ORIGINAL RESEARCH

Effect of protein intake, hyperglycaemia and micronutrients on DNA damage and mitogen responsiveness of peripheral blood lymphocytes

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Abstract

The aim of the study was to comprehensively assess foods differing in protein type (whey vs casein) relative to a low glycaemic index (GI) and high GI carbohydrate (CHO) food for their effects on DNA damage, DNA repair and mitogen response in peripheral blood lymphocytes using the cytokinesis-block micronucleus assay two hours after consumption. We also studied the relationship of these parameters with plasma folate, vitamin B12, zinc, magnesium and selenium immediately before the intervention and change in plasma glucose and plasma insulin during the two-hour period after consumption. There was no effect of protein, CHO or GI on genome damage or immune responsiveness except for a small but significant improvement in resistance to radiation-induced DNA damage in the high-GI CHO food relative to the casein food. Mitogen response was significantly and positively correlated with plasma zinc, magnesium, selenium and folate and baseline genome damage significantly negatively correlated with plasma folate and vitamin B12. There was no significant correlation between plasma micronutrients and change in plasma glucose or insulin except for a positive and significant correlation between change in plasma insulin and plasma selenium. The correlation between change in plasma insulin and change in plasma glucose was weaker. It is apparent from these preliminary data that micronutrient status may have a profound effect on genome stability and immune responsiveness while the effects of short-term changes in glycemia and dietary intake of carbohydrate or protein may have only minor effects on these parameters.

Key words: DNA damage, micronutrients, macronutrients, lymphocytes.

INTRODUCTION

A high rate of damage to DNA is associated with accelerating ageing and increased risk of degenerative diseases such as cancer and Alzheimer disease. Endogenous factors, such as an elevated level of reactive oxygen species (ROS) can cause both base damage and strand breaks in DNA. Repair of such DNA damage depends on the enzymes such as polyADP-ribosepolymerase (PARP) which requires a high level of NAD bioavailability. In situations of excess glucose, NAD is converted to NADH, generating a high energy state, increased ROS generation in cells and depletion of NAD—this may lead to a vicious cycle of increased DNA damage and reduced PARP activity/DNA repair. In addition, it has recently been shown that mice on a medium (20%) or high (33%) protein diet exhibited less DNA damage than mice on a low (7%) protein diet when exposed to ionising radiation as a source of ROS. The protective effect of the high-protein diets could be due to an increased plasma concentration of sulphydryl amino acids, such as cysteine and methionine, which have considerable antioxidant activity, or via increased glutamine which increases tissue antioxidant glutathione. Therefore, it is important to identify foods and diets that do not increase genome instability and/or impair the DNA repair response in cells by depleting NAD and generating excess ROS.

The aim of the study was to comprehensively assess foods differing in protein type (whey vs casein), relative to a low glycaemic index (GI) and high-GI carbohydrate (CHO) food for their post-prandial effects on DNA damage, DNA repair and mitogen response in peripheral blood lymphocytes (PBLs), the latter being a biomarker of immune response. Given the potential impact of micronutrients on genome stability, DNA repair, immune response and insulin resistance and glucose metabolism, we also examined the effect of zinc, magnesium, selenium, vitamin B12 and folate status on these parameters.
These studies could provide insight into the possible mechanism of action of these dietary components on genome stability in PBLs and immune response.

METHODS

Study design and protocol

The study involved overweight men with impaired fasting glucose participating in four acute studies to assess the effect of isocaloric whey protein or casein or low-GI CHO or high-GI CHO on: (i) markers of glycaemic control; (ii) baseline genome damage rate and mitogenic responsiveness; and (iii) the capacity of blood cells to resist radiation-induced DNA damage before and after consumption of different proteins and carbohydrates.

Ten overweight, but otherwise healthy, men with a body mass index (BMI; in kg/m²) of 31.7 ± 1.0 (range: 26.8–40.4), high mean fasting glucose concentration of 6.3 ± 0.1 mmol/L (range: 5.7–7.7 mmol/L) and a mean age of 53.3 ± 1.4 years (range: 41–63 years) were recruited by public advertisement. All subjects had a stable bodyweight during the two months prior to participation, were not seeking to change body weight, nonsmokers and unrestrained eaters. Subjects did not have any medical conditions or take medications known to affect glucose metabolism, gastrointestinal motility or appetite. The study was approved by the Commonwealth Scientific Industrial Research Organisation (CSIRO), Division of Health Sciences and Nutrition Human Ethics Committee. All subjects gave informed, written consent to participate.

