Changes in dietary folate intake differentially affect oxidised lipid and mitochondrial DNA damage in various brain regions of rats in the absence/presence of intracerebroventricularly injected amyloid β-peptide challenge

Ta-Fu Chen[1], Ming-Jang Chiu[1,2], Chou-Tz Huang[3], Ming-Chi Tang[3], Sue-Jane Wang[4,5], Chia-Chuan Wang[4] and Rwei-Fen Syu Huang[3,5]*

[1]*Department of Neurology, National Taiwan University Hospital, College of Medicine, National Taiwan University, Taipei, Taiwan, ROC*
[2]*Neurobiology and Cognitive Science Center, National Taiwan University, Taipei, Taiwan, ROC*
[3]*Department of Nutritional Science, Fu-Jen University, Hsinchuang 242, Taiwan, ROC*
[4]*School of Medicine, Fu-Jen University, Hsinchuang 242, Taiwan, ROC*
[5]*PhD Program in Nutrition and Food Sciences, Fu-Jen University, Hsinchuang 242, Taiwan, ROC*

Abstract

Accumulating evidence suggests that changes in dietary folate intake may modulate the risks of Alzheimer's disease (AD) through as yet unknown mechanisms. The aims of the present study were to investigate how dietary folate affects the brain folate distribution, levels of oxidised lipid and DNA damage in the absence/presence of β-amyloid$_{(25-35)}$ (Aβ) peptide challenge, a pathogenic hallmark of AD. Male Wistar rats were assigned to diets with folic acid at 0 (folate deprivation; FD), 8 (moderate folate; MF) and 8 mg folic acid/kg diet + 0·003 % in drinking-water (folate supplementation; FS) for 4 weeks. A single injection of Aβ peptide (1 mg/ml) or the vehicle solution was intra-cerebroventricularly (icv) administered to rats a week before killing. Brain folate, a marker of oxidative injury, and neuronal death were assayed. In the absence of an Aβ injection, FD rats showed reduced folate levels, and increased 2-thiobarbituric acid-reactive substances and a mitochondrial (mt)DNA 4834 bp large deletion (mtDNA^{4834} deletion) in the hippocampus compared with the counterpart brains of control rats ($P<0·05$). A single icv injection of Aβ peptide potentiated lipid peroxidation in the medulla of FD rats, which was ameliorated by feeding FD rats with the MF and FS diets ($P<0·05$). Feeding the FS diet to Aβ-injected rats enriched brain folate levels and reduced mtDNA^{4834} deletion in the hippocampal and medullary regions compared with corresponding tissues of Aβ + FD rats ($P<0·05$). Aβ + FS rats had reduced rates of neuronal death in the frontal cortex compared with Aβ + FD rats ($P<0·05$). Taken together, our data revealed that folate deprivation differentially depleted brain folate levels, and increased lipid peroxidation and mtDNA^{4834} deletions, particularly, in the hippocampus. Upon Aβ challenge, the FS diet may protect various brain regions against lipid peroxidation, mitochondrial genotoxicity and neural death associated with folate deprivation.

Key words: Dietary folate intake: Mitochondrial DNA large deletion: Lipid peroxidation: Aβ$_{(25-35)}$ peptide: Neuronal death

Folate, one of the B vitamins, plays an important role in the normal functioning of the central nervous system[1]. Folate deficiency, partially due to a poor diet, is associated with cognitive decline, dementia and increased risks of Alzheimer's disease (AD)[2–4]. Although the causal effects of folate insufficiency on AD-associated cognition impairment are not well understood, studies have demonstrated that increased folate intake was correlated with a lower risk of AD[5]. Among cognitively impaired subjects with low folate levels, folate supplementation was associated with improving memory

deficits and performance[6,7]. The 3-year randomised controlled Folic Acid and Carotid Intimamedia Thickness (FACIT) trial confirmed significant effects of folic acid supplementation in improving memory[8]. This contrast with a negative trend in specific test scores of a folate-supplemented group was observed in dementia subjects[9]. In a prospective study with 3718 residents, a faster rate of cognitive decline was associated with high folate intake[10]. The inconsistent results from epidemiological and folate intervention studies underscore a need to investigate how changes in dietary

folate intake mechanistically affect the brain's integrity during the development of neurodegenerative diseases such as AD.

Oxidative damage was widely implicated in the pathogenesis of AD, occurring early in the AD brain, before the onset of plaque pathology and after the deposition of brain fibrillar β-amyloid (Aβ) peptide[11–13]. In brain tissues of AD patients, oxidative insults are frequently observed based on increased levels of lipid peroxidation, proteins and nucleic acids[14]. Nucleic acids, particularly mitochondrial DNA (mtDNA), are the primary target of free radical damage due to a low level of DNA repair and the proximity of reactive oxygen species generated by respiratory chains. Human mtDNA is a double-stranded, circular, 16·5 kb molecule containing genes necessary for the synthesis of the catalytic components of oxidative phosphorylation. Elevated mtDNA oxidative damage was associated with mitochondrial (mt) respiratory dysfunction and vicious reactive oxygen species cycles, which may result in apoptotic cell death[15,16]. Accumulating evidence suggests that AD may be associated with mtDNA aberrations, elevated oxidative stress and mt respiratory dysfunction[17,18]. Impaired energy metabolism and mt abnormalities are observed as a feature of peripheral cells and brain from patients with AD[19,20]. In AD brain specimens, mtDNA of the frontal, parietal and temporal lobes had tenfold higher levels of oxidised bases than nuclear DNA[21]. Increased mtDNA defects and mt dysfunction are considered part of the spectrum of chronic oxidative stress during AD development[22].

Several studies have recently reported that dietary folate may modulate the accumulation of large mtDNA deletions[23–25] – a 4977 bp deletion in humans and a 4834 bp large deletion in rodents. The large mtDNA deletions are commonly found to accumulate in a variety of ageing tissues[26–28]. In particular, such large mtDNA deletions are present at high levels in the brain and heart of ageing human tissues, and are associated with elevated oxidative stress[26,28,29]. We previously demonstrated that dietary folate deprivation (FD) promotes the accumulation of large mtDNA deletions in whole-brain homogenates of rats[25]. Such ageing-associated large mtDNA deletions, however, have not been characterised in various AD-susceptible brain regions in response to different dietary folate levels. We hypothesised that various dietary folate levels may modulate brain folate, thus affecting oxidative lesions and the frequencies of large mtDNA deletions in AD-susceptible brain regions before or after Aβ peptide challenge. The 11-amino acid fragment of the Aβ peptide, Aβ(25–35), located at the C-terminal end of Aβ(1–42) in the hydrophobic domain, was shown to mimic some of the pathological processes in the AD brain[30]. Using an animal model with an experimental design of an intracerebroventricular (icv) injection of Aβ(25–35) peptide to rats fed various levels of dietary folate, changes in brain folate, neuronal death and oxidative modification in lipids as well as mtDNA deletions of various brain tissues were measured to investigate the mechanisms by which dietary folate may affect brain oxidative damage in the absence/presence of Aβ(25–35) peptide challenge.

