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Original Article

Vitamin B_{12} treatment reduces mononuclear DNA damage

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Abstract

Background: DNA damage effects of vitamin B_{12} deficiency were performed *in vitro* and in adults.

Methods: The study group included 32 children (13 girls, 19 boys) with vitamin B_{12} deficiency (mean age 44 ± 58 months) and their 27 mothers (mean age 30.4 ± 5.3 years). The control group contained 30 healthy children and 25 mothers. DNA strand breaks in peripheral blood mononuclear leukocytes were assayed by single-cell alkaline gel electrophoresis (comet assay) before and 8 days after the first injection of vitamin B_{12}.

Results: Mean DNA damage scores in children with vitamin B_{12} deficiency and their mothers were significantly higher before treatment than those after treatment. The DNA damage scores of children after treatment were still significantly higher than controls. There were significant negative correlations between the children and their mothers in terms of vitamin B_{12} levels and DNA damage scores ($r = 0.3$, $P = 0.02$; $r = 0.58$, $P = 0.002$, respectively). There were correlations between the children's and their mothers' DNA damage and the severity of vitamin B_{12} deficiency, suggesting that the children and their mothers may play a role in the scarcity of nutritional vitamin B_{12}.

Conclusion: DNA damage is increased in children with vitamin B_{12} deficiency and in their mothers. DNA damage scores were significantly improved through vitamin B_{12} therapy 8 days after the first injection, however, they were still significantly higher than those of controls.

Key words anemia, comet assay, DNA damage, vitamin B_{12} deficiency.

Vitamin B_{12} is derived from cobalamin in food secondary to production only by prokaryotes. The recommended dietary allowance of cobalamin for adults has been set at 1 μg/day; 1.3 and 1.4 μg daily for lactating and pregnant women, respectively; and 0.1 μg/day for infants.[1] Insufficient intake or disrupted absorption of vitamin B_{12} results in vitamin B_{12} deficiency.[2] Studies of chromosomal damage and uracil misincorporation into DNA in different populations suggest that this level may need to be higher, 7–10 μg/day, to ensure genomic stability.[3,4]

Single-cell gel electrophoresis (also called the comet assay) is a well-established genotoxicity test for the quantitation of DNA damage in individual cells.[5] It has the advantages of speed, simplicity, and the fact that observations are made at the level of peripheral blood mononuclear leukocytes.[6–8] This simple, rapid, and sensitive technique is extremely useful, and has been used for the assessment of the extent of endogenous DNA damage. Furthermore, the comet assay is a potential tool to estimate DNA damage at the single-cell level and it provides information on the presence of DNA damage among individual cells.[8]

As yet, although there are some studies on DNA damage and oxidants in vitamin B_{12} deficiency,[3,9,10] there is no report available on levels of endogenous DNA damage—determined by the comet assay—in peripheral blood lymphocytes DNA damage in children and their mothers. In this study, 32 children with vitamin B_{12} deficiency and their 27 mothers were treated with intramuscular B_{12} and we re-assessed DNA damage scores after 7 days.

Methods

Subjects

Thirty-two children (1–156 months, mean 44 ± 58 months), with a sex ratio of 13:19 (F : M) and their 27 mothers (19–40 years, mean 30.4 ± 5.3 years) were admitted to Harran University Resource Hospital Pediatrics clinic and out-patient clinic because of Vitamin B_{12} deficiency. The mothers' and their children's meat consumption was very low (during 1 month or less): their animal protein requirements were met by poultry products, especially chicken meat. No children or their mothers were reported as being vegetarian. Significant B_{12} deficiency was defined as a vitamin B_{12} concentration of lower than 200 pg/mL in children and mothers.[7] The following definitions were also used: macrocytosis: infant age ≤2 years mean corpuscular volume (MCV) >96 fL, infant age >2 years MCV > 97 fL; neutropenia: <1500/mm^3; and thrombocytopenia: <150 000/mm^2. Vitamin B_{12} deficiency anemia was defined as: infant age ≤2 years hemoglobin <10.5 g/dL, infant age >2 years hemoglobin <11.5 g/dL; mother

