Alleviating Oxidative Damage–Induced Telomere Attrition: a Potential Mechanism for Inhibition by Folic Acid of Apoptosis in Neural Stem Cells

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Abstract
DNA oxidative damage can cause telomere attrition or dysfunction that triggers cell senescence and apoptosis. The hypothesis of this study is that folic acid decreases apoptosis in neural stem cells (NSCs) by preventing oxidative stress–induced telomere attrition. Primary cultures of NSCs were incubated for 9 days with various concentrations of folic acid (0–40 µM) and then incubated for 24 h with a combination of folic acid and an oxidant (100-µM hydrogen peroxide, H2O2), antioxidant (10-mM N-acetyl-L-cysteine, NAC), or vehicle. Intracellular folate concentration, apoptosis rate, cell proliferative capacity, telomere length, telomeric DNA oxidative damage, telomerase activity, intracellular reactive oxygen species (ROS) levels, cellular oxidative damage, and intracellular antioxidant enzyme activities were determined. The results showed that folic acid deficiency in NSCs decreased intracellular folate concentration, cell proliferation, telomere length, and telomerase activity but increased apoptosis, telomeric DNA oxidative damage, and intracellular ROS levels. In contrast, folic acid supplementation dose-dependently increased intracellular folate concentration, cell proliferative capacity, telomere length, and telomerase activity but decreased apoptosis, telomeric DNA oxidative damage, and intracellular ROS levels. Exposure to H2O2 aggravated telomere attrition and oxidative damage, whereas NAC alleviated the latter. High doses of folic acid prevented telomere attrition and telomeric DNA oxidative damage by H2O2. In conclusion, inhibition of telomeric DNA oxidative damage and telomere attrition in NSCs may be potential mechanisms of inhibiting NSC apoptosis by folic acid.

Keywords Folic acid · Apoptosis · Telomere attrition · Oxidative damage · Neural stem cell · In vitro

Abbreviations
CAT Catalase
DAPI 4′,6-Diamidino-2-phenylindole
FPG Formamidopyrimidine DNA-glycosylase
GSH-PX Glutathione peroxidase
GSSG Oxidized glutathione
H2O2 Hydrogen peroxide
LDH Lactate dehydrogenase
LPO Lipid peroxide
MDA Malondialdehyde
MTS 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
8-OxoG 8-Oxoguanine
PBS Phosphate-buffered saline
NAC N-acetyl-L-cysteine
NSC Neural stem cell
ROS Reactive oxygen species
SAMP8 Senescence-accelerated mouse prone 8
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**Materials and Methods**

**Cell Culture**

The Tianjin Medical University Animal Ethics Committee approved all experimental protocols in this study. The hippocampus and striatum were dissected from neonatal (postnatal less than 24 h) Sprague–Dawley (SD) rats (Charles River Laboratories, Beijing, China), and NSCs were prepared as described previously [12]. Hippocampus and striatum tissues were cut into 1-mm³ pieces and digested with 0.25% trypsin at 37 °C for 15 min. This step was followed by agitation, centrifugation, and resuspension of the cells in serum-free Dulbecco’s Modified Eagle’s Medium (DMEM) and nutrient mixture F-12 Ham (F12) (1:1) (Corning, NY, USA), supplemented with 2% B27 supplement (Gibco, USA), 20-ng/mL epidermal growth factor (EGF; PeproTech, USA), 20-ng/mL basic fibroblast growth factor (bFGF; PeproTech), 100-U/mL penicillin and phytoymycin (Slarbio), and 2-mmol/L L-glutamine (Sigma, USA). The resulting cell suspension was plated at 1 × 10⁶ cells/mL in T25 culture flasks (Corning, NY, USA) and then cultured in 95% air and 5% CO₂ at 37 °C, and the culture medium was changed every 2 or 3 days. After purification (7 days), NSCs were incubated for 9 days with various concentrations of folic acid (0–40 µM). Then the cells were exposed for 24 h to medium containing a combination of folic acid and either antioxidant (100-µM hydrogen peroxide, H₂O₂) or antioxidant (10-µM N-acetyl-L-cysteine, NAC) or vehicle. The dosage ranges of H₂O₂ and NAC were selected according to data from the literature [18, 19] and the results of our preliminary experiments. After subjected to the different study conditions for 10 days, cells were harvested to detect intracellular folate concentration, apoptosis, cell proliferation and differentiation, ROS and oxidative damage, antioxidant activities, and telomere attrition.