Materials and methods

Experimental diets and animals

The FD diet was specially formulated by Harlan Teklad (Madison, WI, USA) using an L-amino acid-defined regimen (see Table S1 of the supplementary material, available at http://www.journals.cambridge.org/bjn). The FD diet replenished with a moderate level of folate at 8 mg/kg diet was designated the moderate folate (MF) diet[31]. The MF diet supplemented with folate in drinking-water (0·003 %) was designated the folate-supplemented (FS) diet. All diets contained succinylsulphathiazole (1/100 g) to suppress intestinal microfloral folate production.

Male weaning Wistar rats (n 35) were obtained from the Animal Center of the National Science Council (Taipei, Taiwan, ROC). Rats were housed in stainless-steel wire cages in an air-conditioned room maintained at 25°C and 70 % humidity with a 12 h dark–12 h light cycle. After a 3 d acclimatisation period during which rats were fed a non-purified diet, they were randomly assigned to the FD, MF and FS diets (n 14, 14 and 7 in each respective group) using a pair-fed model as previously described[25]. Access to food and tap water was available ad libitum, and rats were weighed twice a week. The single aggregated Aβ(25–35) peptide (1 mg/ml) or vehicle solution (sterile distilled water) was icv administrated to rats fed the various folate diets 1 week before killing. Rats fed with the MF diet in the absence of Aβ injection were designated as the control group. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Fu-Jen University. Institutional and national guidelines for the care and use of animals were followed.

β-Amyloid(25–35) peptide preparation and surgery

Aβ(25–35) peptides were aggregated as previously described[32]. In brief, the peptide was dissolved in sterile distilled water at a concentration of 1 mg/ml, divided into aliquots in tubes and stored at −20°C. These were 'aged' before the administration by incubation at 37°C for 7 d. Light microscopic observation showed the existence of both birefringent fibril-like structures and globular aggregates.

An icv injection of Aβ(25–35) peptide was given according to operating protocols with some modifications[33]. After 3 weeks of feeding the experimental diets, rats were anaesthetised and positioned in a stereotaxic frame, and a midline sagittal incision was made in the scalp. Holes were drilled in the skull over the lateral ventricles using the following coordinates: anterior-posterior (AP), −0·1 cm; medial-lateral (ML), −0·15 cm; dorsal-ventral (DV), −0·35 cm. All injections were made using a 10 μl Hamilton syringe equipped with a 30S gauge needle. Animals were injected with sterile distilled water (the vehicle group) or the aggregated Aβ(25–35) peptide at a volume of 8 μl into each cerebral lateral ventricle at a rate of 1 μl/min. The needle was left at the site of the injection for an additional 2 min. At 7 d after a single injection, the animals were decapitated. The cortical, hippocampal and medullary tissues were immediately dissected over ice frozen in liquid

N_2 and stored at $-180°C$. The frontal cortical tissues were removed and postfixed in the same fixative solution for 48 h and then embedded in paraffin.

Brain and liver folate assay

Tissue samples for the folate analysis were prepared as previously described[34]. Using a Polytron homogeniser (OMNI 2000; OMNI, Inc., Waterburg, CT, USA), liver and brain samples were homogenised in an extraction solution containing 5 mM-2-mercaptoethanol, 0·1 M-sodium ascorbate, 50 mM-HEPES and 50 mM-2-(N-cyclohexylamino) ethanesulphonic acid/l (pH 7·85). The homogenate was centrifuged at 20 000 **g** for 10 min. The supernatant extract was stored at $-180°C$ in N_2 for later analysis. After incubation of the thawed sample extracts with chicken pancreatic conjugate (4:1, v/v) at 37°C for 6 h, a microbiologic assay was performed using cryoprotected *Lactobacillus casei* in ninety-six-well microtitre plates[35]. The absorbance was detected at 600 nm in an MRX model ELISA reader (Dynatech Laboratories, Billingshurst, West Sussex, UK).

Lipid peroxidation

Lipid peroxidation was quantified by measuring thiobarbituric acid-reactive substance (TBARS) production[36]. The reaction reagents consisted of 3 g sodium dodecylsulphate, 0·1 M-HCl, 10 g phosphotungstic acid and 0·7 g 2-thiobarbituric acid/l. The sample mixture was incubated for 45 min at 95°C, and TBARS were extracted in 2·5 ml of 1-butanol. After centrifugation at 1000 **g** for 10 min, the fluorescence of the butanol layer was measured using a Hitachi F-3000 fluorospectrophotometer (Tokyo, Japan) at 555 nm emission and 515 nm excitation. The TBARS values were expressed as nanomoles of malondialdehyde equivalents per gram of protein using a standard curve of 1,1,3,3-tetraethoxypropane. The protein concentration was determined using a protein dye-binding standard curve[37].

Analysis of the frequencies of accumulated large mitochondrial DNA[4834] deletions

The breakpoints of mtDNA[4834] deletion are at the deletion junction (8103, 12 937–12 952 bp) with two 16 bp repeats that normally flank the wild-type mtDNA. The primers for the detection of large mtDNA deletions were designed to detect PCR products when mtDNA were deleted at the particular junctions to join the flanking region[25]. The quantity of mtDNA[4834] deletion was determined by co-amplifying the mtDNA displacement loop and mtDNA[4834] deletion in a real-time PCR assay. Primers for each have been previously described[25]. The extent of mtDNA[4834] deletions was quantified with a deletion probe (DYXL-5'-(12952) TCACTTT-AATCGCCACATCCATAACTGCTGT (12982)-3' BHQ1) and an mtDNA probe (6FAM (15795) 5'-TTGGTTCATCGTCCATACGT-TCCCCTTA (15822)-3' BHQ1). PCR amplification was carried out in a 20 µl reaction volume consisting of a TaqMan Universal Master Mix (4 µl), 200 nmol/l of each mtDNA[4834] deletion primer, 50 nmol/l of each displacement loop primer and 100 nmol/l of each mtDNA[4834] deletion and displacement loop probes. The cycling conditions included an initial phase of 2 min at 50°C and 10 min at 95°C, and then forty cycles of 15 s at 95°C and 0·5 min at 72°C. The fluorescence spectra were monitored by the LightCycler Detection System with Sequence Detection Software version 4 (LightCycler; Roche Diagnostics, Mannheim, Germany). The cycle at which a statistically significant increase in normalised fluorescence was first detected was designated the threshold cycle number (C_t). The relative frequencies of mtDNA[4834] deletions to mtDNA were calculated using $\Delta C_t = mt\,C_{t\,del}-mt\,C_{t\,D\text{-}loop}$, where D-loop represents the displacement loop. Fewer mtDNA deletions will give rise to a higher C_t number to obtain a detectable fluorescence signal; thus, a smaller ΔC_t value indicates more deletions. The ΔC_t values were used to quantify the relative amount of large mtDNA deletions in percentage with the equation: $R = 2^{-\Delta C_t}$.