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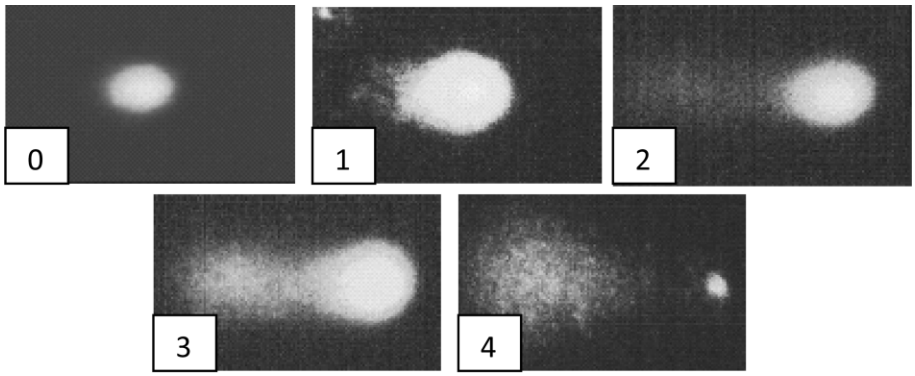

Fig. 1 Alkaline comet assay figures from our study. Images were classified according to fluorescence intensity in the comet tail and were given a value of 0–4 (from undamaged [class 0] to maximally damaged [class 4]).

hemoglobin <12 g/dL, and an MCV > 90 fL. Children with severe congenital malformation, sepsis, hemolytic-type and iron or folate deficiency anemia, and multifactorial anemia, and those with signs and symptoms suggestive of serious illness were excluded from the study.

DNA damage scores were measured in samples obtained from children and their mothers before and 8 days after the first injection of vitamin B_{12} (Dodex 1 mg ampule, Deva Holding Inc.). The children and their mothers that had vitamin B_{12} deficiency were treated for the first 3 days with 10 µg/day injection intramuscularly (i.m.), on day 4 with 100 µg i.m., and on days 5, 7 with 1000 µg/day i.m. Maximum 2.5-mL blood samples were taken per child and their mothers for all parameters. This time was chosen to allow simultaneous sampling with routine hemogram tests, thus avoiding a blood-taking procedure solely for the purpose of the study. The local ethics committee approved this study and the parents gave consent for the newborns' involvement.

Sample preparation

Blood samples were collected from a peripheral vein into vacutainers containing ethylenediaminetetraacetic acid and heparinized tubes. The heparinized tubes were stored at 10°C in the dark to prevent further DNA damage, and were processed within 2 h. Mononuclear leukocyte isolation for the comet assay was carried out by the use of Histopaque 1077 (Sigma); 1 mL of heparinized whole blood was carefully layered over 1 mL of Histopaque and centrifuged for 35 min at 500 × g and 25°C. The interface band containing mononuclear leukocytes was washed with phosphate-buffered saline (PBS) and then collected after 15-min centrifugation at 400 × g. The resulting pellets were resuspended in PBS and the cells were counted in a Neubauer chamber. Membrane integrity was assessed by means of the trypan blue exclusion method. The remaining blood was centrifuged at 1500 × g for 10 min to obtain the plasma. The separated plasma was used to measure vitamin B_{12} and folic acid concentrations, which were measured by an automated chemistry analyzer (Aeroset, Abbott, IL, USA) using commercial kits (Abbott, USA). Our laboratory vitamin B_{12} detection limits are 10–2000 pg/mL. Whole blood count was measured by an automated analyzer (Celldyn 3700, Abbott, IL, USA).

DNA damage determination by alkaline comet assay

Endogenous DNA damage in peripheral mononuclear leukocytes was analyzed by alkaline comet assay according to Singh *et al*. with minor modification.[5,6] All of the analysis steps were conducted under red light or without direct light to prevent additional DNA damage. The images of 100 randomly chosen nuclei (50 nuclei from each of two replicate slides) were analyzed visually for each subject. Each image was classified according to the intensity of the fluorescence in the comet tail and was given a value of 0, 1, 2, 3, or 4 (from undamaged, class 0, to maximally damaged, class 4) (Fig. 1). These samples were randomly chosen from the microscobic slides of our patients, and so the total score of a slide was between 0 and 400 arbitrary units (AU).[6,8] The same biochemistry staff performed all the procedures and a single observer unaware of the subject's group determined the DNA damage score.

