Eur J Nutr 39:106–111 (2000)

ORIGINAL CONTRIBUTION

S. J. Duthie
S. Narayanan
G. M. Brand
G. Grant

DNA stability and genomic methylation status in colonocytes isolated from methyl-donor-deficient rats

Received: 16 February 2000
Accepted: 25 april 2000

S. J. Duthie (✉) · S. Narayanan
G. M. Brand · G. Grant
Rowett Research Institute
Greenburn Road
Bucksburn
Aberdeen AB21 9SB UK
e-mail sd@rri.sari.ac.uk

Summary Background: Epidemiological studies report an inverse relationship between intake of the B vitamin folic acid and colon cancer. Folate is important for DNA synthesis and repair. Moreover, the production of S-adenosylmethionine (SAM), essential for normal DNA methylation and gene expression, is dependent on folic acid. Folate deficiency may increase the risk of malignant transformation by perturbing these pathways.
Aims of the study: The principal aim of this study was to determine the effects of folate deficiency on DNA stability and DNA methylation in rat colonocytes *in vivo*. As the metabolic pathways of folate and other dietary methyl donors are closely linked, the effects of methionine and choline deficiency were also evaluated.
Methods: Male Hooded-Lister rats were fed a diet deficient in folic acid, or in methionine and choline, or in folate, methionine and choline for 10 weeks. DNA strand breakage and misincorporated uracil were determined in isolated colonocytes using alkaline single cell gel electrophoresis. Global DNA methylation was measured in colonic scrapings. Folate was measured in plasma, erythrocyte and liver samples.
Results: Methyl donor deficiency induced DNA strand breakage in colonocytes isolated from all experimental groups. Uracil levels in colonocyte DNA remained unchanged compared with controls. DNA methylation was unaffected either by folate and/or methionine and choline depletion. Rats fed a folate-deficient diet had less folate in plasma, red blood cells and liver than controls.
Conclusions: Folate and methyl deficiency *in vivo* primarily affects DNA stability in isolated colonocytes of rats, without affecting overall DNA methylation.

Key words Folic acid deficiency – methyl-donor deficiency – rat colonocytes – DNA strand breakage – DNA methylation

Introduction

It has been argued that cancer is largely a preventable disease, with inappropriate nutrition accounting for up to one third of cancer deaths (1, 2). Cancer of the colon and rectum is the fourth most prevalent cancer and cause of death from cancer in the world (2). Certain epidemiological studies have reported an inverse relationship between colorectal cancer incidence and dietary intake of the B vitamin folic acid (3, 4). Plasma and red blood cell folate levels are lower in colorectal cancer patients than in normal subjects (5). Moreover, supplementation with folic acid protects against ensuing neoplasia in high-risk patients with ulcerative colitis (6). Folate deficiency is increasingly common

in developed countries (7), affecting more than 50 % of the populace in low socio-economic groups (8, 9).

Folate, as 5-methyltetrahydrofolate, is a cofactor in the metabolic transformation of homocysteine to methionine. Methionine is then metabolised to S-adenosylmethionine (SAM), the principal methyl donor in more than one hundred biochemical reactions, including cytosine methylation in DNA. DNA methylation controls gene expression. Under conditions of folate deficiency, SAM may be limiting, leading to DNA hypomethylation, inappropriate proto-oncogene activation and transcription and malignant transformation (10–12). Animal studies have confirmed that SAM is greatly depleted in liver sampled from rats fed a methyl (folate, choline, methionine and vitamin B_{12}) deficient diet (13). Moreover, severe methyl deficiency induces hepatic DNA hypomethylation and elevates mRNA levels for the proto-oncogenes, c-Ha-*ras*, c-*myc*, and c-*fos* (13). Folate deficiency alone also induces hepatic DNA hypomethylation (14). However, data describing the influence of methyl deficiency on DNA methylation and gene expression in the colon are inconsistent (15, 16).

Normal DNA synthesis and repair requires 5,10-methylenetetrahydrofolate for the manufacture of purines and the pyrimidine thymidine. Folate deficiency is associated with increased DNA instability in cultured cells, manifest as increased DNA strand breakage, uracil misincorporation, chromosomal damage and mutagenesis (17–20). Mice made folate-deficient exhibit chromosomal abnormality (21), while methyl depletion induces both global and proto-oncogene-specific DNA strand breakage in rat liver (22).