Neural cell death by haematoxylin and eosin histological examination

Whole-mount preparations of the frontal tip from rats were scanned using a digital slide system Aperio ScanScope CS (Aperio Technologies, Vista, CA, USA), and all images were analysed by Aperio's ImageScope Viewer software (Aperio Technologies). Morphological features of dead cells in the frontal cortical brain tissues of rats were identified by counting neurons with obviously condensed pyknotic nuclei surrounded by cytoplasmic eosinophilia using haematoxylin and eosin histochemistry as previously described[38]. Damaged and viable neurons were quantified from several randomly selected fields, and the ratios were recorded.

Statistical analysis

Data are presented as means and standard deviations. The effects of dietary folate intake and an Aβ$_{(25-35)}$ injection on animal growth, folate status, lipid peroxidation, DNA oxidative injuries and neuronal death were analysed by one-way ANOVA and Duncan's multiple range test using the general linear model of SAS Institute (Cary, NC, USA). Differences were considered significant at $P < 0.05$. Pearson's correlation coefficients were used to measure the associations between folate levels, lipid peroxidation and mtDNA deletions in various brain tissues and the liver.

Results

Animal growth in response to dietary folate intakes and an intracerebroventricular β-amyloid peptide injection

Growth rates of rats in the experimental groups are presented in Table 1. Among the vehicle groups, the FD rats had a significantly lower weight gain and feed efficiency than did control rats ($P < 0.05$). An icv administration of Aβ$_{(25-35)}$ peptide did not affect the weight gain or feed efficiency of FD or control rats. Among the Aβ$_{(25-35)}$-administered

Table 1. Growth of rats fed diets with various folate concentrations in the presence/absence of an β-amyloid (Aβ) peptide injection*†

(Mean values and standard deviations, n 5, 5, 4, 5 and 7, respectively)

| | Vehicle microinjection | | | | Intracerebroventricular microinjection of Aβ$_{(25-35)}$‡ | | | | | |
| | MF (control) | | FD | | Aβ + FD | | Aβ + MF | | Aβ + FS | |
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Initial weight (g)	74·6^a	4·0	79·0	4·0	74·9	6·3	73·3	4·2	75·8	5·5
Weight gain (g)	176·3^a	3·9	144·4^b	25·9	148·7^b	13·2	172·9^a	13·6	176·3^a	14·0
Total food intake (g)	623·0	19·2	590·9	15·6	602·7	37·5	597·1	45·8	615·8	25·0
Feed efficiency (g gain/g feed)	0·28^{a,b}	0·01	0·24^c	0·04	0·25^{b,c}	0·03	0·29^a	0·02	0·29^a	0·02
Liver weight (g)	7·04^b	0·5	8·60^a	1·12	8·35^a	1·61	7·01^b	0·27	6·99^b	2·30
Brain weight (g)										
Cortex	0·94	0·07	0·94	0·01	0·94	0·01	0·89	0·04	0·92	0·02
Hippocampus	0·11^b	0·02	0·12^{a,b}	0·03	0·10^b	0·03	0·10^b	0·02	0·14^a	0·01
Medulla	0·33	0·09	0·35	0·04	0·35	0·04	0·36	0·03	0·41	0·03

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).
* Data were analysed by one-way ANOVA and Duncan's multiple range test.
† FD, MF and FS indicate no folate (dietary folate deprivation; 0 mg folic acid/kg diet), moderate folate (+8 mg folic acid/kg diet) and folic acid-supplemented (+8 mg folic acid/kg diet and 0·003 % folic acid in drinking-water: 68 μM) diets, respectively. The MF group receiving the vehicle microinjection was designated the control.
‡ Aβ$_{(25-35)}$ administration included an intracerebroventricular microinjection of 8 μl of Aβ$_{(25-35)}$ peptide (1 mg/ml). Vehicle administration included an intracerebroventricular microinjection of 8 μl of the vehicle solution (sterile distilled water).

groups, the Aβ + MF and Aβ + FS groups showed higher weights and feed efficiencies than the Aβ-FD group. The FD and Aβ + FD groups exhibited abnormal increases in liver weight, whereas the Aβ + MF and Aβ + FS groups had liver weights similar to the vehicle control group.

Dietary FD for 4 weeks did not affect the crude brain weight in the cortical, hippocampal or medullary regions (Table 1) in either the vehicle- or Aβ-treated groups. Supplementation of folic acid in Aβ-treated rats significantly increased the crude weight of the hippocampus compared with the Aβ + FD, Aβ + MF and vehicle control groups ($P < 0.05$).

Dietary folate intake modulated folate levels of brain tissues and the liver in the presence/absence of an intracerebroventricular β-amyloid peptide injection

After consuming the FD diet for 4 weeks, folate levels in the hippocampal and medullary tissues of FD rats had significantly dropped by more than 50 % compared with vehicle control rats ($P < 0.05$; Table 2). The cortical folate levels of rats

decreased to a lesser extent after 4 weeks of consuming the FD diet. After the 4-week FD diet, 80 % of hepatic folate was depleted, but without reaching statistical significance due to high measurement variations within the groups. An icv administration of Aβ$_{(25-35)}$ peptide did not further deplete folate levels of the liver or various brain tissues of FD rats. Feeding Aβ$_{(25-35)}$-injected FD rats the FS diet caused a significant supplementation of hepatic and brain folate levels (hippocampal and medullary tissues) back to the levels of the counterpart tissues of vehicle control rats. The MF diet supplemented folate levels of Aβ$_{(25-35)}$ + FD rats only in the liver and medullary tissues ($P < 0.05$).

