



Research paper

Vitamin B₁₂ deficiency results in severe oxidative stress, leading to memory retention impairment in *Caenorhabditis elegans*



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ABSTRACT

Oxidative stress is implicated in various human diseases and conditions, such as a neurodegeneration, which is the major symptom of vitamin B₁₂ deficiency, although the underlying disease mechanisms associated with vitamin B₁₂ deficiency are poorly understood. Vitamin B₁₂ deficiency was found to significantly increase cellular H₂O₂ and NO content in *Caenorhabditis elegans* and significantly decrease low molecular antioxidant [reduced glutathione (GSH) and *L*-ascorbic acid] levels and antioxidant enzyme (superoxide dismutase and catalase) activities, indicating that vitamin B₁₂ deficiency induces severe oxidative stress leading to oxidative damage of various cellular components in worms. An NaCl chemotaxis associative learning assay indicated that vitamin B₁₂ deficiency did not affect learning ability but impaired memory retention ability, which decreased to approximately 58% of the control value. When worms were treated with 1 mmol/L GSH, *L*-ascorbic acid, or vitamin E for three generations during vitamin B₁₂ deficiency, cellular malondialdehyde content as an index of oxidative stress decreased to the control level, but the impairment of memory retention ability was not completely reversed (up to approximately 50%). These results suggest that memory retention impairment formed during vitamin B₁₂ deficiency is partially attributable to oxidative stress.

1. Introduction

After vitamin B₁₂ (B₁₂) is accumulated by living cells, it is converted into two coenzymes, 5'-deoxyadenosylcobalamin and methylcobalamin, which function as the coenzymes for methylmalonyl-CoA mutase (MCM; EC 5.4.99.2) [1] and methionine synthase (MS; EC 2.1.1.13) [2], respectively. MCM catalyzes the conversion of *L*-methylmalonyl-CoA into succinyl-CoA in the catabolic pathway of certain amino acids, odd-numbered fatty acids, cholesterol, and thymine [3]. MS catalyzes methionine synthesis from homocysteine (Hcy) with 5'-methyltetrahydrofolate. During B₁₂ deficiency, the failure of B₁₂-dependent methionine biosynthesis leads to the accumulation of Hcy [4], which has prooxidant activity [4] and can activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to generate reactive oxygen species (ROS) [5]. These observations suggest that B₁₂ deficiency disrupts cellular redox homeostasis to induce oxidative stress, which is implicated in

various human diseases including atherosclerosis and neurodegenerative diseases [6]. However, there is little evidence on the relationship between the symptoms (growth retardation, metabolic disorders, megaloblastic anemia, and neurologic disorders [7]) of B₁₂ deficiency and oxidative stress (or disturbances in the normal redox state).

In humans, insulin appears to regulate learning and memory in the central nervous system as well as energy metabolism in the peripheral tissues [8]. Insulin is synthesized in the brain [9], and its receptors are localized at synapses [10]. The insulin/phosphoinositide 3-kinase pathway in neural and behavioral plasticity is highly conserved in mammals [11], and it regulates various neurological processes. Inhibition of this signaling pathway in the central nervous system causes learning and memory impairment [12].

The nervous system of *Caenorhabditis elegans* comprises 302 neurons, and their neural connections have been extensively studied [13]. Worms sense various chemicals by sensory neurons and exhibit

Abbreviations: Cat, catalase; DNPH, 2,4-dinitrophenylhydrazine; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; Hcy, homocysteine; HPLC, high-performance liquid chromatography; iGluR, ionotropic glutamate receptor; KPB, potassium phosphate buffer; MCM, methylmalonyl-CoA mutase; MDA, malondialdehyde; MMA, methylmalonic acid; MS, cobalamin-dependent methionine synthase; NADPH, nicotinamide adenine dinucleotide phosphate; NGM, nematode growth medium; NMDA, *N*-methyl-*L*-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; SAH, *S*-adenosylhomocysteine; SOD, superoxide dismutase

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several forms of plasticity. Worms that were subjected to prolonged exposure to attractive salt (NaCl) under starvation conditions display a dramatic reduction in chemotaxis to NaCl and eventually a negative chemotaxis. Exposure to NaCl in the presence of food does not reduce of chemotaxis, suggesting that worms change their behavior by integrating two stimuli, NaCl and starvation [14]. Tomioka et al. [11] demonstrated that the insulin/phosphoinositide 3-kinase pathway regulates this NaCl chemotaxis-linked learning and memory in worms.

We demonstrated that *C. elegans* is a suitable model for studying the physiological functions of B₁₂ and the disordered biological processes caused by B₁₂ deficiency [15]. In this study, we used this model animal to clarify the levels of oxidative stress and damage induced during B₁₂ deficiency. Furthermore, we demonstrated that B₁₂ deficiency resulted in severe memory impairment, which was partially attributable to oxidative stress.

2. Methods

2.1. Organisms and preparation of B₁₂-deficient *C. elegans*

The N2 Bristol wild-type *C. elegans* strain was maintained at 20 °C on nematode growth medium (NGM) plates using the *Escherichia coli* OP50 strain as the food source [16]. To induce dietary B₁₂ deficiency, worms were grown on 1.7% (w/v) agar plates containing M9 medium (3 g/L KH₂PO₄, 6 g/L Na₂HPO₄, 0.5 g/L NaCl, 1 g/L NH₄Cl, 1 mmol/L MgSO₄, 50 μmol/L CaCl₂, 2 g/L glucose, 4 mg/L thiamine hydrochloride, and 5 mg/L cholesterol) in 1 L of water. Each plate containing M9-medium supplemented with B₁₂ (100 μg/L CN-B₁₂) received one egg obtained from worms grown on NGM plates with B₁₂-deficient *E. coli* OP50 [15]. Eggs were allowed to hatch and develop into reproductively active adults. Subsequently, adults were then removed from each plate, eggs were collected and then each egg was transferred to a new control plate. After repeating this procedure at least five times, the worms were used as controls. B₁₂-deficient worms were prepared as described previously [15].

2.2. Preparation of a cell homogenate of worms

Control and B₁₂-deficient worms (0.05 g wet weight) were homogenized in 500 μL of 100 mmol/L potassium phosphate buffer (KPB) (pH 7.0) on ice using a hand homogenizer (AS ONE Corp., Osaka, Japan). The homogenates were centrifuged at 15,000×g for 10 min at 4 °C and these supernatants were used as crude enzymes or crude homogenates.