Subjects attended the research clinic on four occasions for this single-blind, randomised, cross-over designed study comparing liquid foods containing whey, casein or low-GI carbohydrate or high-GI carbohydrate. All subjects had a stable bodyweight during the two months prior to participation, were not seeking to change body weight, nonsmokers and unrestrained eaters. Subjects did not have any medical conditions or take medications known to affect glucose metabolism, gastrointestinal motility or appetite. The study was approved by the Commonwealth Scientific Industrial Research Organisation (CSIRO), Division of Health Sciences and Nutrition Human Ethics Committee. All subjects gave informed, written consent to participate.

Subjects attended the research clinic on four occasions for this single-blind, randomised, cross-over designed study comparing liquid foods containing whey, casein, glucose or lactose. There was a seven-day interval between visits. Subjects refrained from exercise, taking paracetamol, paracetamol-containing products or alcohol 24 hours prior to study days and fasted after midnight (water permitted). Upon arrival at the clinic (08:30 hours), subjects' height was measured using a stadiometer (Seca, Germany). Weight was measured (Mettler scales, model AMZ14) in light clothing and without shoes. BMI was calculated by weight (kg) divided by height (m²).

A cannula was inserted into a vein in the lower arm and a fasting blood sample was taken (~15 minutes). The liquid preload was consumed within five minutes at 09:00 hours (time 0) and was immediately followed by 1500 mg of paracetamol (Glaxo Smith-Klein, Sydney, Australia) dissolved in 100 mL of water, as an indirect marker of liquid gastric emptying. Subsequent blood samples were collected at 15, 30, 45, 60, 90, 120 and 180 minutes after time 0 for analysis of glucose and insulin and paracetamol. Genome damage and mitogen responsiveness in control and irradiated cells (3.0-Gy gamma rays) were measured in blood samples collected before consumption of the test food and two hours later.

The order of the test food interventions was fully randomised to avoid effects of habituation to the procedure. Food intake was recorded as were measures of glucose and insulin at eight time points starting immediately before and during the two-hour post-consumption period. Genome damage and mitogen responsiveness in control and irradiated cells (3.0-Gy gamma rays, dose rate 5.0 Gy/minute) were measured in blood samples collected before consumption of the test food and two hours later.

Preloads of liquid food (1MJ) were made from a base of water (100 mL), milk (1% fat; 200 mL), artificially sweetened chocolate syrup (50 g) and either whey protein isolate (MG 2460, Murray Goulburn, Melbourne, Australia), calcium caseinate (MG 2954, Murray Goulburn, Melbourne, Australia), glucose (high-GI carbohydrate; Glucodin, Boots Healthcare, Richmond, Australia) or lactose (low-GI carbohydrate; Ace Chemical Company, Camden Park, Australia) (Table 1). The preloads did not contain dietary fibre and were controlled for energy density, palatability, colour and odour. Preloads were prepared on the morning of consumption (except the casein preload that was prepared 24 hours prior to consumption to improve consistency) and served in an opaque container.

Energy and nutrient composition of food intake was calculated using Food Works Analysis Package 3.1 (Xyris Software, Highgate Hill, Australia) which is based on data from Australian Food Composition Tables. One dietician conducted all nutrient analysis.

<table>
<thead>
<tr>
<th>Table 1 Macronutrient composition of preloads</th>
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<tr>
<td><strong>Preload (liquid food)</strong></td>
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<tr>
<td>Energy (kJ)</td>
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<tr>
<td>Protein (g) (% of energy)</td>
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<td>Fat (g) (% of energy)</td>
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<td>Carbohydrate (g) (% of energy)</td>
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<td>Energy density (kJ/g)</td>
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<sup>(a)</sup> Whey protein isolate (MG 2460, Murray Goulburn).
<sup>(b)</sup> Instant calcium caseinate (MG 2954, Murray Goulburn).
<sup>(c)</sup> Glucodin (Boots Healthcare).
<sup>(d)</sup> Lactose (Ace Chemicals).
**Biochemistry and genome damage assays**

Blood for serum was collected in tubes with no additives and allowed to clot at room temperature for 30 minutes. Blood for plasma was collected in sodium fluoride/EDTA (1 g/L) tubes containing aprotinin (Roche; Indianapolis, IN, USA) 500 KIU/mL blood and stored on ice. Serum and plasma were isolated by centrifugation for 10 minutes at 2000 g (5°C) (Beckman GS-6R Centrifuge, Fullerton, CA, USA) within one hour of collection and aliquots were stored at −80°C. Blood for genome damage assays was collected in lithium heparin tubes.