**Cell Identification**

By the end of 7 days in culture, NSC neurospheres were gently mechanically dissociated and plated on laminin (100 µg/mL) coated coverslips, incubated with 1-mM 5-bromo-2-deoxyuridine (BrdU, Sigma, USA) in proliferative medium for 24 h, and then fixed with 4% paraformaldehyde for 20 min at room temperature. The cells were washed with phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 for 15 min at room temperature, blocked with 10% goat serum for 1 h at 37 °C, and incubated with primary antibodies (mouse anti-BrdU antibody [1:50, Sigma, USA]; rabbit anti-SOX2 antibody

**Introduction**

Oxidative damage contributes to neuronal cell death and therefore to the important public health problems of aberrant aging and neurodegenerative disease [1, 2]. Neural stem cells (NSCs) are the main cell types that produce the central nervous system, which are essential for the study of neural development, neurodegeneration, and nervous system diseases [3]. NSC homeostasis is influenced by oxidative damage; when the balance between proliferation and differentiation are destroyed, adult neurogenesis is disturbed. NSC oxidative damage remains in a tight connection with the occurrence of central nervous system diseases [2]. However, the mechanism of oxidative damage that causes neural cell apoptosis remains to be clarified.

Telomeres are repeating hexameric DNA sequences that protect chromosomes from degradation and end-to-end fusion. Epidemiological studies have revealed that loss of telomere integrity is an important factor in the decay of physiological function associated with aging and several chronic illnesses [4, 5]. Damage to telomeric sequences in stem cells can be repaired by a specialized reverse transcriptase called telomerase [6]. Telomere state depends not only on telomere length but also on the repair that counters telomere loss by adding de novo repeats to the 3′ ends [7]. Oxidative stress has been observed to accelerate telomere attrition in primary astrocytes [8] and to be associated with apoptosis in endothelial cells [9]. Nevertheless, the role of oxidative stress, telomere attrition, and telomerase activity in neurodegeneration is still unclear.

Telomere attrition is associated with aging and genetic and environmental factors such as nutrients [10, 11]. Folate (vitamin B₉) is an essential nutrient that acts as a coenzyme to transfer the one-carbon units that are necessary for deoxythymidylate synthesis, purine synthesis, and many methylation reactions [12]. Folic acid has been observed to stimulate the proliferation and neuronal differentiation of NSCs, as well as to decrease their apoptosis [13, 14]. Folic acid deficiency has been found to increase reactive oxygen species (ROS) levels and DNA damage in the context of cancer [15, 16], while folic acid supplementation inhibits oxidative stress–induced damage, telomere attrition, and apoptosis both in primary astrocytes and a murine model of neurodegenerative disease [8, 17]. However, the relationship between folate, telomerase activity, and telomere attrition in NSCs is still unknown. The hypothesis of this study is that folic acid decreases apoptosis in NSCs by preventing oxidative stress–induced telomere attrition.

| SD       | Sprague–Dawley                        |
|----------|---------------------------------------|
| SOD      | Superoxide dismutase                  |
| T-AOC    | Total antioxidant capacity            |

SD: Sprague–Dawley, SOD: Superoxide dismutase, T-AOC: Total antioxidant capacity
[1:200, Abcam, UK]) overnight at 4 °C. After another washing with PBS, the coverslips were incubated with secondary antibodies (tetramethyl rhodamine isothiocyanate [TRITC]–conjugated anti-mouse antibody, 1:100; fluorescein isothiocyanate [FITC]–conjugated anti-rabbit antibody, 1:100, Jackson, USA) for 1 h at room temperature and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) contained Vectashield (H1200, Vector). The potential capacity of cultured NSC was assessed by immunocytochemistry. NSC neurospheres were gently mechanically dissociated, and the resulting cells were plated on laminin (100 µg/mL) coated coverslips at a density of 10^4 cells/mL in DMEM/F12 medium supplemented with 5% fetal bovine serum (FBS; Gibco), 2% N2 (Gibco), and 100-U/mL penicillin and phytomycin (Gibco), but without B27, EGF, or bFGF [12]. After 6 days of differentiation, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After washing with PBS, the cells were blocked with 10% goat serum for 1 h at 37 °C and incubated with primary antibodies (mouse anti-β-III-tubulin antibody [1:1000, Abcam]; rabbit anti-glial fibrillary acidic protein (GFAP) antibody [1:1000, Abcam]) overnight at 4 °C. After another washing with PBS, the coverslips were incubated with secondary antibodies (tetramethyl rhodamine isothiocyanate [TRITC]–conjugated goat anti-mouse antibody, 1:100; fluorescein isothiocyanate–conjugated goat anti-rabbit antibody, 1:100, Jackson, USA) for 1 h at room temperature and counterstained with DAPI contained Vectashield (H1200, Vector). Immunofluorescence signals were captured using Olympus IX81 microscope (Olympus) and analyzed by Image-Pro Plus 6.0 software.