2.3. Enzyme activity assays

NADPH oxidase activity was determined using the luminescence assay method with lucigenin (Tokyo Chemical Industry Co., Ltd, Tokyo, Japan) as the electron acceptor [17]. The reaction was started by the addition of the crude enzyme and luminescence was monitored using a Luminescencer-Octa AB-2270 (ATTO Corp., Tokyo, Japan).

Total nitric oxide synthase (NOS) activity was assayed using an ultrasensitive colorimetric NOS assay kit (Oxford Biomedical Research, Ink., MI, USA). NOS activity was calculated based on the amount of nitrite that was converted from NO formed in the enzymatic reaction.

Total superoxide dismutase (SOD) was assayed using the SOD assay kit-WST (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Mn-SOD activity was determined in the reaction mixture containing 1 mmol/L KCN, which inhibits both Cu/Zn-SOD and extracellular SOD, resulting in the detection of only Mn-SOD activity [18,19].

Catalase (Cat) activity was determined using an NWLSS™ catalase activity assay kit (Northwest Life Science Specialties, WA, USA). Cat activity was calculated on the basis of the amount of H₂O₂ remaining in the enzymatic reaction mixture, which was monitored by measuring the absorbance at 240 nm using a spectrophotometer (UV-2550, Shimadzu

Corp., Kyoto, Japan).

Glutathione peroxidase (GPx) activity was assayed using a GPx assay kit (Northwest Life Science Specialties). The crude enzyme was diluted 10 fold using 100 mmol/L KPB (pH 7.0) before use. After preincubation of the reaction mixture for 1 min, changes in the absorbance at 340 nm were monitored for 5 min using a Shimadzu spectrophotometer (UV-2550).

NOS, SOD, Cat, and GPx activities were assayed according to the respective manufacturer's instructions.

2.4. Assays of oxidative stress markers

H₂O₂ concentrations were determined using a H₂O₂ assay kit (BioVision, Inc., CA, USA). OxiRed probe reacts with H₂O₂ to produce a product with color (570 nm) in the presence of horseradish peroxidase. The reaction product was determined by measuring the absorbance at 570 nm using a Sunrise Rainbow RC-R microplate reader (Tecan Austria GmbH, Salzburg, Austria).

NO was assayed using an NO (NO₂/NO₃) assay kit (NO₂/NO₃ colorimetric assay kit-C II, Dojindo Laboratories, Kumamoto, Japan). The crude homogenate described previously was heat-treated to remove proteins before use. NO content was calculated based on the amount of NO₂/NO₃ by measuring the absorbance at 540 nm using a microplate reader (Tecan).

Malondialdehyde (MDA) was determined using a TBARS assay kit (ZeptoMetrix Corp., NY, USA). MDA-thiobarbituric acid adducts formed from the reaction of MDA in the sample were determined by measuring the absorbance at 540 nm using a microplate reader (Tecan).

The carbonyl proteins of control and B₁₂-deficient worms were determined using a modified 2,4-dinitrophenylhydrazine (DNPH) method [20]. Carbonyl content was determined by measuring the absorbance at 380 nm and calculated using a molar absorption coefficient of 21,000 (mol/L)⁻¹ cm⁻¹.

All oxidative stress markers were assayed according to the respective manufacturer's instructions or the cited reference.

2.5. Fluorescent staining of H₂O₂ in the living worms

To detect cellular H₂O₂ levels in control and B₁₂-deficient worms, the fluorescent probe BES-H₂O₂-Ac (Wako Pure Chemical Industry, Ltd., Tokyo, Japan) was used. BES-H₂O₂-Ac was dissolved in a small amount of dimethyl sulfoxide, diluted to a final concentration of 200 μmol/L using sterilized M9 medium, and used as a staining solution. Control and B₁₂-deficient worms (approximately 12 worms each) were treated with 150 μL of the staining solution for 1 h under aseptic conditions. To remove the remaining staining solution on the worm body surface, each worm was transferred to a B₁₂-deficient medium without *E. coli* and incubated at 20 °C for 1 h. The washed worms were treated with 20 μL of 1 mmol/L sodium azide solution on a slide glass and observed using an SZX-RFL-2 fluorescence microscope (Olympus, Tokyo, Japan) (λ_{ex}: 485 nm, λ_{em}: 530 nm).

2.6. Assays of antioxidants

Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were determined using a GSH/GSSG assay kit (OXIS International Inc., OR, USA). 5,5'-Dithiobis (2-nitrobenzoic acid) was monitored by measuring at absorbance at 412 nm using a Shimadzu UV-2550 spectrophotometer. GSH and GSSG content were assayed according to the manufacturer's instruction.

L-Ascorbic acid was assayed according to the DNPH method [21]. In brief, ascorbic acid was oxidized to dehydroascorbic acid, derivatized to its osazone, and then assayed using a Shimadzu high-performance liquid chromatography (HPLC) system (SPD-6AV UV-Vis spectrophotometric detector, LP-6A liquid delivery pump, and CTO-6V

Table 1
Primer pairs used for the quantitative polymerase chain reaction (qPCR) analysis.

Gene name	Primer sequences (5' to 3')
<i>sod-1</i>	TCTTCTCACTCAGGTCTCCAAC and TCGGACTTCTGTGTGATCCA
<i>gpx-1</i>	TCGCTTCACAGTGTGGACTT and CGATTTCACACGATGGTTCTCTG
<i>ctl-1</i>	ATTATGCTCGTGGTGGAAACCC and ACAATGTTTGGCGCCCTCAA
<i>gst-10</i>	CGAGGATTCGAGAATACATCC and CTTCAAGCATGGCAACTGAC
<i>act-1</i>	TCCAAGAGAGGTATCCTTACCC and CTCCATATCATCCAGTTGGTG

The primer pairs for qPCR were designed using GENETYX software. A full complement of the homologs of mammalian antioxidant enzymes, including superoxide dismutase (*sod-1*), glutathione peroxidase (*gpx-1*), catalase (*ctl-1*), and glutathione *S*-transferase (*gst-10*), has been identified in the genome of *C. elegans*. For normalization, β -actin (*act-1*) served as the internal standard.

column oven) with a CDS ver. 5 chromato-data processing system (LAsoft, Ltd., Chiba, Japan). Each sample (20 μ L) was chromatographed on a normal phase HPLC column (Senshu pak, ϕ 6.0 \times 150 mm²) and eluted with acetic acid/hexane/ethyl acetate (1:4:5, v/v/v) at 40 $^{\circ}$ C. The flow rate was 1.5 mL/min. The osazone derivatives were monitored by measuring the absorbance at 495 nm.