Statistical analysis

Testing for normality of variances was accomplished using Levene statistical tests. Variances in this assay were homogeneous. The data were compared using the χ^2-test, McNemar, paired and unpaired *t*-tests. Bivariate associations between variables were assessed with Pearson's correlation test. The data were expressed as mean ± SD and the differences were considered statistically significant at $P < 0.05$. Statistical analysis was conducted using SPSS for Windows 11.5 (SPSS, Chicago, IL, USA).

Results

All children had vitamin B_{12} deficiency, 15 (47%) children had profound deficiency of vitamin B_{12} (<120 pg/mL), 19 children (59%) were younger than 24 months, and 10 children's (31%) bodyweight was below the third percentile. Twelve (38%) children had neuromotor retardation, 15 (47%) had atrophic glossitis, 11 (34%) had peripheral hyperpigmentation, and 11 (34%) had thin and sparse hair. Six children (19%) had macrocytosis, 14 (44%) had anemia, seven (22%) had neutropenia, and six (19%) had thrombocytopenia before the vitamin B_{12} therapy. The children and their mothers' laboratory indices before and after the vitamin B_{12} treatment are shown in Table 1. Sixteen (59%) of 27 mothers had deficiency of vitamin B_{12} (<200 pg/mL). There is no significant difference in the hematological laboratory findings

Table 1 Comparison of laboratory findings in children with vitamin B_{12} deficiency and their mothers

	Children			Mothers		
	Before treatment $n = 32$ (%)	After treatment $n = 32$ (%)	P*	Before treatment $n = 27$ (%)	After treatment $n = 27$ (%)	P*
Macrocytosis	5 (16)	3 (9)	0.500	3 (11)	2 (7.4)	1.000
Anemia	14 (44)	16 (50)	0.500	10 (37)	9 (33)	1.000
Neutropenia	7 (22)	4 (13)	0.250	3 (11)	1 (3.2)	0.500
Thrombocytopenia	6 (19)	1 (3)	0.063	2 (7.4)	0	NA

*McNemar test of the difference between paired proportions.

between before and after vitamin B_{12} therapy (Table 1). The plasma mean folic acid level was 12.4 ± 4.7 (range 3–20) ng/mL in children. Vitamin B_{12} levels were 112 ± 51 pg/mL and 191 ± 58 pg/mL in the children and their mothers, respectively (Fig. 2). Plasma vitamin B_{12} levels were greater than 2000 pg/mL in all cases 8 days after the first injection.

Mean DNA damage scores in children with vitamin B_{12} deficiency were significantly higher before treatment than those after treatment (32 ± 23 AU, 19 ± 13 AU, $P < 0.001$, respectively, Fig. 3). Similarly, mean DNA damage scores in mothers were significantly higher before treatment than those after treatment (31 ± 21 AU, 21 ± 16 AU, $P < 0.001$, respectively, Fig. 4). DNA damage scores of children after treatment were still significantly higher than controls (19 ± 13 AU, 6 ± 4 AU, $P < 0.001$, Fig. 3). There were significant negative correlations between the children and their mothers in terms of vitamin B_{12} levels and DNA damage scores (r = 0.3, $P = 0.02$; r = 0.58, $P = 0.002$, respectively, Figs 5,6).

Discussion

In the present study we found that DNA damage was increased in children with vitamin B_{12} deficiency and in their mothers. To the best of our knowledge, all the published studies related to the DNA damage effects of vitamin B_{12} deficiency were performed *in vitro*[2,3,11] and in adults;[12–14] this is the first report showing an association between vitamin B_{12} deficiency and peripheral blood mononuclear leukocytes DNA double-strand breaks assayed by single-cell alkaline gel electrophoresis (comet assay) in children and their mothers.