**Intracellular Folate Assay**

An aliquot of 10^7 cells was centrifuged, suspended in phosphate-buffered saline (PBS), sonicated twice for 10 s each time, and then centrifuged at 5000 × g for 5 min, and the resulting supernatant was collected to detect folate content. Folate was measured using a competitive protein-binding assay (IMMULITE® 2000 Folic Acid kit) with an automated chemiluminescence immunoassay analyzer (IMMULITE 2000 XPi, Siemens Healthcare Diagnostics Inc., Malvern, PA, USA) according to the manufacturer’s specifications. This assay was able to detect folic acid, dihydrofolate, and tetrahydrofolate from 1 to 24 ng/mL. Folate levels in cells were normalized by protein content, as determined by the BCA Protein Assay Kit (Boster, Wuhan, China).

**Cell Apoptosis Assay**

Cell apoptosis was measured by the DeadEnd™ Fluorometric TUNEL System (G2350, Promega, America). This terminal deoxynucleotidyl transferase (TdT)–mediated deoxyuridine monophosphate (dUTP) nick end labeling (TUNEL) assay detects nuclear DNA fragmentation, which is a hallmark of apoptosis. Briefly, NSCs on coverslips were fixed in 4% paraformaldehyde for 25 min, washed twice with PBS, permeabilized with 0.2% Triton X-100 for 5 min, and then incubated in the dark with Equilibration Buffer for 10 min at room temperature. rTdT incubation buffer was added for 60 min at 37 °C. Then terminate the reactions by 2× SSC for 15 min at room temperature. Finally, the cells were incubated with DAPI, washed twice with PBS, and mounted with a fluorescent mounting medium. We detected localized green fluorescence of apoptotic cells (fluorescein-12-dUTP) in a blue background (DAPI) by a fluorescence microscope (Olympus, Tokyo, Japan). Positive cells were counted using Image-Pro Plus 6.0 software.

**Cell Proliferation Assay**

NSC proliferation was measured by BrdU incorporation [20]. Briefly, NSCs on coverslips were incubated with 10-µM BrdU (Sigma, USA) in proliferative medium for 24 h and then fixed in 4% paraformaldehyde for 20 min at room temperature. The cells were washed twice with PBS, permeabilized with 0.1% Triton X-100 for 15 min at room temperature, blocked with 10% goat serum for 1 h at 37 °C, and incubated with primary mouse anti-BrdU antibody (1:100, Sigma) overnight at 4 °C. After another washing with PBS, the coverslips were incubated with secondary TRITC-conjugated goat anti-mouse antibody (1:100, ProteinTech) for 1 h at room temperature and counterstained with DAPI contained Vectashield (H1200, Vector). Immunofluorescence signals were captured using Olympus IX81 microscope (Olympus) and analyzed by Image-Pro Plus 6.0 software.

**Cell Viability Assay**

Cell viability was measured with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA) that uses 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). Cells were seeded into a 96-well plate at a density of 1 × 10^4 cells per well (200 µL/well). Then 20 µL of MTS (5 mg/mL) was added to each well, and the mixture was incubated at 37 °C for an additional 3 h in a humidified, 5% CO2 atmosphere. The absorbance at 490 nm was recorded using a microplate reader (ELX800uv™, BioTek Instruments Inc.).

**Measurement of Telomere Length by Southern Blot**

The telomere restriction fragment (TRF) analysis was performed using a commercial kit (TeloTAGGG Telomere
Length Assay and DNA Molecular Weight Marker II, digoxigenin-labeled, Roche Life Science, Mannheim, Germany), based on the instruction. Briefly, 150-ng genomic DNA sample was digested with HinI and Rsa I for 2 h at 37 °C. Digested DNA was then electrophoresed on 1% agarose pulsed field gels at 6 V/cm for 11 h. The blotting membrane was washed, blocked, incubated with anti-DIG-alkaline phosphatase (1:4000 dilution, Roche, Mannheim, Germany) for 4 h, washed, and exposed with CDP-star (Roche, Mannheim, Germany) for 15 min. After exposure of the blot to an X-ray film, an estimate of the mean TRF length can be obtained by comparing the mean size of the smear to the molecular weight marker with TeloTool.