2.7. Quantitative polymerase chain reaction (qPCR) analysis

Total RNA was prepared from worms using Sephasol[®]-RNA1 (Nacalai Tesque). Poly(A)⁺ mRNA prepared from total RNA using the Poly (A)⁺ Isolation kit from Total RNA (Nippon Gene, Tokyo, Japan) was used to synthesize cDNA using PrimeScript[™] II 1st Strand cDNA Synthesis kit (Takara Bio, Otsu, Japan). Primer pairs used for qPCR analysis were designed using GENETYX software (GENETYX Corp., Tokyo, Japan) to yield 20–22-nucleotide sequences with amplification products of approximately 100 bp (Table 1). A CFX Connect[™] Real-Time System (Bio-Rad) with SYBR Premix Ex Taq (Takara Bio) was used to perform qPCR. The mRNA level β -actin was used as an internal standard. The qPCR experiments were repeated at least three times for each cDNA prepared from three preparations of worms.

2.8. Assay of learning and memory retention in worms

Worms were usually grown in M9 medium containing 0.5 g/L NaCl in the presence or absence of B₁₂ with B₁₂-deficient *E. coli* OP50 as described previously. Changes in learning and memory retention in worms during B₁₂ deficiency were determined using the salt chemotaxis assay method [14,22]. Control and B₁₂-deficient worms were washed using a conditioning buffer (5 mmol/L KPB, pH 6.0, containing 1 mmol/L MgSO₄ and 1 mmol/L CaCl₂) in the presence (+NaCl and -diet, NaCl condition for determining the associative learning effects of NaCl and starvation) or absence (-NaCl and -diet, mock condition for determining NaCl chemotaxis) of 0.06% NaCl and then incubated in the respective buffers at 25 $^{\circ}$ C for 1 h. The treated control and B₁₂-deficient worms were immediately placed into the center (center of B the oval area) of the assay agar plates (9 cm), in which linear NaCl gradients were made from A to C as shown in Fig. 1. Worms located in areas A, B, and C were counted every 10 min during the experimental period (2 h). The chemotaxis index was calculated as (A–B)/(Total–C), where A was the number of worms within 2 cm of the peak spot of NaCl gradient, B was the number of animals that did not move in the center region, and C was the number of worms within 2 cm of the control spot.

Chemotaxis index values (-0.41 ± 0.07) that were determined in the control worms for the first 10 min were defined as normal learning ability. The time (66 ± 7 min) until the chemotaxis index values became 0 was calculated in the control worms and defined as the normal memory retention time.

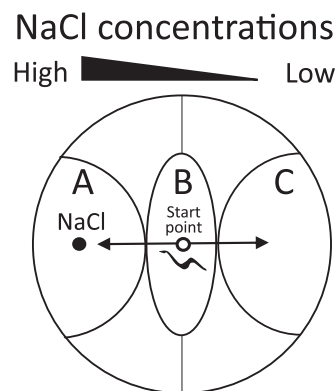


Fig. 1. Learning and memory retention assay. Effects of B₁₂ deficiency on learning and memory retention in worms were determined using the salt chemotaxis assay method. Linear NaCl gradients in agar were made from A to C. After treatment with or without NaCl in the absence of *Escherichia coli* OP50 as a food source, the treated control and B₁₂-deficient worms were immediately placed into the center (center of the B oval area) of the assay agar plates. Worms located in the areas A, B, and C were counted every 10 min throughout the experimental period (2 h).

2.9. Effects of antioxidants on learning and memory retention in worms during B₁₂ deficiency

Each antioxidant (GSH, *L*-ascorbic acid 2-glucoside, or α -tocopherol) was added to the B₁₂-deficient medium at a final concentration of 1 mmol/L and used as the antioxidant-supplemented B₁₂-deficient medium. When worms were grown under B₁₂-deficient conditions for five generations to simulate B₁₂ deficiency as described previously [15], the antioxidant-supplemented B₁₂-deficient medium was used at the indicated times as shown in Fig. 2. The B₁₂-deficient worms treated with each antioxidant during various periods were used for the learning and memory retention assay.

2.10. Protein assay

Proteins were assayed by the Bradford method [23] using ovalbumin as a standard.

2.11. Statistical analysis

The effects of B₁₂ deficiency on various indices of oxidative stress markers in *C. elegans* were evaluated by one-way ANOVA, and a post-hoc analysis was performed using Tukey's multiple comparison tests. Bonferroni-Dunn tests were used as a post-hoc analysis to evaluate the effects of B₁₂ deficiency on gene expression of antioxidant enzymes and the effects of antioxidants on MDA contents during B₁₂ deficiency. Analyses were performed with GraphPad Prism 3 for Windows version 2.01 (GraphPad Software Inc., La Jolla, CA, USA). All data are presented as the mean \pm SEM. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effects of B₁₂ deficiency on ROS, reactive nitrogen species, and other biomarkers of oxidative stress in *C. elegans*

To investigate whether B₁₂ deficiency disrupts redox homeostasis to induce oxidative stress, we determined the levels of various oxidative stress markers in B₁₂-deficient worms (Table 2). H₂O₂ and NO (NO₂⁻+NO₃⁻) contents increased by approximately 2-fold in the B₁₂-deficient worms compared to those in the control worms because the specific activities of NADPH oxidase and NOS in the cell homogenate of control worms were significantly increased during B₁₂ deficiency. Levels of the products of oxidative damage, such as MDA (as a product

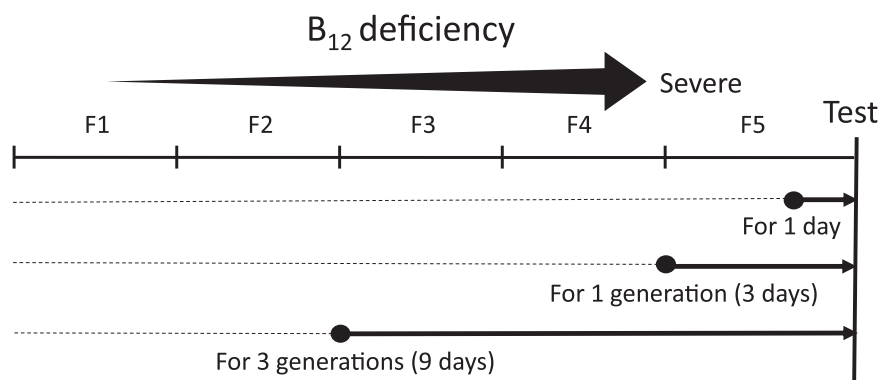


Fig. 2. Scheme of antioxidant feeding experiments during B_{12} deficiency. When worms were grown under B_{12} -deficient conditions for five generations to induce B_{12} deficiency, various antioxidant-supplemented B_{12} -deficient media were used at the indicated times (●). GSH, *L*-ascorbic acid 2-glucoside, or α -tocopherol was used as the antioxidant and added to the B_{12} -deficient medium at a final concentration of 1 mmol/L to create the antioxidant-supplemented B_{12} -deficient medium. The B_{12} -deficient worms treated with each antioxidant for various periods [1, 3 (one generation), or 9 days (three generations)] were used for the learning and memory retention assay.