Serum insulin was measured in singleton using a commercial enzyme immunoassay kit (Mercodia, Uppsala, Sweden). Plasma glucose was determined using an enzymatic kit (Hoffman-LaRoche Diagnostics, Basel, Switzerland) and control sera on a Hitachi 902 Automatic Analyser (Roche Diagnostics, Basel, Switzerland). Serum paracetamol (acetaminophen) was measured using a commercial enzymatic kit (Cambridge Life Sciences, Cambridgeshire, England) adapted for a Cobas Bio centrifugal analyser using reagent volumes compatible with this instrument (Roche Diagnostics, Basel, Switzerland). Minerals in plasma were measured by flame atomic absorption spectrophotometry using a Perkin Elmer 5000 Atomic Absorption Spectrophotometer (Perkin Elmer, Melbourne, Australia). Plasma Folate and vitamin B12 were measured by radioimmunoassay (Bio-Rad Quantaphase II, North Ryde, Australia). Plasma glucose was determined using an enzymatic kit (Bio-Rad; North Ryde, Australia). All biochemical analyses were performed after study completion and all samples for individuals were analysed in the same assay. Intraclass variability was less than 8% for all assays.

Genome stability was measured using the cytokinesis-block micronucleus assay in human peripheral blood lymphocytes. This assay was performed on both untreated lymphocytes and lymphocytes exposed to 3.0-Gy gamma rays to obtain a measure of capacity for DNA repair after oxidative challenge. Lymphocytes were cryopreserved using a standard protocol that preserves viability of cells. Within two months of cryopreservation, the lymphocytes were thawed, washed in culture medium and the CBMN assay performed as previously described. Using this assay, we obtained measures of chromosome breakage and loss (micronucleated, MNed, cells) and mitogen responsiveness (nuclear division index, NDI). We also measured plasma Zn, Mg, Se, vitamin B12 and folate in samples collected before the intervention (i.e. at 0 hour) to take into account the possible effect of these micronutrients on measured results. The study on genome stability was done with 10 subjects which, based on historical data on variation in spontaneous MNed cell frequency, should have resulted in 85% power to detect a 12% change in MNed cells at $P = 0.01$.

**Statistical analysis**

Results for micronutrients, genome damage, NDI, glucose and insulin were normally distributed. We used parametric one-way ANOVA tests to compare effect of treatment followed by Tukey’s test to compare pairs of treatments. Correlation between parameters was measured using Pearson’s test. Differences were considered to be significant at $P < 0.05$ (two-tailed).

**RESULTS**

The area under the concentration versus time curve (AUC) for plasma glucose was significantly greater in the CHO treatments relative to whey and casein, and there was no difference in AUC for plasma insulin (Figure 1).

There was no significant impact of the type of food eaten on baseline genome damage. After correcting for baseline values, it was evident that the observed reduction in radiation-induced genome damage following consumption of the high-GI CHO food was significantly different from the observed change (increase) following consumption of the

![Figure 1](image-url)
The observed changes in radiation-induced genome damage were not significantly correlated with the plasma insulin or plasma glucose AUC (data not shown).

We observed a significant post-prandial reduction in NDI with the casein-based food relative to pretreatment NDI levels \((P < 0.01; \text{data not shown})\). However, although the difference in NDI relative to baseline was greatest in the casein treatment, it was not significantly different from that of the other treatments which also showed a trend to a reduction in NDI post-prandially (Figure 3). Change in NDI values relative to baseline was not correlated significantly with the plasma insulin and plasma glucose AUC (data not shown).

There was: (i) a significant positive correlation between the NDI with plasma Zn, Mg, Se and folate; and (ii) a significant negative correlation between plasma folate and vitamin B12 with baseline MNed cell frequency (Table 2) in fasted samples prior to the intervention. In irradiated cells, only the positive correlation of NDI with plasma magnesium and folate remained significant (Table 3). Also noted were significant correlations between micronutrients such as the positive correlations between Zn, vitamin B12 and folate and the negative correlation between vitamin B12 and magnesium.

Micronutrient status was not related to the post-prandial increase in plasma glucose (measured as the area under the plasma concentration vs time curve, AUC). However, the post-prandial increase in plasma insulin concentration (also measured as AUC) was significantly positively correlated with plasma selenium \((P = 0.003, R = 0.448)\) and glucose AUC \((P = 0.037, R = 0.327)\).