**Measurement of Telomere Length by qPCR**

Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) according to the manufacturer’s instructions and was quantified with a NanoDrop 2000 instrument (Thermo Scientific, USA). qPCR assay was used to determine telomere length as previously used [21]. We used a PCR mixture of 25 µL including 12.5 µL of SYBR Green qPCR Supermix (2x), 1 µL of DNA, 1 µL of 36B4 forward and reverse primers (10 µM) or 1 µL of telomere forward and reverse primers (10 µM), and H₂O (PCR-grade). The PCR processes were stage 1, 5 min at 95 °C; stage 2, two cycles of 15 s at 90 °C and 15 s at 49 °C; and stage 3, 40 cycles of 15 s at 90 °C, 10 s at 62 °C, and 15 s at 74 °C with signal acquisition. PCR was performed using the reference control gene (36B4 single-copy gene) and the telomeric gene. Primers were specific for telomeric gene (forward, 5'-GGTTTTTGGTGGTTGGTTGGTTGGT-3'; reverse, 5'-TCCCCGA CTATCCCTATCCCTATCCCTATCCCTATCCCTA-3') and 36B4 (forward, 5'-CAGCAATGGAAGGGTGAAGGGTGAAGGGT-3'; reverse, 5'-CCCTCTTATCATCAACGGGTACCA-3') [22]. All qPCR reactions were performed in a LightCycler 480 II instrument (Roche Applied Science, Switzerland).

**Immunofluorescence-Fluorescent In Situ Hybridization (IF-FISH)**

Telomeric DNA oxidative damage was measured using IF-FISH, as described previously [23]. NSCs were fixed with 4% paraformaldehyde for 25 min and exposed to 0.5% Triton X-100 for 20 min; Cy3-labeled telomere PNA probe (PNA Bio) hybridization solution was added to the slides. After denaturation (85 °C, 5 min), the slides were left to hybridize overnight at RT in darkness. Next the slides were washed with washing liquor (70% formamide; 1 M Tris–HCl, pH = 7.2) and thrice with Tris-buffered saline with Tween. Blocking was done with 10% goat serum for 1 h, followed by incubation with anti-phosphohistone H2AX (γ-H2AX) (Ser139) (1:500, Millipore, MA, USA) for 10 h at 4 °C and secondary FITC goat anti-mouse antibody (1:500, Spark-Jade) for 1 h. Images of cells were acquired using an inverted fluorescence microscope (Olympus). To determine whether 8-hydroxy-2’-deoxyguanosine (8-OhdG) incorporation caused telomere dysfunction, γ-H2AX was used to mark telomere dysfunction–induced foci (TIFs). The cells were considered TIF-positive if they contained more than two IF foci of both C-rich telomere probe-FITC (TelC-FITC) and the DNA damage response protein γ-H2AX [24].

**Measurement of 8-Oxoguanine (8-OxoG) in Telomeric DNA by qPCR**

DNA was isolated and quantified as described above. Equivalent amounts of DNA (approximately 20 ng/µL) were incubated with formamidopyrimidine DNA-glycosylase (FPG) (NEB, USA) or nuclease-free water for 16 h, followed by a telomere-specific PCR assay [25]. FPG cleaved oxidized guanine-containing DNA to create a fragmentary template for the subsequent PCR, and Ct values increased. The reaction mixtures composed of 12.5 µL of SYBR Green qPCR Supermix (2x), 1 µL of DNA, 1 µL of telomere forward and reverse primers (10 µM), and PCR-grade water were incubated at 95 °C for 10 min, followed by 40 amplification cycles (denaturation, 95 °C for 15 s; annealing and extension, 60 °C for 1 min), and telomere-specific primers (forward, 5'-GGTTTTTGGTGGTTGGTTGGTTGG-3'; reverse, 5'-TCCCCGA CTATCCCTATCCCTATCCCTA-3').

**Measurement of Telomerase Activity**

Telomerase is a ribonucleic acid-protein complex composed of a single long non-coding RNA, called telomerase RNA, and associated proteins. We used a telomerase activity quantification qPCR assay kit (ScienCell, Carlsbad, CA, USA #8928) to measure the products of telomerase activity that are amplified by qPCR. The cell lysis buffer enables the release of telomerase in the native state, and the telomere primer set (TPS) recognizes and amplifies newly synthesized telomere sequences in the assay. Cell proteins were extracted by cell lysis buffer supplemented with PMSF 0.1 M in isopropanol and β-mercaptoethanol. After, cell protein extraction telomerase reaction was performed as described in the product protocol. First, the telomerase reactions (including 0.5 µL cell lysate sample, 4 µL 5 × g telomerase reaction buffer and 15.5 µL nuclease-free H₂O) were incubated at 37 °C for 3 h. The reaction was stopped by heating at 85 °C for 10 min, and the reaction tubes were centrifuged at 1500×g for 10 s. Finally, qPCR was performed to analyze the telomere production by telomerase. The qPCR process
was 10 min at 95 °C, followed by 40 cycles (denaturation, 95 °C for 20 s; annealing, 95 °C for 20 s and extension, 72 °C for 45 s).