Table 2
Effects of B_{12} deficiency on the markers of oxidative stress in *Caenorhabditis elegans*.

	Control	B_{12} deficiency	Recovery
H_2O_2 (nmol/g wet weight)	14.46 ± 0.67^a	34.17 ± 1.48^b	15.85 ± 0.96^a
$NO_2^- + NO_3^-$ (nmol/g wet weight)	33.74 ± 2.76^a	59.88 ± 5.33^b	39.74 ± 6.31^a
NADPH oxidase (RLU/min/mg protein)	0.70 ± 0.13^a	2.15 ± 0.35^b	0.74 ± 0.05^a
NOS (nmol/hour/mg protein)	2.67 ± 0.69^a	6.41 ± 0.36^b	2.41 ± 0.51^a
MDA (nmol/g wet weight)	23.09 ± 3.83^a	38.89 ± 1.33^b	27.26 ± 3.05^a
Carbonyl proteins (nmol/mg protein)	1.86 ± 0.57^a	3.94 ± 0.52^b	2.48 ± 0.23^a

Control and B_{12} -deficient worms were grown on plates containing B_{12} -supplemented (control) and B_{12} -deficient medium, respectively. B_{12} -deficient worms were transferred to B_{12} -supplemented medium for 3 generations and used as the recovery worms. Various biomarkers of oxidative stress were measured in the cell homogenates of each worm. Data represent the mean \pm SEM of three independent experiments. The different letters (a and b) indicate values that are significantly different at $p < 0.05$.

of lipid peroxidation) and carbonyl proteins (as products of protein modification) were approximately 2-fold greater in the B_{12} -deficient worms than in the control worms. The increased levels of H_2O_2 , NO, MDA, and carbonyl proteins completely recovered to the control levels when B_{12} -deficient worms were grown for three generations under B_{12} -supplemented conditions.

When the control and B_{12} -deficient worms were treated with BES- H_2O_2 -Ac as a fluorescent probe for detecting H_2O_2 (Fig. 3), the intestinal tract and its neighboring tissues were stained significantly in the B_{12} -deficient worms, but not in the control worms. The fluorescent intensity in the B_{12} -deficient worms disappeared upon the

addition of B_{12} (Fig. 3). These results indicated that B_{12} deficiency in worms induced severe oxidative stress leading to oxidative damage of various cellular components.

3.2. Effects of B_{12} deficiency on antioxidant contents and antioxidant enzyme activities in *C. elegans*

Table 3 summarizes the antioxidant contents and antioxidant enzyme activities in the control and B_{12} -deficient worms. Control worms possessed approximately 17.5 nmol of GSH per mg of worm proteins and no GSSG was detected. However, the GSH level was significantly reduced in the B_{12} -deficient worms, which displayed a small amount of GSSG (approximately 0.6 nmol/mg protein). The *L*-ascorbic acid level in the B_{12} -deficient worms was decreased to approximately 49% of the control value. The decreased antioxidant levels of B_{12} -deficient worms recovered to the control levels when these worms were grown for three generations under B_{12} -supplemented conditions.

Total SOD, Mn-SOD, and Cat activities in the B_{12} -deficient worms were decreased to approximately 55%, 60%, and 66% of the control levels, respectively. Contrarily, GPx activity was increased by approximately 2-fold relative to the control value. The significantly changed antioxidant enzyme activities completely recovered when B_{12} -deficient worms were grown under B_{12} -supplemented conditions. These results indicated that both low-molecular-weight antioxidants and enzymatic defense systems for oxidative stress were significantly weakened during B_{12} deficiency.

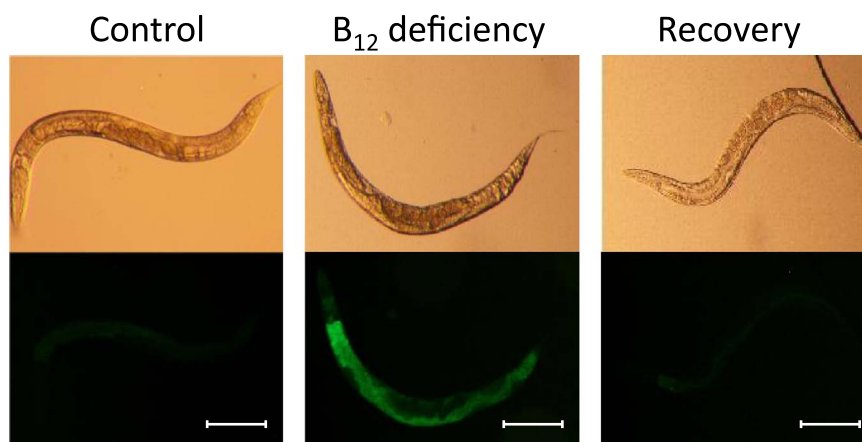


Fig. 3. Accumulation of H_2O_2 in B_{12} -deficient *Caenorhabditis elegans*. Fluorescent images of B_{12} -sufficient worms, B_{12} -deficient worms, and B_{12} -deficient worms grown in B_{12} -sufficient medium for three generations (recovery worms) are shown in Control, B_{12} deficiency, and Recovery, respectively. Scale bars = 250 μ m.

Table 3
Effects of B₁₂ deficiency on antioxidant compounds and antioxidant enzyme activities in *Caenorhabditis elegans*.