Vitamin B_{12} is essential in folate metabolism, and the interaction of the two vitamins is essential for the conversion of homocysteine to methionine, for the synthesis of purines and pyrimidines, for methylation reactions, and for the maintenance of cellular levels of folate.[15] A deficiency of vitamin B_{12} can mimic chemicals in damaging DNA by causing single- and double-strand breaks.[16] When vitamin B_{12} is deficient, then tetrahydrofolate is trapped as methyl-THF; the methylene-THF pool, which is required for methylation of dUMP to dTMP, is consequently diminished. Therefore, B_{12} deficiency, like folic

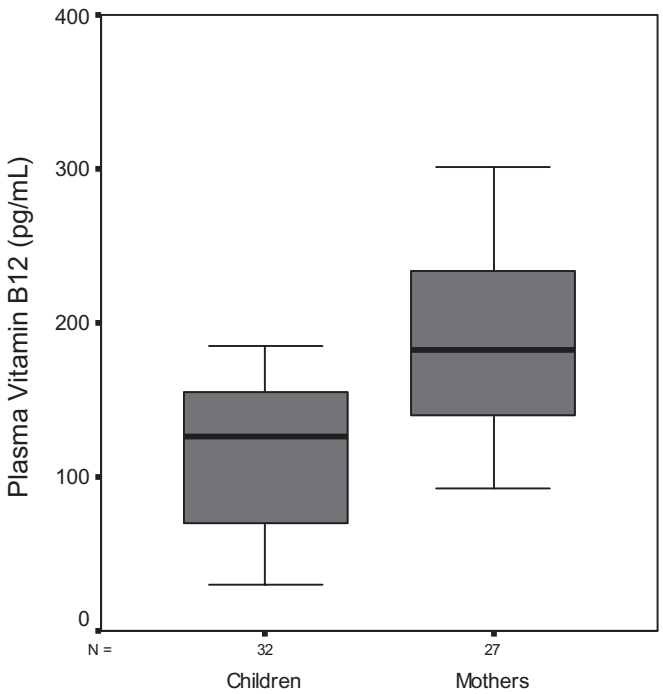

Fig. 2 Boxplot graphic of children and their mothers' plasma vitamin B_{12} levels.

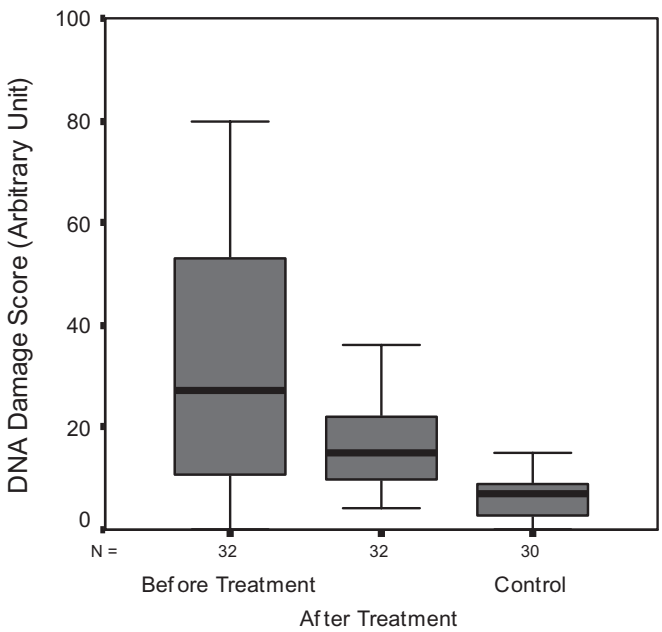

Fig. 3 DNA damage score boxplot graphics in children with vitamin B_{12} deficiency before and after treatment and controls.