**Intracellular ROS Assay**

Intracellular ROS levels were determined by the chemiluminescence method with 2′,7′-dichlorofluorescin diacetate (DCFH-DA; ROS Assay Kit, Nanjing Jiancheng Bioengineering Institute). Briefly, NSCs were incubated with 10-µM DCFH-DA for 30 min in a dark, humidified 5% CO2 atmosphere. The cells were washed twice with PBS to remove extracellular fluorescence probes, then centrifuged, and resuspended in PBS. Fluorescence intensity was quantified by a fluorescence microplate reader (ELX800uv™; BioTek Instruments Inc.) with excitation wavelength at 500 nm and emission wavelength at 525 nm.

**Measurement of Oxidative Damage**

Lactate dehydrogenase (LDH) activity in culture medium, lipid peroxide (LPO), and malondialdehyde (MDA) in cells were assayed using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. The percentage of LDH release was calculated by the equation: LDH release (%) = (Experimental LDH release − spontaneous LDH release)/maximum LDH release. Cellular LPO and MDA concentrations were normalized to the cellular protein content, as determined by a BCA protein assay kit (BosterBio).

**Antioxidant Activity Assays**

The intracellular total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX), and GSH/oxidized glutathione (GSSG) levels were assayed with commercial kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer’s protocol. Cellular T-AOC, SOD, CAT, and GSH-PX activities were normalized to the cellular protein levels, determined using a BCA protein assay kit (BosterBio).

**Statistical Analysis**

Data are expressed as mean±SEM values based on three independent experiments. Two-way analysis of variance was used to evaluate differences between the treatment groups, and the Student–Newman–Keuls test was used for multiple comparisons to determine significant differences among the experimental groups. Correlations were assessed using Pearson’s method. The statistical software package SPSS 24.0 was used to evaluate the differences within groups, and p value less than 0.05 was considered statistically significant.

**Results**

**Cell Identification**

By the end of 7 days in culture, almost all the NSCs in neurospheres had the potential to proliferate as indicated by incorporation of Brdu (Supplementary Fig. 1a), and they also were SOX2-positive (Supplementary Fig. 1a). Then the neurospheres were mechanically dissociated, and the resulting cells were cultured in DMEM/F12 medium supplemented with 5% FBS and 2% N2, but without B27, EGF, or bFGF. After 6 days culture in this differentiation medium, most of the NSCs had differentiated into neurons or astrocytes, which were identified as β-III-tubulin–positive and GFAP-positive, respectively (Supplementary Fig. 1e-h). These results demonstrate that the cultured neurospheres were composed of NSCs with the capacity for self-renewal as well as for neuronal and astrocytic differentiation.

**Intracellular Folate Level**

After 10 days of intervention, folic acid increased the intracellular folate concentration in a dose-dependent manner (F = 233.601, p < 0.001) (Fig. 1). In contrast, NAC had no effect on intracellular folate. H2O2 also had no effect on the intracellular folate when medium contained high level of folic acid. However, when cells were in 0- or 10-µM folic
acid medium. H₂O₂ decreased the intracellular folate concentration ($F = 26.402, p = 0.036; F = 128.259, p = 0.008$).

These data indicate oxidative stress can lower the intracellular folate concentration unless the extracellular supply of the vitamin is high.

**Folic Acid Decreased Cell Apoptosis**

To investigate the cytoprotective effect of folic acid, cell apoptosis rate was assessed using TUNEL assay. Folic acid decreased the cell apoptosis in NSCs in a dose-dependent manner ($F = 71.728, p < 0.001$) (Fig. 2). Folic acid also dose-dependently decreased cell apoptosis when the medium contained H₂O₂ ($F = 423.244, p = 0.002$) or NAC ($F = 1142.353, p = 0.001$). High folic acid (40 µM) decreased cell apoptosis compared with the other three dosages when the medium contained H₂O₂. Meanwhile, 20- or 40-µM folic acid decreased apoptosis compared with 0- or 10-µM folic acid when the medium contained NAC. These data indicated folic acid deficiency increased cell apoptosis, whereas folic acid, especially high level of folic acid, could decrease apoptosis of NSCs.