We report here the influence of folate depletion, alone and in combination with methionine and choline insufficiency, on both DNA methylation and DNA stability in colonocytes isolated from rats fed the deficient diets for 10 weeks.

Materials and methods

Materials

Ultrapure low melting point (LMP) and electrophoresis grade standard melting point (SMP) agarose were from Gibco Life Technologies (Paisley, UK). DAPI (4',6-diamidine–2-phenylindole dihydrochloride) and proteinase K were from Boehringer Mannheim (Lewes, UK). ICN Flow (Irvine, UK) supplied Simultrac Radioassay Kit Vitamin B_{12} [^{57}Co] folic acid [^{125}I] and Dutch Modified RPMI 1640 medium. Nucleospin C&T kits for DNA isolation were from BioGene (Cambridge, UK). ^{3}H-labeled S-adenosyl-L-methionine was from NEN Life Sciences Products (Hounslow, UK). Uracil DNA glycosylase (UDG) was from Helena Biosciences (Sunderland, UK). All other reagents were from Sigma (Poole, UK).

Animals, diets and preparation of blood and tissue samples

Approximately isoenergetic diets containing 112 g casein protein/kg were formulated (Table 1). Mineral and vitamin mixes were in accordance with NRC recommendations and prepared as published previously (23) with the exception that the vitamin mix was devoid of folic acid and choline chloride. Diets were prepared at 2-week intervals and were stored at –20°C.

All procedures were carried out in strict accordance with the requirements of the UK Animals (Scientific Procedures) Act 1986.

Thirty two male Hooded-Lister (Rowett strain) rats were used. Weaned at 19 days, they were group-housed and given free access to a lactalbumin-based semi-synthetic control diet (23) until they weighed 95–100 g [40 days old]. They were subsequently housed individually and fed

Table 1 Experimental diets

Diet (g/kg)	A (control)	B (folate-deficient)	C (choline/methionine-deficient)	D (folate/choline/methionine-deficient)
Folic acid mix	**0.005**	**0**	**0.005**	**0**
L-methionine	**4**	**4**	**0**	**0**
Choline chloride	**2**	**2**	**0**	**0**
Maize starch	382	382	388	388
Casein	112	112	112	112
Potato starch	100	100	100	100
Glucose	150	150	150	150
Corn oil	150	150	150	150
Minerals*	50	50	50	50
Vitamins*	50	50	50	50
Sodium silicate	0.2	0.2	0.2	0.2

* mineral and vitamin mixes were prepared as described (23) with the exception that folic acid and choline chloride were absent.

12 g/d for 5 days of the same control diet. The rats (8 per treatment group) were then given the appropriate experimental diet (Table 1) exclusively for 10 weeks. They were fed a specific amount of diet daily throughout the study; initially 12 g/rat/d, increasing to 15 g/rat/d after 1 week up to 16.5 g/rat/ day from 6 weeks. This was equivalent to 90–100 % of normal free intake of semi-synthetic diet. All food was eaten, water was freely available and the rats were weighed daily.

After 10 weeks the rats were killed by anaesthetic overdose (Halothane) and exsanguination via cardiac puncture, and blood and tissues collected. Blood was collected into EDTA vacutainers, spun at 2400 × g for 15 min at 4°C and the plasma aliquoted into 1 ml plastic tubes, snap-frozen in liquid nitrogen and stored at –80°C for analysis. Erythrocytes reconstituted to initial blood volume with PBS following plasma separation were aliquoted, snap-frozen and stored at –80°C. The liver was removed, cut into 0.5–1 g pieces and snap-frozen. For measurement of folate, liver was thawed, homogenised on ice in 0.02 M phosphate buffer (4°C), and diluted in 0.02 M borate buffer (pH 7.5, 4°C) for immediate analysis. Protein was determined using the Biuret method (24). DNA methylation and comet analysis was carried out on rat colonocytes. To do this the colon was removed, washed 3 times with saline at 4°C and cut longitudinally into two. One half of the colon was spread on a glass plate on ice and the colon mucosa scraped gently off using a glass microscope slide. The scrapings were minced using 60 strokes of sharp scissors, snap-frozen and stored at –80°C before DNA isolation. The second portion of colon was placed in washing buffer at 4°C for colonocyte isolation (described below).

