REVIEW

Application and adaptation of the in vitro micronucleus assay for the assessment of nutritional requirements of cells for DNA damage prevention

Caroline F. Bull, Sasja Beetstra-Hill, Bianca J. Benassi-Evans, Jimmy W. Crott1, Michiyo Kimura2, Theodora Teo, Jing Wu and Michael F. Fenech*

CSIRO Food and Nutritional Sciences, Gate 13 Kintore Avenue, Adelaide, South Australia 5000, Australia, 1Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA 02111, USA and 2Department of Health and Nutrition, Faculty of Health and Welfare, Takasaki University of Health and Welfare, 37-1, Nakaorui-machi, Takasaki City, Gunma Prefecture 370-0033, Japan

DNA damage is a fundamental cause of developmental and degenerative diseases. The in vitro cytokinesis-block micronucleus cytome (CBMN-Cyt) assay is an established comprehensive method for assessing cytostasis and chromosome stability in cells. Originally developed to study the acute effects of single environmental genotoxicants, creative applications and adaptations to the basic protocol have allowed its use in evaluating the impacts of dietary micronutrients and micronutrient combinations (nutriomes) on DNA damage. In this review, we examine some of these studies and the important findings they have generated with respect to nutrient/nutrient, nutrient/genotype and nutrient/genotoxicant interactions. In particular, we examine some of these applications and adaptations and the findings generated; we outline current knowledge gaps and propose some future directions for this field of research.

Introduction

The cytokinesis-block micronucleus cytome (CBMN-Cyt) assay is an established comprehensive method for assessing cytostasis and chromosome stability in cells, the protocol for which has been previously detailed by Fenech (1). In brief, DNA damage events are scored specifically in once-divided binucleated (BN) cells and include micronuclei (MN), a biomarker of chromosome breakage and/or whole chromosome loss, nucleoplasmic bridges (NPBs), a biomarker of DNA misrepair and/or telomere end fusions, and nuclear buds (NBuds), a biomarker of elimination of amplified DNA and/or DNA repair complexes (1). Cytostatic effects are measured via the proportion of mono-, bi- and multinucleated cells and cytotoxicity via necrotic and/or apoptotic cell ratios (1). The standard protocol involves culturing freshly isolated peripheral blood lymphocytes (PBL) for 72 h in complete medium prior to blocking cells during cytokinesis with cytochalasin-B (CytoB) and harvesting onto slides for quantification of chromosomal aberrations. This method was originally developed to study the acute effects of single environmental genotoxicants with exposures ranging from 1 to 24 h and cultures not exceeding 72 or 96 h prior to harvest of cells. Such an approach, however, is not efficacious for evaluating the impacts of micronutrients and micronutrient combinations (nutriomes) as the genomic impacts of micronutrients take at least 7 days to become evident and effects may vary with increasing time and/or genotype. Accordingly, several studies have been conducted whereby adaptations to the standard protocol have provided a means for assessing the impact of micronutrients, in isolation and in combination, in settings that more closely resemble physiological conditions in vivo. These include explorations of nutrient/nutrient, nutrient/genotype and nutrient/genotoxicant interactive effects. In this review, we examine some of these applications and adaptations and the findings generated; we outline current knowledge gaps and propose some future directions for this field of research.

Significant achievements

Adaptations to micronutrient concentrations, experimental time frame and seeding density

Standard culture media can vary enormously between batches with respect to vitamins and minerals, with the concentration often being supraphysiological or deficient relative to human serum, depending on the micronutrient. RPMI-1640 culture medium, one of the most commonly used for culturing human cells, is supraphysiological for folate, methionine and riboflavin (vitamin B2) and deficient for iron, copper, zinc, calcium, magnesium and sulphur. As such, modifications are needed in order to study the true impact of micronutrient deficiency or excess on chromosome stability in vitro. One of the earliest uses of the CBMN-Cyt assay for a physiologically equivalent nutrition study was conducted by Crott et al. (2), whereby folic acid (FA) in medium was modified from supraphysiological concentrations found in complete medium (~3000 nM), to a series of doses within the physiological range (12–120 nM). A further adaptation for this study was the extension of the experimental time frame to 9 days, considerably longer than the standard 72-h protocol, and one more closely reflecting in vivo chronic exposure conditions as occurs due to habitual dietary patterns. Results showed an inverse dose-dependent relationship between FA within the physiological range and increased chromosomal damage biomarkers (2). This and subsequent studies have shown that DNA damage biomarkers are consistently minimised when cells are maintained in culture medium with an FA concentration >60 nM, a concentration greater than that commonly observed in plasma in the general population (<30 nM) (2–7). These important findings, at physiological concentrations, demonstrated the critical nature of adequate FA for cell replication and genomic stability.

