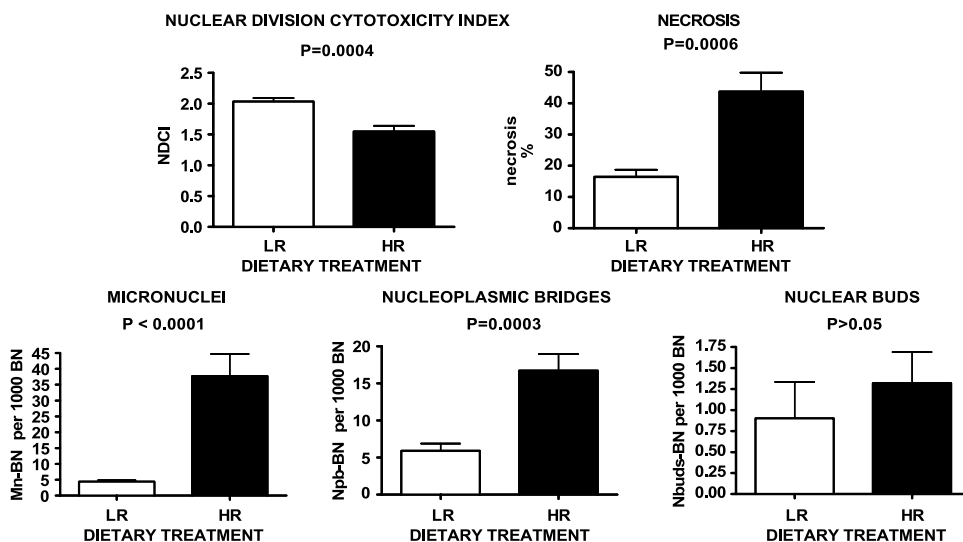


Figure 2. Comparison of CBMN assay cytotoxicity and genotoxicity biomarkers induced by cecal water from rats fed a LR or HR dietary treatment assessed in the WIL2-NS cell line. $n = 10$ rats per group.

It was not possible to obtain a measure of assay variation, as the results from these studies were based on single assays per animal. The percentage coefficients of variation (%CV) for measurements within the LR and HR groups were 30.7 and 58.7 for Mn-BN, 53.3 and 44.2 for Npb-BN, 152.3 and 89.9 for Nbud-BN, 43.9 and 44.2 for necrotic cells, and 8.9 and 19.5 for NDCI, respectively. Based on the fold increase and group CV values, it seems that Mn-BN was the more reliable and sensitive biomarker for detecting cecal water genotoxicity.

Human Fecal Water Study. For fecal water collected from six different subjects, there was a dose-related increase in necrosis and decrease in NDCI such that for 10% fecal water all cells were necrotic and that only 1%

fecal water concentration or lower provided sufficient BN cells to score DNA damage biomarkers reliably (Fig. 3). Mn-BN, Npb-BN, and Nbud-BN all increased significantly at 1% fecal water relative to the control but not at 0.01% or 0.1% fecal water (Fig. 3). From this, it was determined that using 1% fecal water was optimal to measure DNA damage biomarkers in the CBMN assay using the WIL2-NS cell line.

Fold increase (relative to the control) for CBMN-Cyt assay biomarkers was 7.5 (Nbud-BN), 6.5 (Npb-BN), 2.6 (Mn-BN), and 2.2 (necrosis) for the six different subjects and a similar trend of 28.7 (Nbud-BN), 5.9 (Npb-BN), 4.3 (Mn-BN), and 2.4 (necrosis) for results from multiple samples from one individual.