	Control	B ₁₂ deficiency	Recovery
GSH (nmol/mg protein)	17.48 ± 1.05 ^a	12.91 ± 0.15 ^b	17.32 ± 1.89 ^a
GSSG (nmol/mg protein)	N.D.	0.61 ± 0.15	N.D.
L-Ascorbic acid (μg/g wet weight)	11.86 ± 1.23 ^a	5.81 ± 0.53 ^b	9.95 ± 0.46 ^a
Total SOD (U/mg protein)	51.56 ± 1.50 ^a	28.46 ± 3.54 ^b	47.61 ± 4.06 ^a
Mn-SOD (U/mg protein)	12.43 ± 1.54 ^a	7.44 ± 0.24 ^b	10.98 ± 1.04 ^a
Cat (U/mg protein)	9.66 ± 0.24 ^a	6.37 ± 0.57 ^b	8.17 ± 0.89 ^{ab}
GPx (U/mg protein)	2.25 ± 0.14 ^a	4.11 ± 0.32 ^b	2.40 ± 0.35 ^a

Control and B₁₂-deficient worms were grown on plates containing B₁₂-supplemented (control) and B₁₂-deficient medium, respectively. B₁₂-deficient worms were transferred to B₁₂-supplemented medium for 3 generations and used as the recovery worms. Various antioxidant compounds and antioxidant enzyme activities were measured in the cell homogenates of each worm. Data represent the mean ± SEM of three independent experiments. The different letters (a and b) indicate values that are significantly different at $p < 0.05$. N.D. means not detected.

3.3. Effects of B₁₂ deficiency on the levels of mRNAs encoding various proteins involved in cellular antioxidant systems

To evaluate the effects of B₁₂ deficiency on the levels of mRNAs encoding proteins involved in cellular antioxidant systems, the levels of mRNAs encoding SOD (*sod-1*), GPx (*gpx-1*), Cat (*ctl-1*), and GST (*gst-10*) were measured by qPCR (Fig. 4). *gst-10* mRNA expression was significantly decreased in the B₁₂-deficient worms from that in the control worms, but no significant changes in the mRNA levels of the remaining proteins were observed during B₁₂ deficiency. These results suggest that B₁₂ deficiency did not affect the mRNA levels of most proteins involved in cellular antioxidant systems other than GST, which catalyzes the conjugation of GSH to xenobiotic substrates including peroxidized lipids [24]. These results suggested that the significantly decreased activities of SOD and Cat during B₁₂ deficiency were attributable to the oxidative inactivation of enzymes.

3.4. Effects of B₁₂ deficiency on learning and memory retention in *C. elegans*

Although cognitive impairment caused by B₁₂ deficiency has been reported in elderly persons [27], the underlying mechanisms are poorly understood. To clarify effects of B₁₂ deficiency on learning and memory functions involving the insulin/phosphoinositide 3 kinase signaling pathway in the central nervous system, the *C. elegans* salt (NaCl) chemotaxis associative learning assay system was used. B₁₂ deficiency did not affect NaCl chemotaxis and learning ability in the worms (Fig. 5). However, the memory retention time (63 ± 6 min) of control worms was significantly decreased to approximately 58% (37 ± 4 min) during B₁₂ deficiency (Fig. 5). When B₁₂-deficient worms were grown under B₁₂-supplemented conditions for three generations, the shortened memory retention time of B₁₂-deficient worms completely recovered to control levels. These results indicated that B₁₂ deficiency did not affect learning ability but led to severe impairment of memory retention in *C. elegans*.

To investigate the effects of antioxidants on the memory retention impairment of worms during B₁₂ deficiency, worms were treated with GSH, L-ascorbic acid 2-glucoside, and α-tocopherol (1 mmol/L, each) for 1, 3 (one generation), or 9 days (three generations) under B₁₂-deficient conditions (Fig. 6). No antioxidants affected NaCl chemotaxis and learning ability in B₁₂-deficient worms (data not shown). In B₁₂-deficient worms treated with a water-soluble antioxidant (GSH or L-ascorbic acid 2-glucoside) for 1, 3, or 9 days, the memory retention times were 44 ± 8 min and 41 ± 5 min, respectively; 46 ± 4 min and 43 ± 4 min, respectively; and 48 ± 5 min and 51 ± 6 min, respectively. The addition of the fat-soluble antioxidant α-tocopherol similarly ameliorated the impaired memory retention ability (39 ± 3 min for 1 d, 48 ±