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Another example of a study which incorporated several protocol adaptations to more closely resemble the physiological state is that of Wu et al. (8), whereby PBL were cultured over the long term (9 days) in a selenium (Se) dose–response study. Key questions examined were genotoxicity and cytotoxicity of different Se doses, the optimal Se concentration for genome stability and whether adequate Se improved resistance to γ-radiation-induced genome damage (8). One adaptation to the CBMN-Cyt protocol was the use of selenium-l-methionine (Se-Met) as the Se source. Se-Met is an important dietary form of Se in humans and is commonly used in cancer prevention studies (8). RPMI-1640 medium was prepared at six dietary forms of Se in humans and is commonly used in cancer treatment increased. To achieve this, each batch of medium effect of changing Met concentrations as Se-Met in each methionine (Met)-free medium as a base. A further modification was also made to minimise the potentially confounding effect of changing Met concentrations as Se-Met in each treatment increased. To achieve this, each batch of medium was supplemented with the appropriate amount of l-Met to ensure a consistent concentration of 50 μM, a level closer to that observed in plasma (20–30 μM) (8) and considerably lower than the supraphysiological concentration in standard culture medium (100 μM) (5). A final adaptation and protocol advance used in this study was the use of low cell seeding density in microwell cultures (150 000 cells/ml in 200 μl culture) that allowed continuous growth of PBL without the need for subculture (8). Using this physiologically relevant study design, it was concluded from the observed results that chromosomal instability was minimised at 100–430 μg/l Se, with cells from these cultures displaying significantly lower frequencies of both NBuds and NPBs (8). Previous studies had suggested an in vivo plasma concentration of 120 μg Se/l for minimisation of cancer risk (9). Supraphysiological concentrations (>1880 μg/l), on the other hand, resulted in a sharp increase in cell death and reduced nuclear division index. No protective effect was observed for Se-Met with challenge by γ-irradiation (8).

Investigations of nutrient–gene interactions on chromosomal damage

In their 2001 paper discussed above, Crott et al. (2) also examined whether the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism impacted on DNA damage levels when cultured in physiological FA concentrations (12–120 nM). This study found no difference between CC and TT genotypes. A related study, also by Crott et al., tested whether human PBL obtained from TT donors were more able to resist uracil incorporation into DNA than controls (CC donors), when cultured in medium containing FA in the physiological range (10). Again, no effect of genotype was observed. New evidence at the time then showed that riboflavin (vitamin B2), the immediate precursor to the MTHFR cofactor, flavin adenine dinucleotide (FAD), was an independent determinant of plasma homocysteine (Hcy) levels, but only in individuals with the C677T polymorphism. Vitamin B2 (B2) in RPMI-1640 is 530 nM, considerably higher than the 5–50 nM found in plasma, and may therefore raise intracellular FAD levels to a degree that would increase the activity of the slow variant MTHFR 677 TT homozygotes to a degree that made them indistinguishable from the fast variant (CC) genotype (5,10,11). As such, the authors speculated that excess B2 in standard culture medium may have negated the influence of the T677 allele on MTHFR activity and on uracil incorporation into DNA, thus making it impossible to detect the effect of this polymorphism. The concentration of Met in culture medium was another possible confounder in these studies with RPMI containing 100 μM compared with 20–30 μM normally found in vivo. Elevated Met concentrations may have caused increased intracellular S-adenosyl methionine, a known inhibitor of MTHFR activity. As such, the authors concluded that supraphysiological concentrations of B2 and Met may have caused differences in MTHFR activity to be undetectable in this in vitro system (10).

Combining these concepts, Kimura et al. (5) then repeated these experiments to study the differential impact of MTHFR genotype on chromosome stability using a lower physiological concentration of Met (50 μmol/l), in combination with deficient or sufficient concentrations of B2 (0 and 500 nM) and FA (20 and 100 nM). Results using this model showed significant differences in biomarkers of chromosome damage between the TT and CC genotypes. The effect of B2 was small compared with that of FA. MN were 21% higher in TT cells than in CC cells (P < 0.05) and 42% lower in the high FA medium than in the low FA medium (P < 0.0001). The NBud levels were 27% lower in TT cells than in CC cells (P < 0.05) and 45% lower in the high FA medium (P < 0.0001). High B2, on the other hand, was found to increase NBud levels by 25% compared with low B2 in the FA-deficient condition (20 nM). An interactive effect was recorded between FA and B2 that impacted on NBud levels (P = 0.04) (5).