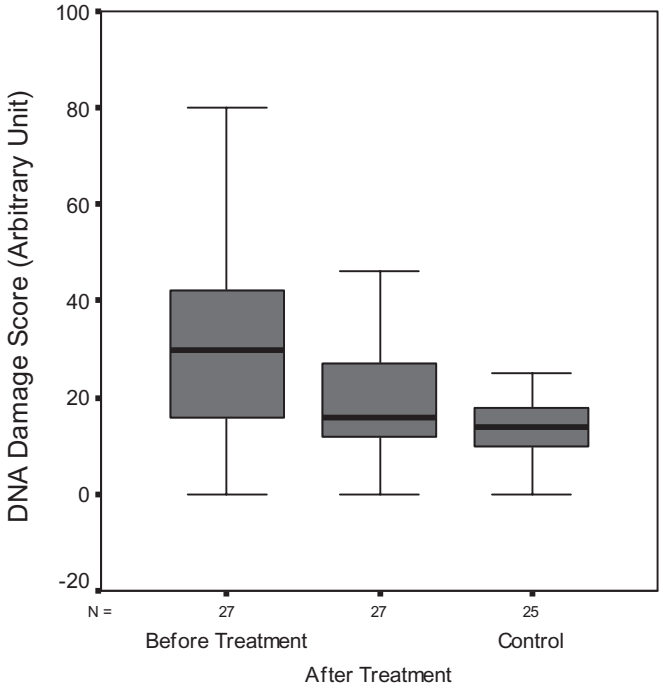

Fig. 4 DNA damage score boxplot graphics in mothers with vitamin B_{12} deficiency before and after treatment and controls.

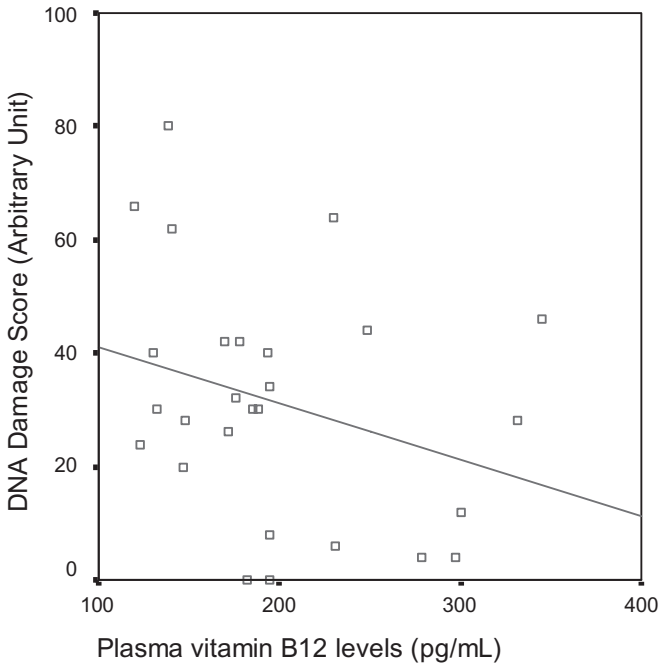

Fig. 6 DNA damage score and plasma vitamin B_{12} levels correlation graphic in mothers with vitamin B_{12} deficiency.

acid deficiency, causes uracil to accumulate in DNA, chromosome breaks, excessive uracil in DNA, micronucleus formation and DNA hypomethylation[3,14,17] The two deficiencies may act synergistically.[3,18] The mechanism of chromosome breaks has been shown to be deficient methylation of uracil to thymine, and subsequent incorporation of uracil into human DNA (4 million/cell).[9,16] In our cases, plasma folic acid levels were normal; therefore there was increased DNA damage because of vitamin B_{12} and other macro-micronutrient deficiency. There was a negative correlation between DNA damage scores and vitamin B_{12} levels ($r = 0.3$, $P = 0.02$). In addition, although this significant correlation between the DNA damage and vitamin B_{12}, as DNA strand breaks, can result from various causes, the lower correlation coefficient suggests other factors influencing these end-points. However, this study also shows that DNA damage is improved by vitamin B_{12} in 7 days. All these observations, combined with the results of our study, lead us to emphasize that further studies are needed to more clearly identify the effects at later time-points on the DNA repair.