Dietary folate intake modulated lipid peroxidation in the brains of rats in the presence/absence of β-amyloid peptide challenge

In the absence of an Aβ challenge, FD rats had significantly elevated TBARS levels in the hepatic and hippocampal tissues compared with vehicle control rats (Table 3). An icv

Table 2. Folate status of rats fed diets with various folate concentrations (nmol/g tissue) in the presence/absence of an intracerebroventricular administration of the β-amyloid (Aβ) $_{(25-35)}$ peptide*†

(Mean values and standard deviations, n 5, 5, 4, 5 and 7, respectively)

| | Vehicle microinjection | | | | Intracerebroventricular microinjection of Aβ$_{(25-35)}$‡ | | | | | |
| | MF (control) | | FD | | Aβ + FD | | Aβ + MF | | Aβ + FS | |
Rat tissues	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Folate levels (nmol/g tissue)										
Liver	115·1^{b,c}	48·4	24·11^c	22·83	7·80^c	4·91	142·5^b	37·7	305·1^a	148·9
Cortex	11·88^{a,b}	5·17	7·13^b	3·16	9·51^{a,b}	3·38	8·75^{a,b}	2·97	13·78^a	3·40
Hippocampus	10·62^{a,b}	2·54	5·24^c	1·48	6·35^{b,c}	2·42	7·07^{a,b,c}	2·53	11·27^a	4·64
Medulla	7·26^{a,b}	3·31	2·32^c	1·47	4·20^{b,c}	1·32	8·50^a	2·13	8·49^a	3·79

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).
* Data were analysed by one-way ANOVA and Duncan's multiple range test.
† Control, FD, MF and FS indicate no folate (dietary folate deprivation), moderate folate (+8 mg folic acid/kg diet; same folate levels as in the control diet) and folic acid-supplemented (+8 mg folic acid/kg diet and 0·003 % folic acid in drinking-water: 68 μM) diets, respectively. The MF group receiving the vehicle microinjection was designated the control.
‡ Aβ administration included an intracerebroventricular microinjection of 8 μl of Aβ$_{(25-35)}$ peptide (1 mg/ml). Vehicle administration included an intracerebroventricular microinjection of 8 μl of the vehicle solution (sterile distilled water).

Table 3. Dietary folate-modulated thiobarbituric acid-reactive substance levels in various brain regions of rats in the presence/absence of an β-amyloid (Aβ) $_{(25-35)}$ peptide challenge*†

(Mean values and standard deviations, n 5, 5, 4, 5 and 7, respectively)

| | Vehicle microinjection | | | | Intracerebroventricular microinjection of Aβ$_{(25-35)}$‡ | | | | | |
| | MF (control) | | FD | | Aβ + FD | | Aβ + MF | | Aβ + FS | |
Rat tissues	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Malondialdehyde equivalents (nmol/g protein)										
Liver	0·05^b	0·01	0·16^a	0·08	0·14^a	0·06	0·12^a	0·07	0·13^a	0·04
Cortex	1·43	0·44	1·44	0·56	1·47	0·35	1·47	0·67	1·59	0·61
Hippocampus	1·20^b	0·21	2·39^a	1·05	2·69^a	0·44	1·91^{a,b}	0·88	1·78^{a,b}	0·49
Medulla	2·20^b	0·45	3·57^b	1·92	4·94^a	1·09	2·47^b	0·47	2·69^b	0·58

^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* Data were analysed by one-way ANOVA and Duncan's multiple range test.

† FD, MF and FS indicate no folate (dietary folate deprivation), moderate folate (+8 mg folic acid/kg diet) and folic acid-supplemented (+8 mg folic acid/kg diet) and 0·003 % folic acid in drinking-water: 68 μM) diets, respectively. The MF group receiving the vehicle microinjection was designated the control.

‡ Aβ administration included an intracerebroventricular microinjection of 8 μl of Aβ$_{(25-35)}$ peptide (1 mg/ml). Vehicle administration included an intracerebroventricular microinjection of 8 μl of the vehicle solution (sterile distilled water).

Aβ injection did not further aggravate lipid peroxidation of FD rats in either tissue. In the presence of Aβ challenge, feeding the MS or FS diets did not significantly affect hepatic or hippocampal TBARS levels of FD rats. The medullary TBARS levels of rats increased after 4 weeks of consuming the FD diet, but without reaching statistical significance. An icv Aβ injection significantly elevated lipid peroxidation in the medullary tissue of FD rats ($P<0·05$). Both the MF and FS diets significantly diminished the medullary TBARS levels of Aβ + FD rats to vehicle control values ($P<0·05$). Neither changes in dietary folate levels nor Aβ challenge affected lipid peroxidation in the cerebral cortex.

Dietary folate intake modulated mitochondrial DNA^{4834} deletions in the brains of rats in the presence/absence of β-amyloid peptide challenge

In the absence of an Aβ challenge, FD rats had increased frequencies of mtDNA^{4834} deletions in the hippocampal region compared with the counterpart tissue of control rats (Table 4). A similar elevation with a less extent of accumulated mtDNA deletions was observed in the medulla of FD rats

($P<0·05$). An icv administration of Aβ$_{(25-35)}$ did not augment accumulated mtDNA^{4834} deletions in various brain regions of FD rats. Compared with Aβ + FD rats, rats fed the FS diet had reduced levels of mtDNA^{4834} deletions in hippocampal, medullary and hepatic tissues ($P<0·05$), alleviating the brain mtDNA damage of Aβ + FD rats to background levels of the vehicle control group. Similar changes in mtDNA deletions in response to dietary folate intake were observed in the cortical tissue of Aβ + FD rats, but without statistical significance.

Relationships between folate levels and mitochondrial DNA^{4834} deletions in various brain regions and liver of rats

We pooled the data of the vehicle- and Aβ-injected groups to study the relationships between tissue folate levels and mtDNA deletions in brain regions and the liver. Fig. 1 shows that increased frequencies of mtDNA^{4834} deletions were significantly associated with reduced folate levels in hippocampal (r −0·593, $P=0·001$), medullary (r −0·345, $P=0·042$) and hepatic tissues (r −0·547, $P=0·002$). Such a folate level–mt genotoxicity relationship was not observed in the cortex tissue (r −0·242, $P=0·133$).