Another nutrient/gene study using an adapted form of the CBMN-Cyt assay investigated the effect of FA deficiency in PBL with the BRCA1 and BRCA2 mutation to test the hypothesis that carriers of BRCA1 or BRCA2 pathogenic mutations are more susceptible to the DNA-damaging effects of folate deficiency (12). In this study by Beetstra et al., genome stability was compared between carriers of the BRCA1 and BRCA2 mutations with and without breast cancer and non-carrier cancer-free relatives. The study was conducted in PBL over the long term (9 days) in complete medium containing either 12 or 120 nM FA (12). Results showed that moderate folate deficiency had a greater influence on genomic stability than BRCA1 or BRCA2 mutations. The authors suggested that genetic effects may have been corrected or masked by supraphysiological nutrient concentrations in culture medium, including B2 and Met (12), while other nutrients (including Se) and natural antioxidant phenolic compounds are lacking in standard culture medium (12). Interestingly, an in vitro study conducted at a similar time had shown that 1–3 months of Se supplementation significantly reduced elevated levels of bleomycin-induced chromosome breakage in carriers of the BRCA1 mutation, resulting in damage levels similar to those of non-carriers (13). Accordingly, Beetstra et al. (12) proposed that culture medium containing nutrient and antioxidant concentrations which more closely reflect in vivo levels, as well as a larger cohort allowing for more effective genotype matching between groups, may provide a more accurate picture of phenotypic differences caused by these mutations in a low folate background.

Adaptations to examine nutrient/genotoxicant interactions on genome stability

The CBMN-Cyt assay is widely recognised as a comprehensive and sensitive tool for assessing the impact of a single compound (endogenous or exogenous) on genomic stability. In the vast majority of studies conducted, including toxicology testing for novel therapeutics, the standard protocol is adopted whereby cells are maintained in standard culture medium for
Interactive effects of micronutrient and phytonutrient factors with ionising radiation and alcohol

Several studies have been conducted whereby cells (PBL or cell lines) cultured over the long term in media containing micronutrients in the physiological range have been challenged with \( \gamma \)-irradiation. In the case of Se, exposure to 1.5 Gy of irradiation following 9 days in Se-containing culture medium showed no significant protective effect (8). FA, on the other hand, was shown to offer significant protection against radiation-induced damage in WIL2-NS cells (14). Long-term (9 days) cultures were conducted with 0.2, 2, 20 and 200 nM FA. A dose-dependent reduction in MN and NPBs was observed in the unirradiated cells, as well as in those exposed to 1.5 Gy \( \gamma \)-irradiation (interaction \( P = 0.004 \)) (14).

Vrinda and Uma Devi (15) used the CBMN-Cyt assay to demonstrate a protective effect of the antioxidant flavonoids Orentin (Ot) and Vicenin (Vc), extracted from the leaves of Indian holy basil \textit{Ocimum sanctum}, against ionising radiation-induced MN induction. Whole blood cultures were established using complete medium, with a solution of Ot/Vc added for 30 min prior to exposure to \( \gamma \)-irradiation. Thereafter, cells were maintained in culture for 72 h, the final 24 h of which was in the presence of CytoB. PBL were then isolated, fixed and binucleated cells were scored. Results showed that these nutritional compounds were highly protective against ionising radiation-induced MN formation and that this effect was likely associated with antioxidant activity of Ot and Vc (15).

Other adaptations of the CBMN-Cyt assay have effectively demonstrated the impact of alcohol and individual components of wine on genomic stability (16,17). Teo and Fenech (16) examined the interactive effect of FA and ethanol (EtOH) using WIL2-NS cells over a 15-day time course. For this \( 3 \times 4 \) factorial experiment cells were maintained in one of three concentrations of FA (20, 200 or 200 nM), each of which also contained either 0, 0.09, 0.36 or 1.34\% v/v EtOH. The latter concentrations were based on blood alcohol readings that have been recorded following binge drinking (18). Findings from this interactive study suggested a protective effect of FA against alcohol-induced DNA damage (specifically MN and NPBs), with a detrimental interactive effect recorded between low FA and high EtOH (16).