**Folic Acid Increased Cell Proliferative Capacity**

Cell proliferation and cell viability were assessed using BrdU incorporation and MTS, respectively. Folic acid increased cell proliferation in NSCs in a dose-dependent manner ($F = 192.746, p < 0.001$) (Fig. 3a–e). Folic acid also dose-dependently increased cell proliferation when the medium contained H₂O₂ ($F = 26,530.421, p < 0.001$) or NAC ($F = 11,580.757, p < 0.001$).
Fig. 3 Cell proliferative capacity and cell viability. NSCs were incubated as described in Fig. 1. a–d Representative micrographs of the BrdU incorporation assay. Representative images of NSCs were stained with markers of BrdU (red), cell nuclei were stained with DAPI (blue), the scale bar is 50 µm. e Quantification of BrdU-positive cells/total number of DAPI-stained nuclei. f Cell viability detected by MTS assay. The plotted values represent the mean±SEM values of three independent experiments. Statistical analysis was performed using two-way ANOVA. *, p < 0.05 compared with 0-µM folic acid. #, p < 0.05 compared with 10-µM folic acid. &, p < 0.05 compared with 20-µM folic acid. $, p < 0.05 compared with the group without H2O2 or NAC at the same folic acid level.
Folic acid dose-dependently increased cell viability in the absence ($F = 59.457, p < 0.001$) and presence of exogenous H$_2$O$_2$ ($F = 961.137, p = 0.001$) (Fig. 3f). However, in the presence of NAC, only the highest dose of folic acid (40 µM) increased cell viability ($F = 4510.192, p < 0.001$). These results showed that folic acid deficiency decreased NSC proliferation and cell viability and folic acid supplementation could increase cell proliferative capacity.

**Folic Acid Inhibited Telomere Shortening**

Southern blot analysis of telomere length revealed that high folic acid (40 µM) inhibited telomere shortening compared with the other three folic acid dosages ($F = 5.285, p = 0.009$) (Fig. 4a). The representative image of Southern blot was shown in Supplementary Fig. 2. Furthermore, relative telomere length assayed by qPCR was shortened by folic acid deficiency and increased by high folic acid concentrations (20 and 40 µM) in a dose-dependent manner ($F = 33.822, p < 0.001$) (Fig. 4b). Similar effects of folic acid occurred when the medium contained H$_2$O$_2$ or NAC (Fig. 4b). The results of telomere length indicated folic acid especially high level of folic acid (40 µM) could inhibit telomere attrition of NSCs.

**Folic Acid Inhibited Telomeric DNA Oxidative Damage**

High folic acid (40 µM) decreased the ratio of TIF-positive cells compared with the other three folic acid dosages with or without H$_2$O$_2$ ($F = 26.683, p < 0.001$) (Fig. 5a–e). The incorporation ratio of 8-OxoG within telomeric DNA was increased by folic acid deficiency and decreased by folic acid in a dose-dependent manner ($F = 35.746, p < 0.001$) (Fig. 5f). These data showed folic acid, especially high level of folic acid (40 µM), could inhibit telomeric DNA oxidative damage of NSCs.

**Folic Acid Increased Telomerase Activity**

The expression of telomerase RNA was too low to detect (defined by the kit manufacturer’s instructions as a Cq value higher than 33) in 3 treatment groups, namely, 0-µM folic acid with or without H$_2$O$_2$ and 10-µM folic acid with H$_2$O$_2$ (Table 1). Folic acid (10–40 µM) increased the telomerase activity dose-dependently ($F = 35.746, p < 0.001$).

The oxidant H$_2$O$_2$ decreased the telomerase activity in the NSCs that received 0- and 10-µM folic acid (Table 1), whereas the antioxidant NAC increased the telomerase activity in folic acid–deficient NSCs (0-µM folic acid, Table 1).

**Folic Acid Decreased Oxidative Damage and Increased Antioxidant Enzyme Activities**

Folic acid decreased ROS levels in NSCs in a dose-dependent manner ($F = 170.325, p < 0.001$) (Fig. 6). Similar effects of folic acid occurred when the medium contained H$_2$O$_2$ or NAC (Fig. 6). H$_2$O$_2$ increased ROS levels across the range of folic acid doses ($F = 3147.793, p < 0.001$), but NAC decreased ROS levels when folic acid is lower than 10 µM only ($F = 2163.538, p < 0.001$) (Fig. 6a).