Table 4. Dietary folate-modulated mitochondrial DNA (mtDNA)^{4834} deletions in various brain regions of rats in the presence/absence of β-amyloid (Aβ) $_{(25-35)}$ peptide challenge*†

(Mean values and standard deviations, n 5, 5, 4, 5 and 7, respectively)

| | Vehicle microinjection | | | | Intracerebroventricular microinjection of Aβ$_{(25-35)}$ | | | | | |
| | MF (control) | | FD | | Aβ + FD | | Aβ + MF | | Aβ + FS | |
Rat tissues	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
$\Delta C_t (= $ mt $C_{t\,del} - $ mt $C_{t\,D\text{-loop}})$‡										
Liver	1·96^{a,b}	0·25	1·67^b	0·15	1·68^b	0·36	2·23^{a,b}	0·69	2·55^a	0·73
Cortex	3·23	1·19	2·19	0·29	2·40	1·10	2·86	1·24	3·26	1·28
Hippocampus	4·05^a	1·29	1·65^b	1·06	1·75^b	0·66	2·93^{a,b}	0·86	5·11^a	2·64
Medulla	2·19^{a,b}	0·66	1·13^c	0·28	1·73^{b,c}	0·82	1·94^{a,b}	0·40	2·56^a	0·44

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* Data were analysed by one-way ANOVA and Duncan's multiple range test.

† FD, MF and FS indicate no folate (folate deprivation), moderate folate (+8 mg folic acid/kg diet) and folic acid-supplemented (+8 mg folic acid/kg diet and 0·003 % folic acid in drinking-water: 68 μM) diets, respectively. The MF group receiving the vehicle microinjection was designated the control. Aβ administration included an intracerebroventricular microinjection of 8 μl of Aβ$_{(25-35)}$ peptide (1 mg/ml). Vehicle administration included an intracerebroventricular microinjection of 8 μl of the vehicle solution (sterile distilled water).

‡ The quantity of mtDNA^{4834} deletions was determined by co-amplifying the mtDNA D-loop and mtDNA^{4834} deletion in a real-time PCR assay. The relative frequencies of mtDNA^{4834} deletions to mtDNA were calculated using $\Delta C_t = $ mt $C_{t\,del} - $ mt $C_{t\,D\text{-loop}}$; a smaller ΔC_t value indicates more deletions.

### Dietary folate modulated neuronal death of rats in the presence/absence of an β-amyloid peptide challenge

To investigate whether dietary folate intake and/or Aβ challenge may involve in alternative mechanisms to affect the cortex region, the neuronal death of the frontal cortical brain tissues of rats fed various folate diets was assayed. Fig. 2 shows that neuronal cell death was only occasionally observed in the cortex brain of control rats (Fig. 2(A)). Neuronal death in brain tissues of FD rats significantly increased by 2·5-fold compared with the counterpart tissue of the control group (Fig. 2(B) and (E)). Administration of Aβ$_{(25-35)}$ did not aggravate the neuronal death rate of FD rats (Fig. 2(C) and (E)). Aβ-treated rats fed the FS diet had significantly reduced neuronal death compared with Aβ + FD rats (Fig. 2(D) and (E)). When compared with the control group, feeding rats the FS diet completely prevented Aβ + FD-promoted neuron death (Fig. 2(E)).

Discussion

Our data demonstrated that hippocampal, as opposed to cortical and medullary, tissue was the most vulnerable brain region for oxidative lipid damage in response to dietary FD. Feeding rats an FD diet for 4 weeks significantly elevated TBARS levels in the hippocampus tissue compared with the counterpart tissue of control rats. The hippocampal oxidative

injuries of FD rats might have been partially, if not totally, due to rapid folate depletion, as 4 weeks of the FD diet significantly decreased hippocampal folate levels by 50 % without affecting cortical folate levels (Table 2). Folate was proposed to possess *in vitro* and *in vivo* antioxidant capabilities to scavenge free radicals[39,40]. Numerous investigations have shown that folate deficiency promotes H_2O_2 generation and lipid peroxidation in human cells and rodent tissues[41-43]. Alternatively, a folate deficiency induced elevated homocysteine levels in neuronal cells, a well-known pro-oxidant to elicit lipid peroxidation and elevated oxidative stress[42-45]. Consistently, we have previously shown that feeding rats an FD diet for 4 weeks elevated serum homocysteine levels, which were associated with brain oxidative DNA damage as well as increased hepatic lipid peroxidation[25,43]. Although elevated brain homocysteine levels were not measured in the present study, our data showed that 4 weeks of the FD diet significantly promoted higher levels of TBARS in the liver of rats, in parallel with FD-induced hippocampal folate depletion and oxidative lipid damage (Tables 2 and 3). Collectively, the findings suggest that rats fed an FD diet attained various degrees of brain folate depletion, which may be partially ascribed to the different magnitude of lipid peroxidation in brain regions.