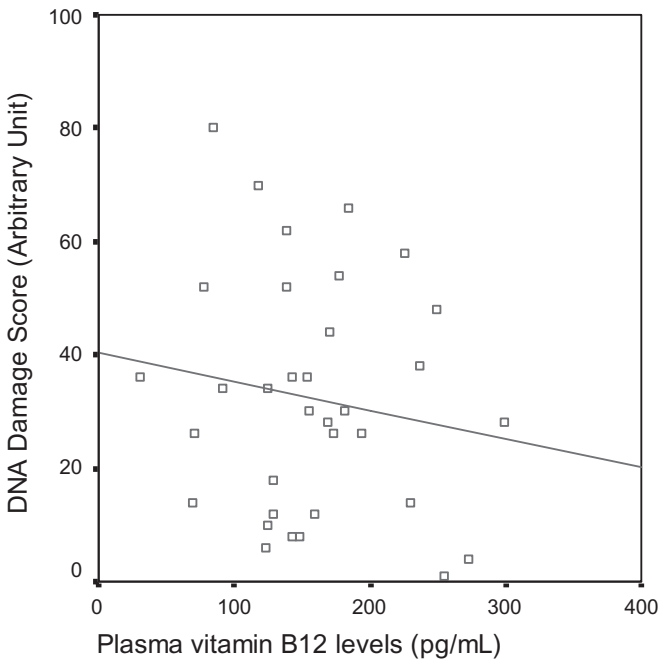

Fig. 5 DNA damage score and plasma vitamin B_{12} levels correlation graphic in children with vitamin B_{12} deficiency.

Because all the vitamin B_{12} needs of humans are met through the diet, inadequate intake causes deficiency.[2] Typical diets in developed countries contain more than adequate amounts of vitamin B_{12}, although vitamin B_{12} deficiency may occur in vegans, who consume no animal products whatever.[4] Elsewhere, vitamin B_{12} deficiency may occur frequently in populations whose diet contains little meat. Low vitamin B_{12} levels have also been reported in populations in the Middle East, elsewhere in Asia, Africa, Mexico, and Central and South America.[1] Koc *et al*. reported that vitamin B_{12} deficiency was highly prevalent in pregnant women and that 41% of infants are born with deficient vitamin B_{12} storage in the Sanliurfa province, which is in the same area of Turkey that our study was performed in.[19] The important causes of the high frequency of vitamin B_{12} deficiency in this region are inadequate consumption of animal products due to local tradition, poverty and poor hygienic conditions.[19] Simi-

larly, in this study, the mothers' and their children's meat consumption was very low: animal protein requirements were met by poultry products, especially chicken meat. Infants' vitamin B_{12} source is their mothers' vitamin B_{12} pool, intimately involved in maintaining vitamin B_{12} in the body, and so the positive association between infants' vitamin B_{12} concentration and their mothers' vitamin B_{12} levels that we found was to be expected.[20]

Vitamin B_{12} deficiency is known to cause neuropathy due to demyelization and loss of peripheral neurons.[9] Low plasma B_{12} concentrations are common, although up to 90% may not manifest clinical signs of vitamin B_{12} deficiency.[12] Nevertheless, these people may be at risk from progressive neurological and/or hematologic diseases.[20,21] Neurological symptoms have been reported to occur in the absence of anemia in up to 28% of cases. It has been reported that developmental delay is greater than 56% in infants with vitamin B_{12} deficiency.[21,22] In our study, neuromotor retardation was 38% in children. It is probable that the causes of the difference depend on children's age, the severity and duration of the deficiency, and many other factors. Macrocytosis and other hematologic parameters were according to the literature.[22]

The major limitation of this study was that important plasma antioxidants and antioxidant enzymes, such as vitamin C, vitamin E, selenium glutathione peroxidase, and superoxide dismutase, were not determined.

In conclusion, vitamin B_{12} deficiency causes endogenous mononuclear leukocyte DNA damage and significantly reverses several days after vitamin B_{12} therapy in children and their mothers, but DNA damage scores were significantly higher in vitamin-B_{12}-deficient subjects than in controls.