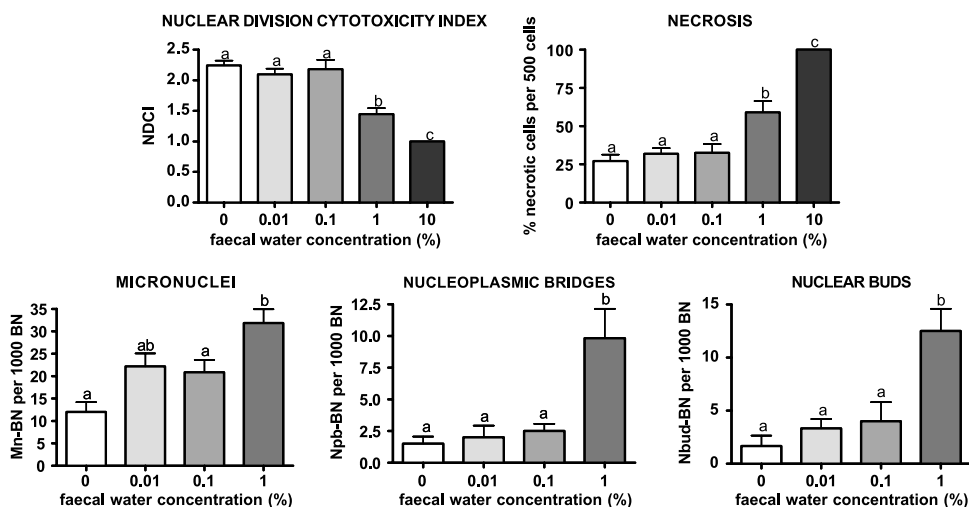


Figure 3. Dose-response effect of fecal water concentration on CBMN assay cytotoxicity and genotoxicity biomarkers assessed in the WIL2-NS cell line. Columns, mean of six fecal water samples (the result for each sample was derived from two replicate cultures); bars, SE. Values not sharing the same letter are significantly different from each other ($P < 0.05$).

The intraindividual CVs of CBMN-Cyt biomarkers were smaller than the interindividual CVs, particularly for Mn-BN (7.7% versus 23.8%) and Npb-BN (19.1% versus 57.7%), whereas the %CV did not differ substantially for Nbud (39.6% versus 41.3%), respectively.

Discussion

This pilot study explored the performance of the CBMN-Cyt assay to determine cecal and fecal water genotoxicity. It is uncertain whether genotoxins in the colon are generated before entry into the colon or whether passage through the colon is required for genotoxins to be generated. The rat study shows that genotoxic activity is detectable in cecal water, indicating that passage through the colon is not essential for genotoxins to be generated. More importantly, the rat study showed that cecal water genotoxicity was affected strongly by the type of dietary regimen such that the HR diet, known to promote colon cancer in rats (22), caused a large increase in Mn-BN and Npb-BN, biomarkers associated with increased cancer risk (23, 24). This indicates that measurement of the genotoxic potential of gut contents could be a useful biomarker of cancer risk potential.

To our knowledge, this is the first study to use the CBMN-Cyt assay for evaluation of DNA damage potential of human fecal water, and as such, the optimal dilution of fecal water to be used needed to be determined. Previous studies showed that undiluted fecal water was cytotoxic to HT-29 cells (25), which agrees with the extreme cytotoxicity we observed with a 10% dilution of fecal water. The study further illustrated that a 1% dilution of fecal water was required for use in a cell line *in vitro* with sufficient viability, suggesting that the effective concentration of fecal water solutes reaching the colonic cells *in vivo* is likely to be of an order of ~1% or less; however, this has yet to be tested directly.

Before this study, the sensitivity and extent of variation of the CBMN-Cyt biomarkers to fecal water exposure were unknown. The data indicate that the CBMN-Cyt assay using the WIL2-NS cell line to measure fecal water genotoxicity is a reliable and reproducible method in that the CV was smaller for repeated measures of multiple samples from one individual compared with CV of samples from different individuals. The results also suggest that the extent of fecal water-induced CBMN-Cyt assay biomarkers for an individual who maintains a relatively unchanged dietary pattern is unlikely to vary substantially from 1 week to the next.

It is interesting to note in the rat cecal water study that the lowest fold increase response to the HR diet was Nbud-MN biomarker; however, in the human fecal water study, Nbud-MN showed the highest sensitivity to fecal water. These differences suggest that fecal and cecal water might affect genome instability through different mechanisms or that they may also have been affected by the different study assay protocols. More work is required to validate these methods, but it seems reasonable to consider using both the short-term and long-term exposure protocols in the same way that pharmaceuticals are tested for genotoxicity.

In conclusion, this study provides preliminary validation data for the CBMN-Cyt assay using the WIL2-NS

cell line as a comprehensive and reliable method to measure the genotoxic and cytotoxic potential of fecal water within and between individuals. The fecal water CBMN-Cyt assay can be used to study the effect of different dietary patterns on genotoxicity of the contents in the distal bowel and the associated risk with colorectal cancer. The use of suitable animal models remains necessary to examine the effect of diet on genotoxicity of cecal and fecal water to determine correlations between measurements at different loci with DNA damage in the colon.

Acknowledgments

We thank the volunteers who donated samples for this study and Sylvia Usher for the isolation of fecal water in the human study.

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