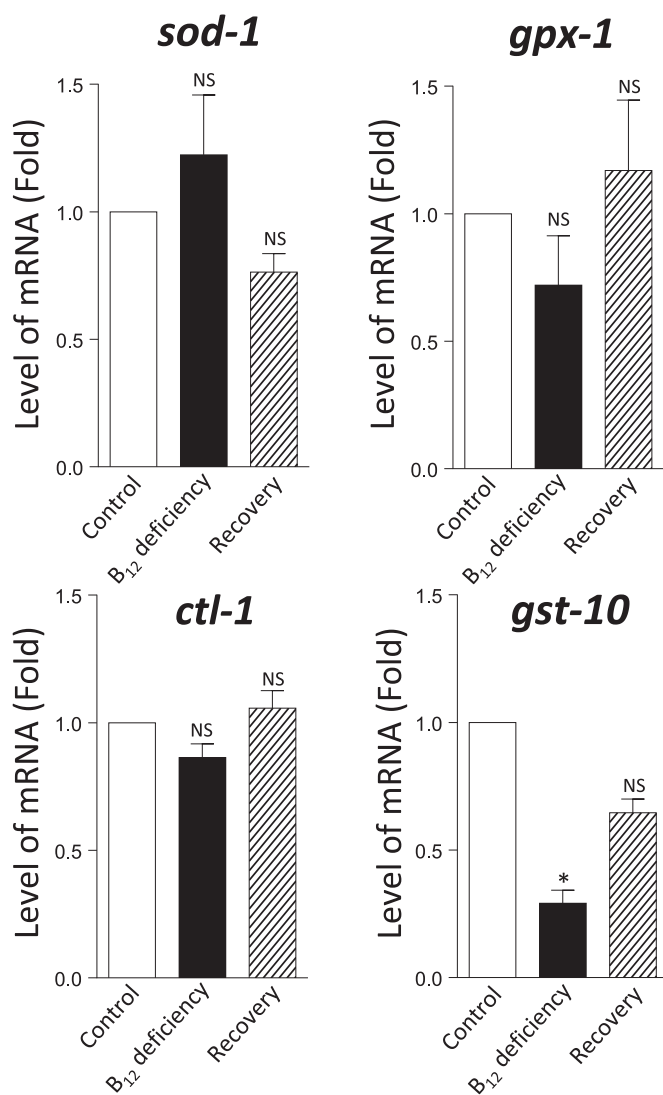


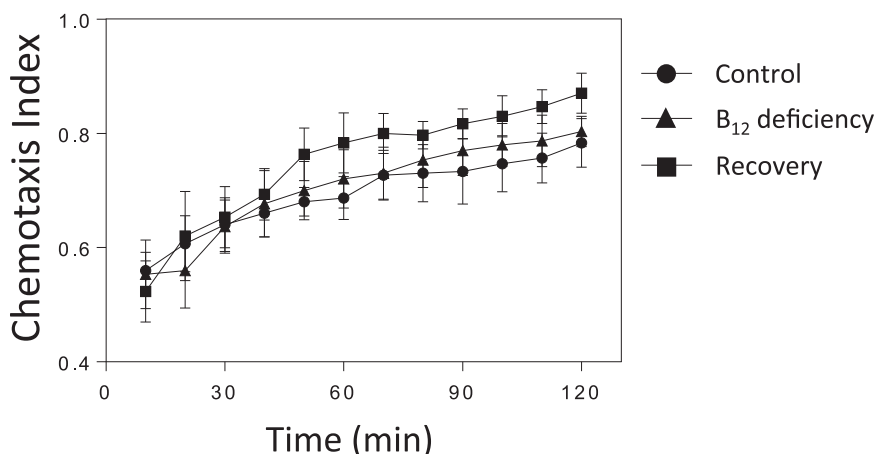
Fig. 4. Levels of mRNAs encoding enzymes involved in oxidant defense systems in control and B₁₂-deficient worms. Using qPCR, we determined the levels of mRNAs encoding superoxide dismutase (*sod-1*), glutathione peroxidase (*gpx-1*), catalase (*ctl-1*), and glutathione S-transferase (*gst-10*). Control, control worms (white bar); B₁₂ deficiency, B₁₂-deficient worms (black bar); and Recovery, worms recovered from B₁₂ deficiency (shaded bar). Data represent the mean ± SEM of three independent experiments. * $P < 0.05$ versus Control group. NS represents no significant differences.

4 min for 3 days, and 46 ± 7 min for 9 days). These results indicated that the memory retention time (37 ± 4 min) in the worms during B₁₂ deficiency was partially recovered to 46 ± 7 min, 48 ± 5 min, and 51 ± 6 min (approximately 50% recovery) by treatment with α-tocopherol, L-ascorbic acid 2-glucoside, and GSH, respectively, for three generations. As shown in Fig. 7, B₁₂ deficiency significantly increased the cellular MDA level. In the B₁₂-deficient worms treated with GSH, L-ascorbic acid 2-glucoside, and α-tocopherol for three generations, the MDA levels were identical to control levels. These results suggest that approximately 50% of the impairment of memory retention induced by B₁₂ deficiency in *C. elegans* was attributable to oxidative stress.

4. Discussion

Using B₁₂-deficient worms, we demonstrated for the first time that B₁₂ deficiency leads to severe oxidative stress, which significantly impaired memory retention. Hcy was significantly accumulated in B₁₂-deficient worms as well as B₁₂-deficient mammals [15]. Hcy is well known to be readily self-oxidized to produce ROS [4]. Moreover,

NaCl chemotaxis



Learning and memory retention abilities

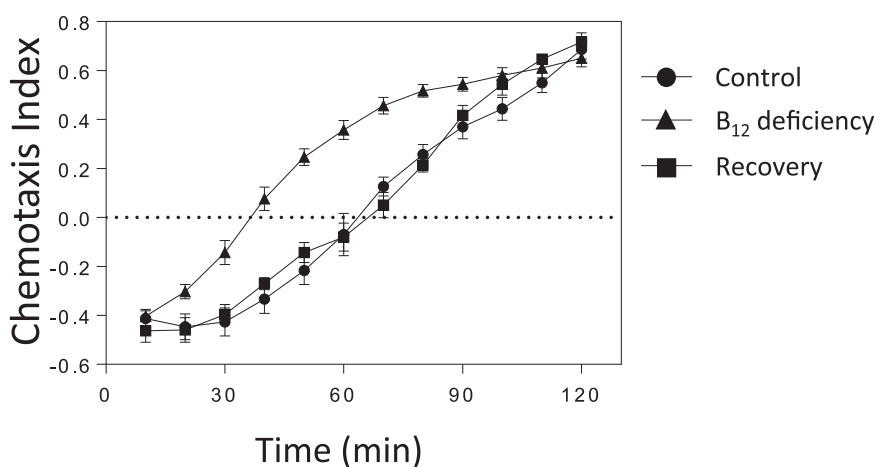


Fig. 5. Effects of B₁₂ deficiency on learning and memory retention in *Caenorhabditis elegans*. Learning and memory retention in worms during B₁₂ deficiency were examined using the salt chemotaxis assay method. Control and B₁₂-deficient worms were treated with a conditioning buffer in the presence (+NaCl and -diet, NaCl condition for assessing learning ability associated with NaCl and starvation) or absence (-NaCl and -diet, mock condition for determining NaCl chemotaxis) of NaCl. Worms located in the areas A, B, and C of Fig. 1 were counted every 10 min throughout the experimental period (2 h). The chemotaxis index was calculated as (A-B)/(Total-C). Control, Control worms (●); B₁₂ deficiency, B₁₂-deficient worms (▲); and Recovery, worms recovered from B₁₂ deficiency (■). Data represent the mean ± SEM of three independent experiments.

Hcy can activate NADPH oxidase to generate ROS [5]. Yaw et al. [5] reported that Hcy stimulates the phosphorylation of NADPH oxidase subunits by protein kinase C β in monocytes to enhance oxidase activity. NADPH oxidase is also stimulated by SAH accumulated during B₁₂ deficiency [6]. Our unpublished data indicate that B₁₂ deficiency causes the accumulation of SAH in *C. elegans* as well as mammals. These results suggested that Hcy or SAH (or both) accumulated during B₁₂ deficiency and resulted in severe oxidative stress. Particularly, the accumulation of H₂O₂ in the intestinal muscle of B₁₂-deficient worms was remarkable (Fig. 3).

NO produced from *L*-arginine by NOS is an important signaling molecule in multicellular organisms. In the present study, B₁₂ deficiency significantly increased both cellular NO levels and NOS activity in *C. elegans* (Table 2). However, using bioinformatics, a full complement of homologs of NOS has not been identified in the genome of *C. elegans*. Recently Gusarov et al. [28] demonstrated that NO produced by bacteria (as a food source) in the worm gut can diffuse into its tissues and initiate a signaling cascade. When we assayed NOS activity in the cell homogenates of *E. coli* OP50 grown under B₁₂-sufficient and B₁₂-deficient conditions, B₁₂ deficiency did not affect the NOS activity of the bacterium as a food source (121.4 ± 31.7 and 145.6 ± 26.7 nmol/

h/mg protein, respectively). These results suggest that B₁₂-deficient worms may eat larger amounts of *E. coli* OP50 than their controls. As NO reportedly inhibited the activities of MCM and MS [29,30], excessive levels of NO produced in response to B₁₂ deficiency would lead to further reductions in the activities of both B₁₂-dependent enzymes.