Greenrod and Fenech (17) approached alcohol consumption and genomic stability in a more holistic manner, using a suite of experiments to examine the contribution of individual components that might exert a protective effect of moderate alcohol consumption. To achieve this, PBL were exposed \textit{in vitro} to components of white wine, individually or in combination, prior to exposure to the DNA-damaging and cytotoxic effects of hydrogen peroxide (H\(_2\)O\(_2\)) or \( \gamma \)-irradiation (17). These were measured against control cultures in which cells were maintained either in white wine stripped of polyphenols or diluent (Hank’s balanced salt solution) (17). The components tested were EtOH, glycerol, tartaric acid and the polyphenols catechin and caffeic acid (individually and combined). Each component, or mixture, was incorporated into medium at a final concentration of either 2.5 or 10\% with the former corresponding approximately to the theoretical average concentration of wine components to be found in the blood of a 60-kg person after consuming 300 ml of white wine. Following a 30-min incubation in the presence of each wine component or mixture, PBL either in autologous plasma or whole blood were exposed to H\(_2\)O\(_2\) challenge (750 \( \mu \)M) for 30 min or 1.5 Gy \( \gamma \)-irradiation. Cells were then cultured in complete medium for 72 h, including 28 h in the presence of CytoB. Harvested cells were examined for biomarkers of chromosomal damage in the CBMN-Cyt assay (17).

Findings of the H\(_2\)O\(_2\) challenge showed that all wine components, except tartaric acid, significantly reduced DNA damage (17). Interestingly, each component was equally effective in reducing damage, and the mixture failed to offer a synergistic protective effect compared with individual components. This suggested that the protective effects may function through a similar mechanism which is already at its maximal level at the individual concentrations examined. This theory is also supported by the fact that protection was not significantly increased in the 10\%, compared with the 2.5\%, dilutions (17). With respect to \( \gamma \)-irradiation, all components were found to be protective, with the greatest effect afforded by the polyphenolic compounds. This was also consistent with the reduced effect observed with the phenolic-striped wine. EtOH was the only individual component that caused an increase in baseline frequency of cells with MN, and this effect was neutralised in the mixture (17). In this case, adaptations of the CBMN-Cyt assay allowed the detailed study of interactive and protective effects of wine components, in a physiologically relevant \textit{in vitro} system. Findings showed that the main components of wine can reduce the DNA-damaging effects of two important oxidants, H\(_2\)O\(_2\) and ionising radiation (17).

Impact of dietary intake on genotoxic potential of the contents of the bowel

A final example of an adaptation whereby the CBMN-Cyt assay has been applied to examine the effect of nutritional factors on genomic stability is demonstrated in a study by Benassi \textit{et al}. (19). In this model, using WIL2-NS cells \textit{in vitro}, the direct physiological impact of a whole dietary regimen on DNA damage and cancer risk was assessed by testing the genotoxicity and cytotoxicity of faecal water from humans and caecal water from rats following consumption of specific diets (19). For this adapted protocol, cells were exposed \textit{in vitro} to caecal water from rats (1-h incubation with 10\% dilution) or faecal water from faeces of free-living human volunteers (24 h in 1\% dilution) (19). The rats had been fed for 2 weeks on either a high colorectal cancer risk diet (rich in fat, low in calcium and fibre, with barbecued red meat as the protein source) or a low colorectal cancer risk diet (high in fibre and calcium, low in fat, with casein as the protein source) (19).

Findings from the human study showed that 1\% (or lower) faecal water concentration provided sufficient BN cells to score DNA damage biomarkers reliably. In the cultures containing 1\% concentration, significant 2.6-, 6.5-, 7.5- and 2.2-fold increase in BN cells with MN, NPB, NBuds and necrotic cells, respectively, were observed compared with controls (0\%) (\( P < 0.05 \)). Damage was not significantly increased in the 0.1 or 0.01\% concentrations. Caecal water from rats fed the high-risk diet showed significant 7.6-, 1.8- and 4.0-fold increases in BN cells with MN, NPB and necrotic cells compared with caecal water from those which had consumed the low-risk diet (\( P < 0.001 \)) (19). This adaptation of the assay provides
a powerful experimental tool whereby potentially harmful (or protective) total dietary patterns can be effectively tested for the generation of carcinogenic environments in the colon (19).