Measurement of folate and vitamin B_{12}

Plasma (200 µl), erythrocyte (100 µl) and hepatic (100 µl homogenate) folate were determined using a commercially available kit (Simultrac Radioassay Kit Vitamin B_{12} [^{57}Co] folic acid [^{125}I]).

DNA isolation and genomic DNA methylation status

DNA was isolated from rat colon mucosa using a NucleoSpin C&T kit. The DNA concentration was determined spectrophotometrically at 260/280 nm. Methylation was measured in total genomic DNA after the method of Balaghi and Wagner (14). This assay measures the incorporation of methyl groups from ^{3}H-labeled S-adenosyl-L-methionine at specific cytosine residues in genomic DNA using the bacterial enzyme Sss1 methylase. DNA methylation status is inversely related to the degree of radioactive incorporation, i. e. the lower the methylation of the DNA the higher the DPM.

Preparation of isolated rat colonocytes

Rat colonocytes were isolated by a method based on that of Brendler-Schwaab et al (25). One half of the rat colon was immediately removed into washing buffer [Ca^{2+}/Mg^{2+}-free HBSS containing 10 mM HEPES, pH 7.4] at 4°C on ice. The washing buffer was aspirated off and the colon gently agitated in digestion buffer [Ca^{2+}/Mg^{2+}-free HBSS containing proteinase K (50U/ml), pH 7.4] at 37°C for 15 min. The tissue was discarded from the tube and the remaining colonocyte cell suspension centrifuged at 200 × g for 10 min at 4°C. The supernatant was decanted, the cells counted using a Neubauer Improved Haemocytometer and the pellet washed in cold RPMI 1640 medium and centrifuged as before for 3 min. The cells were resuspended in 85 µl LMP agarose for comet analysis. Cell integrity (typically greater than 75 %) was determined by Trypan Blue exclusion.

Comet analysis (single cell gel electrophoresis)

DNA strand breakage and misincorporated uracil were measured using the alkaline comet assay or single cell gel electrophoresis (17). Isolated rat colonocytes were suspended in 85 µl of 1 % LMP agarose (w/v in PBS, pH 7.4) and pipetted onto a frosted glass microscope slide precoated with 95 µl of 1 % SMP agarose. The agarose was set for 10 min at 4°C and the slide incubated for 1 h at 4°C in lysis solution [2.5 M NaCl, 10 mM Tris, 100 mM Na_2EDTA and 1 % (v/v) Triton X–100, (NaOH to pH 10.0)]. The slides were washed 3 times for 5 min each in buffer [60 mM Tris-HCl, 1 mM EDTA, 0.1 mg/ml BSA, pH 8.0], gently blotted dry and the gel covered with 50µl of either buffer or uracil DNA glycosylase (0.1 unit/gel in buffer). The slides were incubated for 1 h at 37°C in a moist atmosphere. The slides were subsequently aligned in a horizontal gel electrophoresis tank (260 mm wide) containing electrophoresis buffer [0.3 M NaOH and 1 mM Na_2EDTA, pH 12.7] for 40 min before electrophoresis at 25 V for 30 min. The slides were washed 3 times for 5 min each at 4°C in neutralizing buffer [0.4 M Tris-HCL, pH 7.5] and stained with DAPI.

DAPI-stained nucleoids [5 µg/ml] were scored visually (on the basis of the intensity of fluorescence in the comet tail). DNA damage due to strand breakage is estimated using the score obtained from buffer-treated gels alone. Misincorporated uracil is estimated by subtracting the visual score obtained from the buffer-treated gels from the score obtained after incubation with enzyme (17). This method of visual classification has been extensively validated using computerised image analysis [Komet 3.0, Kinetic Imaging Ltd, Liverpool, UK] (17).

Table 2 The influence of a methyl-deficient diet on folate status and body weight

Group	A (control)	B (folate-deficient)	C (choline/methionine-deficient)	D (folate/choline/methionine-deficient)
Initial body weight (g)	108 ± 2.4	105 ± 3.1	108 ± 1.0	110 ± 4.0
Final body weight	381 ± 14.7	376 ± 8.1	342 ± 5.8*	346 ± 7.0*
Plasma folate (ng/ml)	58.1 ± 4.1	30.5 ± 4.2* [52.4 ± 7.3]	89.5 ± 7.3* [153.9 ± 12.6]	58.8 ± 5.3 [101.2 ± 9.1]
Erythrocyte folate (ng/ml)	9.2 ± 0.4	6.7 ± 0.7* [72.9 ± 7.7]	8.2 ± 0.4 [89.0 ± 4.1]	5.9 ± 0.4* [65.1 ± 3.9]
Liver folate (ng/mg)	81. 2 ± 6.2	60.2 ± 4.7* [74.1 ± 5.8]	70.9 ± 5.4 [87.3± 6.7]	57.6 ± 4.9* [70.9 ± 6.1]