Folic acid deficiency increased medium LDH levels, intracellular LPO, and MDA levels, indicating that oxidative damage occurred in those NSCs (Fig. 6b–d). In contrast, folic acid supplementation dose-dependently decreased medium LDH ($F = 67.288, p < 0.001$), intracellular LPO ($F = 80.331, p < 0.001$), and MDA ($F = 61.352, p < 0.001$) (Fig. 6b–d). Exogenous H$_2$O$_2$ increased medium LDH, intracellular LPO, and MDA levels, whereas NAC decreased them (Fig. 6b–d).
The effect of folic acid on improving antioxidant capacity in NSCs was evident in the data showing that the vitamin increased the levels of SOD, CAT, GSH-PX, and the ratio of GSH/GSSG (all $p < 0.001$) (Fig. 6e–h). Furthermore, $\text{H}_2\text{O}_2$ decreased antioxidant capacity in NSCs. NAC increased SOD and CAT levels at 10- and 20-µM folic acid and increased GSH-PX level only at 20-µM folic acid (Fig. 6e–h).

**Discussion**

The results of the present study showed that folic acid deficiency in NSCs decreased intracellular folate concentration, cell proliferative capacity, telomere length, and telomerase activity but increased apoptosis, telomeric DNA oxidative damage, and intracellular ROS levels. In
contrast, folic acid supplementation dose-dependently increased intracellular folate concentration, cell proliferative capacity, telomere length, and telomerase activity but decreased apoptosis, telomeric DNA oxidative damage, and intracellular ROS levels. Exposure to H$_2$O$_2$ aggravated telomere attrition and oxidative damage, whereas NAC alleviated the latter. High doses of folic acid prevented telomere attrition and telomeric DNA oxidative damage by H$_2$O$_2$.

Neurodegenerative diseases are characterized by a chronic and selective process of neuronal cell death. Folic acid is an essential nutrient that is vitally important to neural cell survival [14]. Folic acid deficiency has obvious impact on neural cell proliferation and could induce cell apoptosis [26]. In vitro studies have found that folic acid supplementation decreases apoptosis in astrocytes [8] and stimulates cell proliferation in embryonic NSCs [27]. In vivo studies have discovered that folic acid supplementation delays age-related neurodegeneration in the cerebral cortex and hippocampal CA1 region of senescence-accelerated mouse prone 8 (SAMP8) mice [17], whereas folic acid deficiency worsens neural cell injury in the hippocampus following ischemia/reperfusion injury [28]. Furthermore, in a recent study of Chinese patients with Parkinson’s disease, relatively higher homocysteine and lower folate levels correlated with white matter hyperintensities [29]. The present study provided further insight by showing in primary NSCs that folic acid deficiency increased apoptosis and decreased cell proliferative capacity, whereas folic acid supplementation did the opposite.

Oxidative stress, resulting from an imbalance between ROS production and antioxidant defenses, contributes to aging and the pathogenesis of numerous diseases including neurodegenerative diseases [30–33]. SOD, CAT, and GSH-PX are antioxidant enzymes that contribute importantly to cells’ capacity for surviving oxidative stress [34, 35]. The severity of oxidative stress in cultured cells can be assessed by measuring intracellular ROS, LPO, and MDA, as well as by measuring the release of LDH from cells to the medium. In the present study, folic acid decreased medium LDH concentrations, lowered intracellular LPO and MDA levels, and increased the intracellular SOD, CAT, GSH-PX, and T-AOC activities of NSCs. Taken together, these results indicated that sufficient amounts of folic acid could enhance antioxidant capacity and protected cells from oxidative damage.