Dietary FD significantly increased the accumulation of large mtDNA deletions in the hippocampal and medullary regions of rats, but not in the cortex tissue. The frequencies of
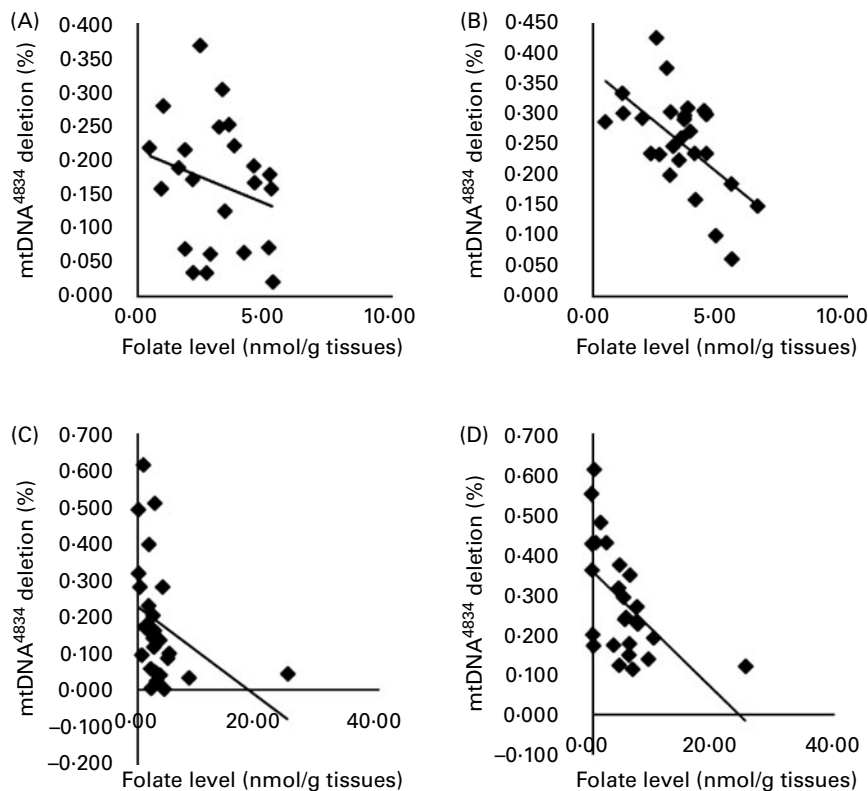
Fig. 1. Pearson's correlation coefficients for the relationships between tissue folate status and the accumulation of mitochondrial DNA^{4834} deletions in (A) cortical (r −0·242, P=0·133), (B) hippocampal (r −0·593, P=0·001) and (C) medullary tissues (r −0·345, P=0·042) and (D) liver homogenates of rats fed diets with various folate concentrations in the presence/absence of an β-amyloid peptide challenge (r −0·547, P=0·002). The quantity of mtDNA^{4834} deletions was determined by co-amplifying the mtDNA displacement loop (D-loop) and mtDNA^{4834} deletion in a real-time PCR assay, as described in 'Materials and methods'. $\Delta C_t = $ mt $C_{t\,del} - $ mt $C_{t\,D-loop}$ were used to quantify the relative amount of large mtDNA deletions in percentage with the equation: $R = 2^{-\Delta C_t}$.
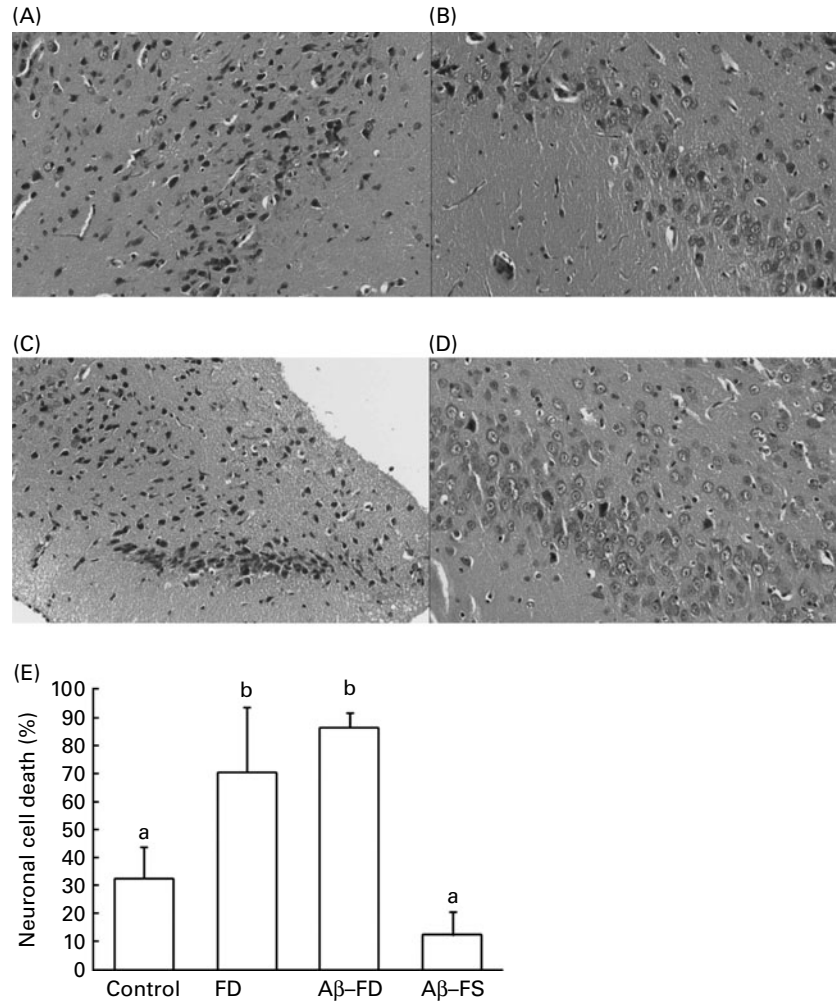
Fig. 2. Representative microphotographs of the neuronal death of rats fed the moderate folate (MF) (A), folate deprivation (FD) (B and C) and folate supplementation (FS) diets (D) for 4 weeks in the absence (A and B) and presence of an amyloid β-peptide (Aβ)$_{(25-35)}$ injection (C and D). The FD, MF and FS diets indicate diets with folic acid at 0, 8 mg and 8 mg folic acid/kg diet + 0.003 % folic acid in drinking-water levels, respectively. The MF group receiving the vehicle microinjection was designated the control. Brain sections of the frontal cortex were stained with haematoxylin and eosin. Cell death was measured as described in 'Materials and methods'. (E) Quantification of neuronal death in the experimental groups. Neuronal death was expressed as the death ratio (numbers of dead neurons/total number of neurons in the fixed field). Values are means, with standard deviations represented by vertical bars (n 4). Data were analysed by one-way ANOVA and Duncan's multiple range test. ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$).

mtDNA deletions in the hippocampal and medullary regions were significantly and inversely correlated with their respective brain folate levels (Fig. 1). This finding is in accordance with our previous observation that an FD diet depleted brain folate levels and promoted the accumulation of large mtDNA deletions in whole-brain homogenates of rats[25]. A study has revealed that FD impaired mt antioxidant status, promoted superoxide overproduction and elevated mt protein oxidative damage[46]. As reactive oxygen species production can lead to the loss of mtDNA molecules[47], the FD-elicited oxidative stress inside FD neurons may be attributable to increased mtDNA deletions of the hippocampal and medullary regions. Such oxidative injuries in mtDNA, however, were not found in the cortex tissue of rats fed with the 4-week FD diet. As the 4-week FD did not significantly affect cortex folate levels (Table 2), the lack of oxidative mtDNA damage in the FD cortex region further supports the antioxidant role of

folate. It has been reported that feeding rats a folate/methyl-deficient diet for 16–18 weeks induced oxidative stress and DNA damage in the cortex of the brain[48], suggesting a long-term effect of FD on cortical oxidative injuries.