Analyses were performed at the end of the experiment (week 10).
Results are mean ± SEM for n=8.
*P< 0.05, where significance refers to differences between Group A and Groups B–D
Folate depletion, expressed as a percentage of control values, is indicated in parenthesis [ ]

Statistical analysis

Significant differences between all experimental groups were analysed by ANOVA followed by Tukey's HSD *post hoc* test using "Statistical Package for Social Sciences" (SPSS version 8).

Results

Rats were fed an experimental diet deficient in folic acid (folate-free) and/or in the methyl donors choline (choline-free) and methionine (70 % of requirements) for 10 weeks (Table 1). Folate deficiency (Group B) had no significant effect on final body weight (Table 2). Rats fed a methionine and choline deficient diet (Group C) were approximately 10 % lighter than controls. Rats deficient in all three methyl donors (Group D) showed no further weight loss (Table 2).

Plasma folate was depleted by approximately 50 % in rats fed only a folate-deficient diet (Group B, Table 2). Conversely, plasma folate was increased 50 % after 10 weeks on a choline and methionine deficient diet (Group C, Table 2). Feeding a combined folate, choline and methionine deficient diet had no net effect on plasma folate levels (Group D, Table 2). Red blood cell and liver folate in groups B and D (folate deficient) were decreased by 25 % (Table 2). Choline and methionine deficiency alone (Group C) did not significantly alter erythrocyte or liver folate stores (Table 2).

Folate deficiency (Group B) increased DNA strand breakage more than 30 % in isolated rat colonocytes (Fig. 1a). Choline and methionine deficiency, either alone (Group C) or in combination with folate deficiency (Group D), induced a similar increase in strand breakage (Fig. 1a).

None of the experimental diets altered uracil misincor-

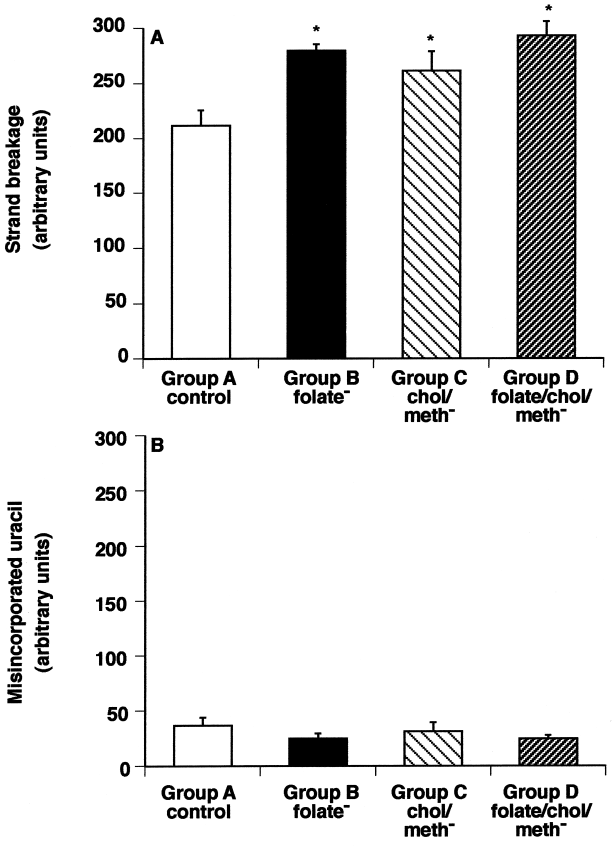

Fig. 1 The effect of methyl donor deficiency on DNA strand breakage (a) and misincorporated uracil (b) in isolated colonocytes of rats. Rats were either fed a control diet (Group A) or a diet deficient in folic acid (Group B), choline and methionine (Group C) or a diet deficient in all 3 methyl donors (Group D) for up to 10 weeks. DNA strand breakage or uracil misincorporation was measured in rat isolated colonocytes. Results are mean ± SEM (n=8). *P< 0.01, where significance [ANOVA followed by Tukey's HSD test] refers to differences between Group A and Groups B–D after 10 weeks on the diet