Taken together, the applications of the CBMN-Cyt assay discussed above clearly demonstrate the need for experimental culture medium to match physiological micronutrient concentrations to enable better assessment of the true nutrient/nutrient, nutrient/genotype and nutrient/genotoxictant interactions which will provide greater confidence to the extrapolation of such in vitro data to likely in vivo effects. These studies also demonstrate the adaptability of the in vitro CBMN-Cyt assay to measure the generation of genotoxic environments in body fluids generated by complex dietary patterns.

Important knowledge gaps and recommendations for future research

Research findings highlight the need for culture medium for in vitro nutritional studies to be physiologically relevant to the in vivo situation. The current difficulty in producing culture medium that has physiologically relevant concentrations of all micronutrient components based on body fluid composition is a significant limitation for this field of research. The time-, cost- and labour-intensive nature of preparing medium from individual components increases the possibility of variability due to different reagents, laboratory equipment and the chance of human error. As such, a major priority for research in this field is the development of a physiologically correct culture medium production system so that commercially produced stocks may be customised for research purposes. Additionally, this raises the question as to whether the assay in its current form is appropriate for toxicological safety studies for novel therapeutic products. It will be interesting to examine whether genotoxicity testing data using the CBMN-Cyt assay (as well as other methods) is significantly modified if physiological culture medium is used in the future, instead of current standard medium. For example, would current negative genotoxicity tests with chemicals become positive if folate concentration in culture medium was reduced from the supraphysiological (~3000 nM) level to a physiological concentration of 20 nM?

Dose–response curves for the impact of many individual nutrients on DNA damage are yet to be established. To date, only folate and selenium have been studied adequately. While it is well documented that the MN index differs with age and gender, the impact of these factors on bioavailability and metabolism of specific nutrients, in individuals with varying genetic backgrounds, has not yet been determined and is essential for the advancement of personalised nutrition. The ultimate goal is to find for each individual the ‘nutriome’ (the complete nutrient combination) that best suits their genome so that cellular function, genome and epigenome maintenance are optimised. The key to unravelling this question lies in developing nutrient arrays in microculture systems such that multiple nutriomes can be simultaneously tested, while taking into consideration the impact of dosage (Figure 1).

When conducting nutrient dose–response studies, an additional factor that should be taken into consideration is the time required for ‘washout’ prior to the collection of data. It has been shown that intracellular stores of folate take up to 4 days to be depleted, thus it is important that long-term (minimum of 9 days) studies be conducted to minimise the effect of residual intracellular stores (20). A related issue is that of the conditions for the cells prior to being placed into experimental medium. It is yet to be determined whether intracellular stores of different nutrients vary depending on whether cells have been cultured immediately following isolation from blood or whether they have been cultured beforehand in medium with extremely high-or low-micronutrient concentrations. As such, the time allowed for intracellular nutrient depletion may need to be adapted depending on the pre-experimental conditions. It is also important that cultures are maintained at appropriate oxygen concentrations. Physiological oxygen tension is at least two to four times lower than that of atmospheric oxygen typically used in cell culture incubators; it has been shown that cells grown under physiological oxygen conditions experience less oxidative stress and paradoxically grow more slowly compared to cells in atmospheric oxygen incubators (21,22).

Finally, it is not yet known how applicable existing findings are to the longer term in vivo setting. Protocols for longer term studies (i.e. >10 days) need to be validated to determine chronic effects of both severe and mild nutrient deficiencies, interactive effects of nutrients with other factors and fluctuations in micronutrient levels which are likely to occur in vivo due to dietary changes. Some of the studies discussed above involved only brief exposure (<24 h) to the food component or genotoxictant and while these experiments have relevance from the perspective of a bolus (particularly in the case of food components) longer term chronic exposure studies are also necessary. This issue has particular relevance with the entry of dietary supplements and functional foods into the food supply chain, which have been created to deliver higher than normal concentrations of specific phytounutrients. These products highlight a need for long-term testing of the interactive biological effects of elevated antioxidant compounds and vitamin/nutrient content. While the CBMN-Cyt assay is ideally placed for investigation of such interactive effects of multiple nutrient combinations, this will also necessitate the development of an efficient robust high-throughput automated system to efficiently generate statistically meaningful data.
In conclusion, with some creative adaptations, the CBMN-Cyt assay has been demonstrated to provide a highly sensitive and effective measure of the impact of nutrient/nutrient, nutrient/gene or nutrient/genotoxicant interactions on chromosomal stability. Further adaptations, namely the development of longer term protocols, physiologically equivalent culture medium and high-throughput automation will further enhance the relevance of this method for optimising genome maintenance into the future.

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