**DISCUSSION**

The results of the present study do not support the hypothesis that reduced protein intake relative to carbohydrate or increased glycemic load leads to increased genome instability; diminished DNA repair capacity and reduced immune responsiveness in the short-term post-prandial period. Although the results suggest an aggravating effect of casein intake on radiation-induced genome damage and immune responsiveness relative to high-GI CHO, the effects are relatively small.

The lack of an effect of glycemic load on genome stability may be due to insufficient time to alter the NAD/NADH ratio in the target PBL cells which are quiescent in **vivo**. A longer-term intervention coinciding with the **in vivo** turnover rate of lymphocytes may be required to produce a significant alteration in NAD/NADH ratio sufficient to impact on PARP activity. Another possible explanation could be more than adequate nicotinic acid status in the subjects studied which may compensate for any slight changes in the NAD/NADH ratio. However, we did not measure nicotinic acid or NAD/NADH ratio, which is a weakness of this preliminary study. The data suggest a marginally improved resistance to radiation-induced genome damage in the high-GI CHO treatment, which, although contrary to expectations, may reflect an adaptive response to low-level increase in oxidative damage that is hypothesised to increase with high-GI diets. Adaptive responses in DNA repair have been observed with pretreatment small doses of radiation or oxidative stress within the time frame of the study protocol (i.e. two hours) using the same model.\(^{13}\) However, we have no evidence that oxidative stress was actually increased in the high-GI CHO

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**Figure 2** Difference in genome damage induced by 3.0Gy radiation challenge in lymphocytes from blood samples collected two hours post consumption of intervention foods relative to results for lymphocytes from pre-consumption blood samples. DNA damage was measured in peripheral blood lymphocytes using the cytokinesis-block micronucleus assay. Frequency of cells containing micronuclei (i.e. MNed cells) was measured in 1000 binucleated cells. With the exception of casein, all treatments showed a small reduction in radiation-induced DNA damage in samples post consumption. The results also showed that the change in radiation-induced DNA damage post consumption of HI GI CHO was significant relative to the results for casein. \(n = 10\) per group. \(*P < 0.05\) versus casein. GI, glycaemic index; HI, high; LO, low.

**Figure 3** Difference in nuclear division index (NDI) of peripheral blood lymphocytes measured using the cytokinesis-block micronucleus assay two hours after consumption of test food relative to baseline at time 0 hour. The results show a trend for a reduced NDI in post-prandial samples relative to pre-consumption samples, but differences between groups were not statistically significant. \(n = 10\) per group.

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**Table 3** Micronutrient status was not related to the post-prandial increase in plasma glucose (measured as the area under the plasma concentration vs time curve, AUC). However, the post-prandial increase in plasma insulin concentration (also measured as AUC) was significantly positively correlated with plasma selenium \((P = 0.003, R = 0.448)\) and glucose AUC \((P = 0.037, R = 0.327)\).
The observed significant positive correlations between plasma levels of zinc, magnesium, selenium and folate with NDI are in keeping with numerous studies indicating the stimulating effect of these micronutrients on immune function. The negative correlation of plasma folate and B12 with MNed cell frequency is in agreement with our studies showing that folate and its bioavailability are important determinants of chromosomal instability and micronucleus formation in human lymphocytes, and that supplementation with folate and vitamin B12 reduces MNed cell frequency.

There may be some concern that baseline genome damage rates may have been confounded by the use of paracetamol as a marker of gastric emptying; however, our results show no evidence of increase in MNed cell frequency post food consumption and results from an independent study also showed no effect of paracetamol on MNed cell frequency.

The significant positive correlation of plasma selenium concentration with insulin response fits with results from a study on glutathione peroxidase (GPx) overexpression murine model associated with increased insulin resistance and obesity. It is also consistent with the reported positive association between erythrocyte GPx1 activity and levels of insulin resistance in normal pregnant women that are pregnant for at least 16 weeks. It has been hypothesised that increased antioxidant GPx activity may interfere with ROS required for an insulin sensitivity response.

It is apparent from these preliminary data that micronutrient status may have a profound effect on genome stability and immune responsiveness while the effects of short-term changes in glycemia and dietary intake of carbohydrate or protein may have only minor effects on these parameters.

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CONFLICT OF INTEREST
No conflict of interest has been declared by M. Fenech, M. Noakes, J. Bowen or P. Clifton.
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