Antioxidant enzyme (SOD and Cat) activities were significantly reduced in B₁₂-deficient worms (Table 3). However, no significant changes in the mRNA levels of these enzymes were observed during B₁₂ deficiency (Fig. 3). Many studies [31–33] have demonstrated that SOD is potentially inhibited by H₂O₂, which accumulated during B₁₂ deficiency. Cat and SOD are susceptible to oxidative inactivation [34].

As shown in Table 3, GSH levels were significantly reduced in B₁₂-deficient worms, whereas a slight increase in GSSG levels was found. GSH is the most abundant low-molecular-weight antioxidant and GSH/GSSG is the major redox index in living cells. GSH mainly plays important roles in oxidant defense systems, as the compound both effectively scavenges ROS and serves as the substrate for GPx, which catalyzes the detoxification of H₂O₂ and other peroxides. B₁₂ deficiency significantly increased GPx activity, but not its mRNA expression in *C. elegans*. We do not have any evidence to explain this activation

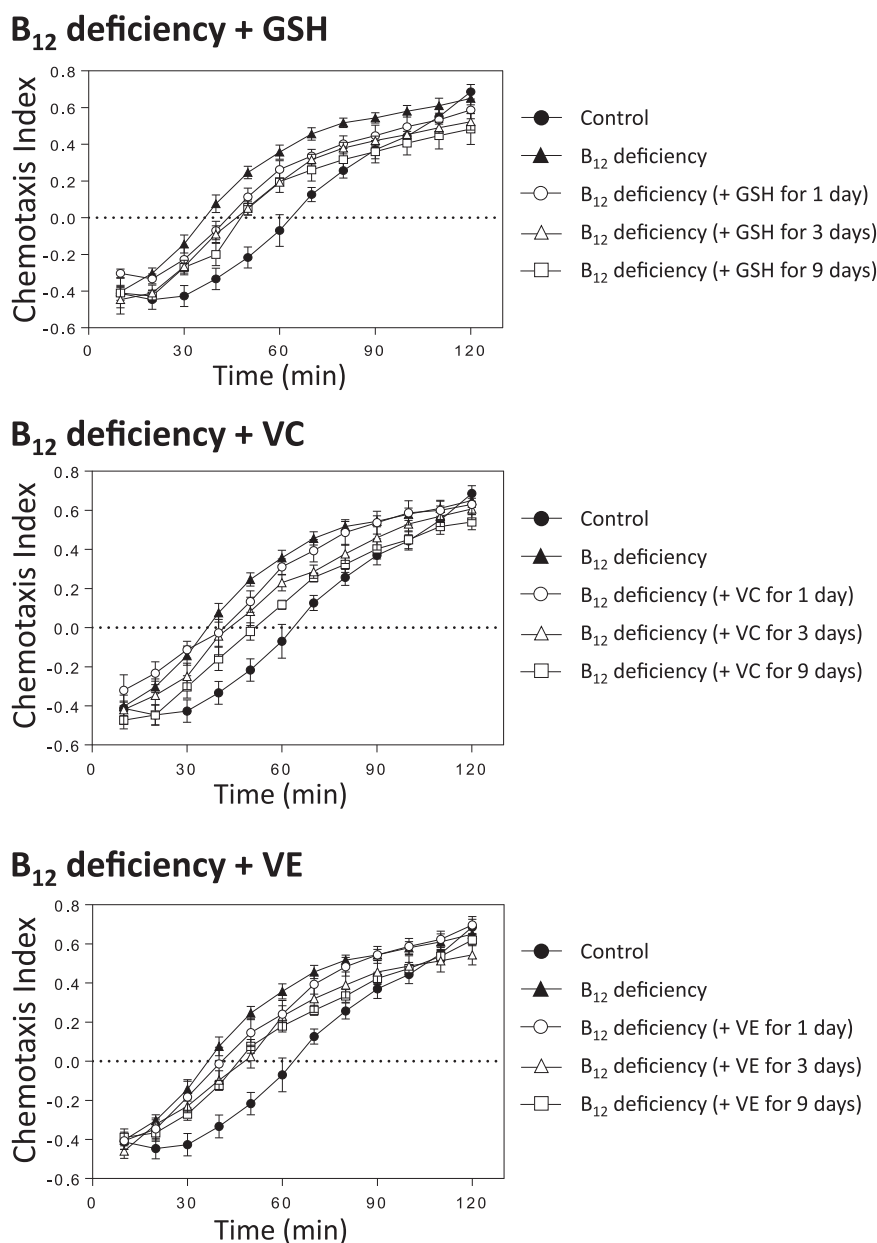


Fig. 6. Effects of various antioxidants on memory retention impairment caused by B₁₂ deficiency in *Caenorhabditis elegans*. Each antioxidant [GSH, L-ascorbic acid 2-glucoside (VC), or α -tocopherol (VE)] was added to B₁₂-deficient medium at a final concentration of 1 mmol/L to create the antioxidant-supplemented B₁₂-deficient medium. After worms were grown under B₁₂-deficient conditions for five generations to induce B₁₂ deficiency, the B₁₂-deficient worms were treated with each antioxidant for various periods [1 d (○), 3 days or one generation (△), or 9 days or three generations (□)] and used for the learning and memory retention assay. Data for control worms (●) and B₁₂-deficient worms (▲) as described in Fig. 5 were adopted. Data represent the mean \pm SEM of three independent experiments.

mechanism of GPx. Conversely, the mRNA level of GST, which catalyzes the conjugation of GSH to detoxify lipid peroxides and various metabolites, was significantly decreased during B₁₂ deficiency. Lipid peroxides induce further oxidative modification of cellular components [35]. These observations previously described and the results of this study indicate that B₁₂ deficiency disrupts cellular redox homeostasis to induce severe oxidative stress.