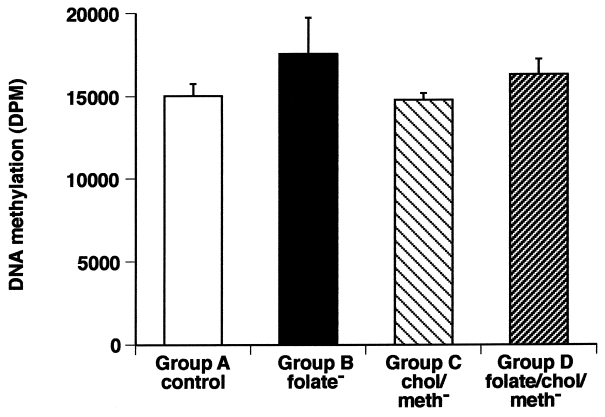

Fig. 2 The effect of methyl donor deficiency on genomic DNA methylation of isolated colonocytes of rats. Rats were either fed a control diet (Group A) or a diet deficient in folic acid (Group B), choline and methionine (Group C) or a diet deficient in all 3 methyl donors (Group D) for up to 10 weeks. Results are mean DPM ± SEM (n=8)

poration in rat isolated colonocytes (Fig. 1b). Moreover, genomic DNA methylation in rat colonocytes was not affected by folate or methyl deficiency either alone or in combination (Fig. 2).

Discussion

Deficiencies in folic acid and dietary methyl groups have been implicated in the aetiology of colorectal cancer (3, 4, 26). There appear to be two principal mechanisms through which folic acid deficiency could modulate malignant transformation, firstly by altering DNA methylation and proto-oncogene expression and secondly by negatively affecting DNA stability and repair.

Rats fed methionine and choline deficient diets develop spontaneous hepatocellular carcinoma (27, 28). Also, folate and methyl deficiency decreases global DNA methylation in rat liver (13) and induces transcription of mRNA for the proto-oncogenes c-*fos*, c-*myc* and c-Ha-*ras* (13). Moreover, hepatic SAM is depleted and the ratio of SAM to S-adenosylhomocysteine (SAH), critical for sustaining normal cellular/biochemical methylation reactions, is severely reduced (13, 14, 16).

In this study folate deficiency and/or methionine and choline deficiency had no effect on global methylation status in DNA isolated from rat colonic scrapings. This is in agreement with data from Kim and colleagues (16) who found that both genomic and c-*myc* methylation, and SAM and the SAM:SAH ratio remained unaltered in rat colon from folate-deficient rats. This was the case, even in the presence of a significant depletion in liver SAM, suggesting that hypomethylation in the colon may be relatively resistant to dietary modulation (16).

DNA strand breakage if left unrepaired can induce chromosome instability, altered gene expression and neoplastic transformation. Single cell gel electrophoresis has been used to determine the influence of short chain fatty acids, flavonoids and lactic acid-producing bacteria on both DNA strand breakage and oxidative base damage in human and rodent colonocytes (29–31). Thus, a modified comet assay was employed in this study to measure DNA strand breakage and uracil misincorporation concurrently in isolated rat colonocytes (17).

DNA strand breakage in the present study was increased in colonocytes isolated from methionine and choline and/or folate deficient rats (Fig. 1). Using quantitative PCR, Pogribny et al. have shown that after 9 weeks on a combined methionine, choline and folate deficient diet, gene-specific DNA strand breakage was induced in exon 5 of the p53 proto-oncogene isolated from liver (22).

There was no change in the level of uracil detected in colonocyte DNA in any of the deficient groups. Folate depletion *in vitro* increases uracil misincorporation specifically and dose-dependently in human lymphocytes (17). Likewise, uracil and micronuclei frequencies are increased in blood cells from folate-deficient human subjects (32). However, quiescent lymphocytes express very low levels both of deoxyuridine triphosphatase (dUTPase) and UDG, which control the levels of uracil in the nucleotide pool and in DNA (33). Moreover, the activities of these enzymes are dramatically increased in proliferating cells (33). The endogenous level of these enzymes may be higher in rat colonocytes compared with lymphocytes, ensuring that even under conditions of nutritional stress, excess deoxyuridine triphosphate does not accumulate and misincorporated uracil is removed from the DNA molecule.