Dietary FD was shown to sensitise neurons to amyloid toxicity[45], a hallmark of AD. Our data extend this previous finding to demonstrate that a single icv injection of the aggregated Aβ peptide augmented lipid peroxidation in the medullary tissue of folate-deprived rats, which could be ameliorated by feeding FD rats with the MF and FS diets. This observation supports the antioxidant role of folate against Aβ-promoted oxidative injuries. Furthermore, we found that the FS diet, but not the MF diet, significantly diminished mtDNA deletions in the hippocampus and medulla of FD rats upon Aβ challenge. It suggests a threshold effect of dietary folate levels, which protect against FD-associated mtDNA degeneration. Consistently, results of several studies have shown that high

levels of folic acid supplementation may counteract mtDNA oxidative injuries and mt dysfunction in macrophage cells and hepatocytes[46,49]. By folic acid supplementation, the mt respiratory function and mt-associated death signalling were modulated to reduce apoptotic death[49,50]. Given the fact that feeding the FS diet significantly reduced cortical neuronal death of Aβ-treated and folate-deprived rats (Fig. 2), the finding implied a folate-associated death signalling involved in the neuronal death of FD rats. Whether or not this is the alternative mechanism behind the reported association between folate and cortical neuronal death remains to be definitively established.

The present study had a number of limitations. The relatively small sample size due to the premature death of animals receiving injection surgery may reduce the statistical power for subgroup analysis. Several groups[30,33] used $Aβ_{(35-25)}$, an 11-amino acid with a sequence that was the reversal of ordering of $Aβ_{(25-35)}$, as a control to test the specificity of the toxic amino acid sequence of $Aβ_{(25-35)}$. Whether the oxidative stress of brain regions induced by $Aβ_{(25-35)}$ injection was specific to the toxic effect of $Aβ_{(25-35)}$ remains to be clarified. As haematoxylin–eosin staining used in the present study is not a good assay to evaluate apoptotic death, alternative measurements for chromatin condensation and caspase activation will be used for further studies.

Given the assumptions and limitations of the in vivo study, the results in the present study suggest that dietary FD differentially depleted brain folate, and increased lipid peroxidation and mtDNA^{4834} deletions in various brain regions. The hippocampal tissue was the most vulnerable brain region for oxidised lipid and mtDNA damage in response to dietary FD. The FS diet was biologically effective at enriching brain folate, and protecting AD-susceptible brains against FD-induced mt genotoxicity and neurotoxicity upon Aβ challenge. Whether the effects of folic acid supplementation found in the present in vivo study have physiological relevance in humans remains to be clarified.

References

1. Reynolds E (2006) Vitamin B$_{12}$, folic acid, and the nervous system. Lancet Neurol 5, 949–960.
2. Engelborghs S, Vloeberghs E, Maertens K, et al. (2004) Correlations between cognitive, behavioural and psychological findings and levels of vitamin B$_{12}$ and folate in patients with dementia. Int J Geriatr Psychiatry 19, 365–370.
3. Reynolds EH (2002) Folic acid, ageing, depression, and dementia. BMJ 324, 1512–1515.
4. Ramos MI, Allen LH, Mungas DM, et al. (2005) Low folate status is associated with impaired cognitive function and dementia in the Sacramento area Latino study on aging. Am J Clin Nutr 82, 1346–1352.
5. Luchsinger JA, Tang MX, Miller J, et al. (2007) Relation of higher folate intake to lower risk of Alzheimer disease in the elderly. Arch Neurol 64, 86–92.
6. Fioravanti M, Ferrario E, Massaia M, et al. (1998) Low folate levels in the cognitive decline of elderly patients and the efficacy of folate as a treatment for improving memory deficits. Arch Gerontol Geriatr 26, 1–13.
7. Bryan J, Calvaresi E & Hughes D (2002) Short-term folate, vitamin B$_{12}$ or vitamin B$_6$ supplementation slightly affects memory performance but not mood in women of various ages. J Nutr 132, 1345–1356.
8. Durga J, van Boxtel MP, Schouten EG, et al. (2007) Effect of 3-year folic acid supplementation on cognitive function in older adults in the FACIT trial: a randomized, double blind, controlled trial. Lancet 369, 208–216.
9. Sommer BR, Hoff AL & Costa M (2003) Folic acid supplementation in dementia: a preliminary report. J Geriatr Psychiatry Neurol 16, 156–159.
10. Morris MC, Evans DA, Bienias JL, et al. (2005) Dietary folate and vitamin B$_{12}$ intake and cognitive decline among community-dwelling older persons. Arch Neurol 62, 641–645.
11. Duyckaerts C (2004) Looking for the link between plaques and tangles. Neurobiol Aging 25, 735–739.
12. Thal DR, Rüb U, Orantes M, et al. (2002) Phases of A beta-deposition in the human brain and its relevance for the development of AD. Neurology 58, 1791–1800.
13. Velliquete RA, O'Connor T & Vassar R (2005) Energy inhibition elevates beta-secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: possible early events in Alzheimer's disease pathogenesis. J Neurosci 25, 10874–10883.
14. Behl C & Moosmann B (2002) Antioxidant neuroprotection in Alzheimer's disease as preventive and therapeutic approach. Free Radic Biol Med 33, 182–191.
15. Chinnery PF, Samuels DC, Elson J, et al. (2002) Accumulation of mitochondrial DNA mutations in ageing, cancer, and mitochondrial disease: is there a common mechanism? Lancet 360, 1323–1325.
16. Ravagnan L, Roumier T & Kroemer G (2002) Mitochondria, the killer organelles and their weapons. J Cell Physiol 192, 131–137.
17. Reddy PH & Beal MF (2005) Are mitochondria critical in the pathogenesis of Alzheimer's disease? Brain Res Rev 49, 618–632.
18. Maruszak A, Gaweda-Walerych G, Soltyszewski I, et al. (2006) Mitochondrial DNA in pathogenesis of Alzheimer's and Parkinson's diseases. Acta Neurobiol Exp 66, 153–176.
19. Bosetti F, Brizzi F, Barogi S, et al. (2002) Cytochrome c oxidase and mitochondrial F1F0-ATPase (ATP synthase) activities in platelets and brain from patients with Alzheimer's disease. Neurobiol Aging 23, 371–376.
20. Mancuso M, Filosto M, Bosetti F, et al. (2003) Decreased platelet cytochrome c oxidase activity is accompanied by increased blood lactate concentration during exercise in patients with Alzheimer disease. Exp Neurol 182, 421–426.
21. Wang J, Xiong S, Xie C, et al. (2005) Increased oxidative damage in nuclear and mitochondrial DNA in Alzheimer's disease. J Neurochem 93, 953–962.