Surprisingly, B₁₂-deficient worms displayed significantly decreased cellular L-ascorbic acid levels (Table 3). Recently, Alexander et al. [36] reported that *C. elegans* can synthesize L-ascorbic acid *de novo*. L-ascorbic acid serves as a potent antioxidant and participates in collagen biosynthesis in most mammals [37], but the function of L-ascorbic acid in *C. elegans* is not understood. We reported that some B₁₂-deficient worms exhibit a specific morphological abnormality [15] similar to a “dumpy” mutant phenotype that is induced by disordered cuticle collagen biosynthesis [25,26]. In *C. elegans*, collagen mainly functions

as a component of the cuticle [38], and it is involved in other cellular events [39,40]. To investigate the effects of B₁₂ deficiency on collagen proteins in *C. elegans*, collagen protein levels were assayed in B₁₂-deficient worms. Collagen protein content in B₁₂-deficient worms was decreased to approximately 57% of the control level (unpublished data). However, the mRNA expression of the prolyl 4-hydroxylase complex responsible for collagen biosynthesis was not changed during B₁₂ deficiency (unpublished data). These results suggested that the biosynthesis of worm collagen proteins was disordered by the reduction of cellular L-ascorbic acid levels during B₁₂ deficiency. Siwik et al. [41] reported that oxidative stress can activate metalloproteinases involved in the degradation of collagen in rat cardiac fibroblasts, suggesting that oxidative stress induced in B₁₂-deficient worms stimulated the degradation of collagen proteins.

Lipid peroxidation and protein oxidation are detected in the tissues of patients with Alzheimer disease [32,33], which is classified as a

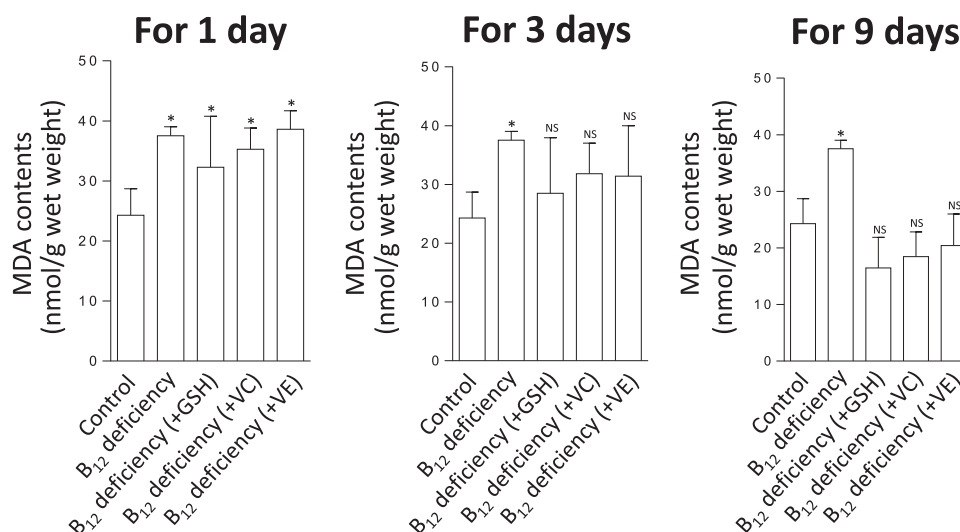


Fig. 7. Effects of various antioxidant treatments on MDA formed during B₁₂ deficiency in *Caenorhabditis elegans*. The B₁₂-deficient worms treated with each antioxidant [GSH, L-ascorbic acid 2-glucoside (VC), or α -tocopherol (VE)] for 9 days (three generations) were used for the MDA assay. The experimental procedures were identical to those described in Fig. 6. Control, control worms; B₁₂ deficiency, B₁₂-deficient worms; B₁₂ deficiency (+GSH), B₁₂-deficient worms treated with GSH; B₁₂ deficiency (+VC), B₁₂-deficient worms treated with VC; and B₁₂ deficiency (+VE), B₁₂-deficient worms treated with VE. Data represent the mean \pm SEM of three independent experiments. * $P < 0.05$ versus Control group. NS represents no significant differences.

neurodegenerative disorder associated with various symptoms, such as memory loss and cognitive impairment. The associative learning assay method revealed that B₁₂ deficiency in *C. elegans* resulted in disordered memory retention (Fig. 6). INS-1, an insulin-like peptide found in *C. elegans*, is secreted from AIA sensory neurons and then transferred to ASER sensory neurons, which function in associative learning. Tomioka et al. [11] demonstrated that certain oxidative modifications of peptides including INS-1 and lipids by ROS result in defective associative learning in *C. elegans*. Thus, B₁₂ deficiency would inhibit the INS-1 signaling pathway essential for associative learning.

Although lipid peroxidation (MDA) levels in B₁₂-deficient worms were completely recovered to the control level by treatment with various antioxidants for three generations (Fig. 7), the memory retention impairment found in B₁₂-deficient worms recovered only to approximately 50% of the control level (Fig. 6); additionally, identical results were observed even in worms treated for five generations (not shown data). These results suggest that memory retention impairment induced by B₁₂ deficiency is partially (approximately 50%) attributable to oxidative stress, whereas the remaining factor is unclear at present.

The neurotransmitter glutamate signals via ionotropic glutamate receptors (iGluRs), which are linked to learning and memory formation [42,43]. Kano et al. [44] demonstrated that two genes, *nmr-1* and *nmr-2*, are predicted to encode the subunits of an N-methyl-D-aspartate (NMDA)-type iGluR that is necessary for memory retention in *C. elegans*. *nmr-1*- and *nmr-2*-defective mutants exhibit degeneration of memory retention [44]. Hcy and SAH reportedly activate NMDA receptors involved in signal transmission between nerve cells [45,46] and then induce an excitotoxic neurodegenerative process [47]. These observations and the findings of this study indicate that Hcy and SAH accumulation due to the reduction of MS activity during B₁₂ deficiency is closely related with such neuropathy.

The results presented in this study suggest that the daily intake of antioxidants would considerably prevent the development of symptoms of neuropathy during B₁₂ deficiency and protect against the development of severe megaloblastic anemia and various metabolic disorders associated with this deficiency. High intakes of folic acid reportedly mask the megaloblastic anemia of B₁₂ deficiency [48]. Vegetarians have a higher risk of B₁₂ deficiency than non-vegetarians [49]. Vegetarian diets are usually rich in carotenoids, folic acid, vitamin C, and vitamin E but are significantly deficient in B₁₂ because B₁₂ is found only in animal-derived foods [50]. These observations suggest that vegetarians

represent a high-risk group for B₁₂ deficiency.

Author contributions

T.B. and T.M. performed most experiments. T.K. supervised the experimental techniques for *C. elegans* and discussed the results. T.I. contributed to the design of the oxidative stress experiments and discussed the results. T.B., Y.Y., and F.W. designed the experiments, interpreted the results, and wrote the manuscript. All authors commented on the manuscript and approved the final version.

Notes

The authors declare no competing financial interests.

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