In summary, DNA strand breakage increased significantly in colonocytes isolated from rats fed a folate-deficient and/or a methionine and choline-deficient diet for 10 weeks. In contrast, there was no change in the overall global methylation status of the colonocytes. Two different forms of folate are used either for synthesis of thymine from uracil [5,10-methylenetetrahydrofolate] or for cytosine methylation in DNA [5-methyltetrahydrofolate]. When the dietary methyl supply is restricted and SAM is depleted, folate metabolism is shifted to maintain an adequate supply of 5'-methyltetrahydrofolate at the expense of several other folate metabolites (34). This may create a relative deficiency in 5,10-methylenetetrahydrofolate, disrupting normal DNA synthesis and repair. Such a disruption in DNA repair could account for the increase in strand breakage. While it cannot be discounted that methyl donor deficiency may induce specific oncogene hypomethylation in the colon, it may be that nutritional inadequacy initially affects the genome, by decreasing DNA stability.

Acknowledgments The World Cancer Research Fund and The Scottish Executive Rural Affairs Department (SERAD) support this work.

References

1. Doll R, Peto R (1981) The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. J Natl Cancer Inst 66:1192–1265
2. World Cancer Research Fund (1997) Food, nutrition and the prevention of cancer: a global perspective. American Institute for Cancer Research, Washington USA
3. Benito E, Stiggelbout A, Bosch FX, Obrador A, Kaldor J, Mulet M, Munoz N (1991) Nutritional factors in colorectal cancer risk: a case-control study in Majorca. Int J Cancer 49:161–16.
4. Giovannucci E, Stampfer MJ, Colditz GA, Rimm EB, Trichopoulos D, Rosner BA, Speizer FE, Willet WC (1993) Folate, methionine and alcohol intake and risk of colorectal adenoma. J Natl Cancer Inst 85:875–884
5. Porcelli B, Frost B, Rosi F, Arezzine L, Civitelli S, Tanzini G, Marinello E (1996) Levels of folic acid in plasma and in red blood cells of colorectal cancer patients. Biomed Pharmacother 50:303–305
6. Lashner BA, Heidenreich PA, Su GL, Kane SV, Hanauer SB (1989) Effect of folate supplementation on the incidence of dysplasia and cancer in chronic ulcerative colitis. Gastroenterology 97:255–259
7. DeBree A, van Dusseldorf M, Brouwer IA, van het Hof KH, Steegers-Theunissen RPM (1997) Folate intake in Europe: recommended, actual and desired intake. Eur J Clin Nutr 51:643–660
8. Senti RF, Pilch SM (1985) Analysis of folate data from the 2nd national health and nutritional examination survey (NHANES-II). J Nutr 115: 1398–1402
9. Blount BC, Ames BN (1994) Analysis of uracil in DNA by gas chromatography-mass spectrometry. Anal Biochem 219:195–200.
10. Feinberg AP, Vogelstein B (1983) Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 301:89–92
11. Fang J-Y, Zhu S-S, Xiao S-D, Jiang S-J, Shi Y, Chen X-Y, Zhou X-M, Qian L-F (1996) Gastric cancer: clinical and laboratory studies: studies on the hypomethylation of c-*myc*, c-Ha-*ras* oncogenes and histopathological changes in human gastric carcinoma. J Gastroenterol Hepatol 11:1079–1082
12. Fang J-Y, Xiao S-D, Zhu S-S, Yuan J-M, Qiu D-K, Jiang S-J (1997) Relationship of plasma folic acid and status of DNA methylation in human gastric cancer. J Gastroenterol 32:171–175
13. Wainfain E, Poirier LA (1992) Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. Cancer Res 52:2071s–2077s
14. Balaghi M, Wagner C (1993) DNA methylation in folate deficiency: use of CpG methylase. Biochem Biophys Res Commun 193:1184–1190
15. Kim Y-I, Pogribny IP, Salomon RN, Choi S-W, Smith DE, James SJ, Mason JB (1996) Exon-specific DNA hypomethylation of the p53 gene of rat colon induced by dimethylhydrazine: modulation by dietary folate. Am J Clin Pathol 149:1129–1137