References

1 Stabler SP, Allen RH. Vitamin B_{12} deficiency as a worldwide problem. *Annu. Rev. Nutr.* 2004; **24**: 299–326.
2 Herrmann W, Obeid R. Causes and early diagnosis of vitamin B_{12} deficiency. *Dtsch. Arztebl Int.* 2008; **105**: 680–5.
3 Fenech M. The role of folic acid and Vitamin B_{12} in genomic stability of human cells. *Mutat. Res.* 2001; **475**: 57–67.
4 Blount BC, Mack MM, Wehr CM *et al*. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. *Proc. Natl. Acad. Sci. U. S. A.* 1997; **94**: 3290–5.
5 Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 1988; **175**: 184–91.
6 Kocyigit A, Keles H, Selek S, Guzel S, Celik H, Erel O. Increased DNA damage and oxidative stress in patients with cutaneous leishmaniasis. *Mutat. Res.* 2005; **585**: 71–8.
7 Aslan M, Horoz M, Kocyigit A *et al*. Lymphocyte DNA damage and oxidative stress in patients with iron deficiency anemia. *Mutat. Res.* 2006; **601**: 144–9.
8 Faust F, Kassie F, Knasmüller S, Kevekordes S, Mersch-Sundermann V. The use of the alkaline comet assay with lymphocytes in human biomonitoring studies. *Mutat. Res.* 2004; **566**: 209–29.
9 Ames BN. DNA damage from micronutrient deficiencies is likely to be a major cause of cancer. *Mutat. Res.* 2001; **475**: 7–20.
10 Fenech M. Micronucleus frequency in human lymphocytes is related to plasma vitamin B_{12} and homocysteine. *Mutat. Res.* 1999; **428**: 299–304.
11 Walker PR, Smith B, Carson C *et al*. Induction of apoptosis in neoplastic cells by depletion of vitamin B_{12}. *Cell Death Differ.* 1997; **4**: 233–41.
12 Goringe A, Ellis R, McDowell I *et al*. The limited value of methylmalonic acid, homocysteine and holotranscobalamin in the diagnosis of early B_{12} deficiency. *Haematologica* 2006; **91**: 231–4.
13 Fenech M, Dreosti IE, Rinaldi JR. Folate, vitamin B_{12}, homocysteine status and chromosome damage rate in lymphocytes of older men. *Carcinogenesis* 1997; **18**: 1329–36.
14 Fenech M, Aitken C, Rinaldi J. Folate, vitamin B_{12}, homocysteine status and DNA damage in young Australian adults. *Carcinogenesis* 1998; **19**: 1163–71.
16 Ames BN. Micronutrient deficiencies. A major cause of DNA damage. *Ann. N. Y. Acad. Sci.* 1999; **889**: 87–106.
15 Glader B. Vitamin B_{12} (Cobalamin) deficiency. In: Kliegman RM, Behrman RE, Jenson HB, Stanton BF (eds). *Nelson Textbook of Pediatrics*, 18th edn. Saunders, Philadelphia, PA, 2007; 2013–4.
17 Fenech M. Micronutrients and genomic stability: a new paradigm for recommended dietary allowances (RDAs). *Food Chem. Toxicol.* 2002; **40**: 1113–7.
18 Kapiszewska M, Kalemba M, Wojciech U, Milewicz T. Uracil misincorporation into DNA of leukocytes of young women with positive folate balance depends on plasma vitamin B_{12} concentrations and methylenetetrahydrofolate reductase polymorphisms. A pilot study. *J. Nutr. Biochem.* 2005; **16**: 467–78.
19 Koc A, Kocyigit A, Soran M *et al*. High frequency of maternal vitamin B_{12} deficiency as an important cause of infantile vitamin B_{12} deficiency in Sanliurfa province of Turkey. *Eur. J. Nutr.* 2006; **45**: 291–7.
20 Weiss R, Fogelman Y, Bennett M. Severe vitamin B_{12} deficiency in an infant associated with a maternal deficiency and a strict vegetarian diet. *J. Pediatr. Hematol. Oncol.* 2004; **26**: 270–1.
21 Dror DK, Allen LH. Effect of vitamin B12 deficiency on neurodevelopment in infants: current knowledge and possible mechanisms. *Nutr. Rev.* 2008; **66**: 250–5.
22 Zengin E, Sarper N, Caki Kiliç S. Clinical manifestations of infants with nutritional vitamin B deficiency due to maternal dietary deficiency. *Acta Paediatr.* 2009; **98**: 98–102.