22. Markesbery WR & Lovell MA (2006) DNA oxidation in Alzheimer's disease. *Antioxid Redox Signal* **8**, 2039–2045.

23. Branda RF, Brooks EM, Chen Z, *et al.* (2002) Dietary modulation of mitochondrial DNA deletions and copy number after chemotherapy in rats. *Mutat Res* **501**, 29–36.

24. Crott JW, Choi SW, Branda RF, *et al.* (2005) Accumulation of mitochondrial DNA deletions is age, tissue and folate-dependent in rats. *Mutat Res* **570**, 63–70.

25. Chou YF, Yu CC & Huang RFS (2007) Changes in mitochondrial DNA deletion, content, and biogenesis in folate-deficient tissues of young rats depend on mitochondrial folate and oxidative DNA injuries. *J Nutr* **137**, 2036–2042.

26. Cortopassi GA, Shibata DD, Soong NW, *et al.* (1992) A pattern of accumulation of a somatic deletion of mitochondrial DNA in aging human tissues. *Proc Natl Acad Sci U S A* **89**, 7370–7374.

27. Wei YH (1992) Mitochondrial DNA alterations as ageing-associated molecular events. *Mutat Res* **275**, 145–155.

28. Lee HC, Pang CY, Hsu HS, *et al.* (1994) Differential accumulations of 4,977 bp deletion in mitochondrial DNA of various tissues in human ageing. *Biochim Biophys Acta* **1226**, 37–43.

29. Lee HC & Wei YH (2007) Oxidative stress, mitochondrial DNA mutation, and apoptosis in aging. *Exp Biol Med* **232**, 592–606.

30. Sun MK & Alkon DL (2002) Impairment of hippocampal CA1 heterosynaptic transformation and spatial memory by beta-amyloid$_{25-35}$. *J Neurophysiol* **87**, 2441–2449.

31. Walzem RL & Clifford AJ (1988) Folate deficiency in rats fed diets containing free amino acid or intact proteins. *J Nutr* **118**, 1089–1096.

32. Wilkinson B, Koenigsknecht-Talboo J, Grommes C, *et al.* (2006) Fibrillar beta-amyloid-stimulated intracellular signaling cascades require Vav for induction of respiratory burst and phagocytosis in monocytes and microglia. *J Biol Chem* **281**, 20842–20850.

33. Stepanichev MY, Moiseeva YV, Lazareva NA, *et al.* (2003) Single intracerebroventricular administration of amyloid-beta$_{(25-35)}$ peptide induces impairment in short-term rather than long-term memory in rats. *Brain Res Bull* **61**, 197–205.

34. Varela-Moreiras G & Selhub J (1992) Long-term folate deficiency alters folate content and distribution differentially in rat tissues. *J Nutr* **122**, 986–991.

35. Horne DW & Patterson D (1988) *Lactobacillus casei* microbiological assay of folic acid derivatives in 96-well microtiter plates. *Clin Chem* **34**, 2357–2359.

36. Fraga CG, Leibovitz BE & Tappel AL (1988) Lipid peroxidation measured as thiobarbituric-reactive substances in tissue slices: characterization and comparison with homogenates and microsomes. *Free Radic Biol Med* **4**, 155–161.

37. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.

38. Tocco G, Musleh W, Sakhi S, *et al.* (1997) Complement and glutamate neurotoxicity: genotypic influences of C5 in a mouse model of hippocampal neurodegeneration. *Mol Chem Neuropathol* **31**, 1–12.

39. Joshi R, Adhikari S, Patro BS, *et al.* (2001) Free radical scavenging behavior of folic acid: evidence for possible antioxidant activity. *Free Radic Biol Med* **30**, 1390–1399.

40. Doshi SN, McDowell IFW, Moat SJ, *et al.* (2001) Folate improves endothelial function in coronary artery disease. An effect mediated by reduction of intracellular superoxide? *Arterioscler Thromb Vasc Biol* **21**, 1196–1202.

41. Chen YH, Huang RFS, Wei JS, *et al.* (2001) Folate deficiency-mediated down regulation of intracellular glutathione and antioxidant enzymes increases susceptibility of human hepatoma HepG2 cells to various oxidant stress-induced cytotoxicity. *J Biomed Lab Sci* **13**, 52–57.

42. Chern CL, Huang RFS, Chen YH, *et al.* (2001) Folate deficiency-induced oxidative stress and apoptosis are mediated via homocysteine-dependent overproduction of hydrogen peroxide and enhances activation of NF-κB in human HepG2 cells. *Biomed Pharmacother* **55**, 434–442.

43. Huang RFS, Hsu YC, Lin HL, *et al.* (2001) Folate depletion and elevated plasma homocysteine promote oxidative stress in rat livers. *J Nutr* **131**, 33–38.

44. Ho PI, Ashline D, Dhitavat S, *et al.* (2003) Folate deprivation induces neurodegeneration: roles of oxidative stress and increased homocysteine. *Neurobiol Dis* **14**, 32–42.

45. Kruman II, Kumaravel TS, Lohani A, *et al.* (2002) Folic acid deficiency and homocysteine impair DNA repair in hippocampal neurons and sensitize them to amyloid toxicity in experimental models of Alzheimer's disease. *J Neurosci* **22**, 1752–1762.

46. Chang CM, Yu CC, Lu HT, *et al.* (2007) Folate deprivation promotes mitochondrial oxidative decay: DNA large deletions, cytochrome *c* oxidase dysfunction, membrane depolarization and superoxide overproduction in rat liver. *Br J Nutr* **97**, 855–863.

47. Suematsu N, Tsutsui H, Wen J, *et al.* (2003) Oxidative stress mediates tumor necrosis factor-alpha-induced mitochondrial DNA damage and dysfunction in cardiac myocytes. *Circulation* **107**, 1418–1423.

48. Bagnyukovaa TV, Powell CL, Oleksandra Pavliv O, *et al.* (2008) Induction of oxidative stress and DNA damage in rat brain by a folate/methyl-deficient diet. *Brain Res* **1237**, 44–51.

49. Huang RFS, Yaong HC, Chen SC, *et al.* (2004) *In vitro* folate supplementation alleviates oxidative stress, mitochondria-associated death signalling and apoptosis induced by 7-keto-cholesterol. *Br J Nutr* **92**, 887–894.

50. Ye YL, Chan YT, Liu HC, *et al.* (2010) Depleted folate pool and dysfunctional mitochondria associated with defective mitochondrial folate proteins sensitize Chinese ovary cell mutants to *tert*-butyl hydroperoxide-induced oxidative stress and apoptosis. *J Nutr Biochem* **21**, 793–800.