16. Kim Y-I, Christman JK, Fleet JC, Cravo ML, Salomon RN, Smith D, Ordovas J, Selhub J, Mason JB (1995) Moderate folate deficiency does not cause global hypomethylation of hepatic and colonic DNA or c-*myc*-specific hypomethylation of colonic DNA in rats. Am J Clin Nutr 61:1083–1090
17. Duthie SJ, Hawdon A (1998) DNA stability (strand breakage, uracil misincorporation, and defective repair) is increased by folic acid depletion in human lymphocytes *in vitro*. FASEB J 12:1491–1497
18. Libbus BL, Borman LS, Ventrone CH, and Branda RF (1990) Nutritional folate deficiency in Chinese hamster ovary cells: chromosomal abnormalities associated with perturbations in nucleic acid precursors. Cancer Genet Cytogenet 46:231–242
19. James SJ, Basnakian AG, Miller BJ (1994) *In vitro* folate deficiency induces deoxynucleotide pool imbalance, apoptosis and mutagenesis in Chinese hamster ovary cells. Cancer Res 54:5075–5080
20. Branda RF, LaFayette AR, O'Neill JP Nicklas JA (1997) Effect of folate deficiency on mutations at the *hprt* locus in Chinese hamster ovary cells exposed to monofunctional alkylating agents. Cancer Res 57:2586–2588
21. MacGregor JT, Schlegel R, Wehr CM Alperin P, Ames BN (1990) Cytogenetic damage induced by folate deficiency in mice is enhanced by caffeine. Proc Natl Acad Sci USA 87: 9962–9965
22. Pogribny IP, Basnakian AG, Miller BJ Lopatina NG, Poirier LA, James SJ (1995) Breaks in genomic DNA and within the p53 gene are associated with hypomethylation in livers of folate/methyl deficient rats. Cancer Res 55:1894–1901
23. Grant G, Dorward PM, Pusztai A. (1993) Pancreatic enlargement is evident in rats fed diets containing raw soyabean (*Glycine max*) or cowpea (*Vigna unguiculata*) for 800 days but not in those given diets based on kidney bean (*Phaseolus vulgaris*) or lupinseed (*Lupinus angustifolius*). J Nutr 123:2207–2215
24. Gornall AG, Bardawill CJ, David MM (1949) Determination of serum proteins by means of the Biuret reaction. J Biol Chem 177:751–766
25. Brendler-Schwaab SY, Schmezer P, Liegibel U, Weber S, Michalek K, Tompa A, Pool-Zobel BL (1994) Cells of different tissues for *in vitro* and *in vivo* studies in toxicology: compilation of isolation methods. Toxic *In Vitro* 8:1285–1302
26. Glynn SA, Albanes D, Pietinen P, Brown CC, Rautalahti M, Tangrea JA, Gunter EW, Barrett MJ, Virtamo J, Taylor PR (1996) Colorectal cancer and folate status: a nested case-control study among male smokers. Cancer Epidemiol Biomarkers and Prevention 5:487–494.
27. Ghoshal AK, Farber E (1984) The induction of liver cancer by dietary deficiency of choline and methionine without added carcinogens. Carcinogenesis 5:1367–1370
28. Henning SM, Swendseid ME, Coulson WF (1997) Male rats fed methyl- and folate-deficient diets with or without niacin develop hepatic carcinomas associated with decreased tissue NAD concentrations and altered poly (ADP) polymerase activity. J Nutr 127:30–36
29. Abrahamse SL, Pool-Zobel BL, Rechkemmer G (1999) Potential of short chain fatty acids to modulate the induction of DNA damage and changes in the intracellular calcium concentration by oxidative stress in isolated rat distal colon cells. Carcinogenesis 20:629–634
30. Wollowski I, Ji ST, Bakalinsky AT, Neudecker C, Pool-Zobel BL (1999) Bacteria used for the production of yoghurt inactivate carcinogens and prevent DNA damage in the colon of rats. J Nutr 129:77–82
31. Duthie SJ, Dobson VL (1999) Dietary flavonoids protect human colonocyte DNA from oxidative attack *in vitro*. Eur J Nutr 38:28–34
32. Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, Wang G, Wickramasinghe SN, Everson RB, Ames BN (1997) Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. Proc. Natl. Acad. Sci USA 94:3290–3295
33. Vilpo JA, Autio-Harmainen H (1983) Uracil DNA glycosylase and deoxyuridine triphosphatase: studies of activity and subcellular location in human normal and malignant lymphocytes. Scand J Clin Invest 43:583–590
34. Rogers AE (1995) Methyl donors in the diet and responses to chemical carcinogens. Am J Clin Nutr 61 (suppl): 659s–665s