Telomeres are dynamic nucleoprotein-DNA structures that cap and protect linear chromosome ends [36]. Critically short telomeres trigger cellular senescence in mature cells, which contribute to aging-related degenerative diseases [37, 38]. While numerous genetic and environmental factors are associated with telomere attrition, oxidative stress has been identified as an underlying mechanism [39]. Telomeric DNA is particularly vulnerable to oxidative damage because of its high level of guanine in the 5′-TTAGGG-3′ repeat sequence [40, 41]. Guanine is the most susceptible of the natural bases which contribute to aging-related degenerative diseases [37, 38]. While numerous genetic and environmental factors are associated with telomere attrition, oxidative stress has been identified as an underlying mechanism [39]. Telomeric DNA is particularly vulnerable to oxidative damage because of its high level of guanine in the 5′-TTAGGG-3′ repeat sequence [40, 41]. Guanine is the most susceptible of the natural bases which contribute to aging-related degenerative diseases [37, 38]. While numerous genetic and environmental factors are associated with telomere attrition, oxidative stress has been identified as an underlying mechanism [39]. Telomeric DNA is particularly vulnerable to oxidative damage because of its high level of guanine in the 5′-TTAGGG-3′ repeat sequence [40, 41]. Guanine is the most susceptible of the natural bases which contribute to aging-related degenerative diseases [37, 38]. While numerous genetic and environmental factors are associated with telomere attrition, oxidative stress has been identified as an underlying mechanism [39]. Telomeric DNA is particularly vulnerable to oxidative damage because of its high level of guanine in the 5′-TTAGGG-3′ repeat sequence [40, 41]. Guanine is the most susceptible of the natural bases which contribute to aging-related degenerative diseases [37, 38]. While numerous genetic and environmental factors are associated with telomere attrition, oxidative stress has been identified as an underlying mechanism [39]. Telomeric DNA is particularly vulnerable to oxidative damage because of its high level of guanine in the 5′-TTAGGG-3′ repeat sequence [40, 41]. Guanine is the most susceptible of the natural bases which contribute to aging-related degenerative diseases [37, 38]. While numerous genetic and environmental factors are associated with telomere attrition, oxidative stress has been identified as an underlying mechanism [39]. Telomeric DNA is particularly vulnerable to oxidative damage because of its high level of guanine in the 5′-TTAGGG-3′ repeat sequence [40, 41]. Guanine is the most susceptible of the natural bases which contribute to aging-related degenerative diseases [37, 38]. While numerous genetic and environmental factors are associated with telomere attrition, oxidative stress has been identified as an underlying mechanism [39]. Telomeric DNA is particularly vulnerable to oxidative damage because of its high level of guanine in the 5′-TTAGGG-3′ repeat sequence [40, 41]. Guanine is the most susceptible of the natural bases which contribute to aging-related degenerative diseases [37, 38]. While numerous genetic and environmental factors are associated with telomere attrition, oxidative stress has been identified as an underlying mechanism [39]. Telomeric DNA is particularly vulnerable to oxidative damage because of its high level of guanine in the 5′-TTAGGG-3′ repeat sequence [40, 41]. Guanine is the most susceptible of the natural bases which contribute to aging-related degenerative diseases [37, 38]. While numerous genetic and environmental factors are associated with telomere attrition, oxidative stress has been identified as an underlying mechanism [39].

Table 1  Telomerase activity in NSCs

| FA (µM) | H$_2$O$_2$ (+/−) | NAC (+/−) | Cq (mean ± SEM) | ΔCq (mean ± SEM) | Activity hTERT (Mean ± SEM) |
|--------|-----------------|----------|-----------------|-----------------|---------------------|---------------------|
| 0      | −               | −        | >33             | −               | No activity*        |
| +      | −               | −        | >33             | −               | No activity*        |
| −      | +               | 31.74 ± 0.32 | 1.91 ± 0.28 | 0.29 ± 0.06 |                     |
| 10     | −               | −        | 29.83 ± 0.54  | 0.00            | 1.00 (Ref) a        |
| +      | −               | >33      | −               | −               | No activity*        |
| −      | +               | 29.43 ± 0.28 | −0.40 ± 0.28 | 1.75 ± 0.21 b   |
| 20     | −               | −        | 29.64 ± 0.45  | −0.19 ± 0.22   | 1.47 ± 0.23 a       |
| +      | −               | 29.89 ± 0.85 | 0.06 ± 0.40  | 1.32 ± 0.40    |
| −      | +               | 29.07 ± 0.52 | −0.76 ± 0.44  | 1.93 ± 0.50 b   |
| 40     | −               | −        | 29.16 ± 0.13  | −0.67 ± 0.61   | 2.04 ± 0.84 a       |
| +      | −               | 29.91 ± 0.67 | 0.08 ± 0.40  | 1.51 ± 0.92    |
| −      | +               | 28.91 ± 0.34 | −0.92 ± 0.24  | 2.26 ± 0.17 b   |

ΔCq is quantification cycle value obtained from qPCR assay

* In accordance with the kit manufacturer’s instructions, telomerase activity was recorded as no telomerase activity (i.e., activity too low to detect) if the Cq value was higher than 33

a, p < 0.05 compared with 0-µM folic acid. b, p < 0.05 compared with 10-µM folic acid

Folate is essential for nucleotide synthesis and maintaining redox status [44]. A recent study showed that folic acid...
deficiency increases intracellular ROS levels and aggravates telomere attrition in primary cultures of astrocytes [8]. There is evidence that a similar mechanism may occur in vivo since folic acid supplementation decreases ROS levels and alleviates both telomere attrition and telomeric DNA damage in SAMP8 mice [17]. The present study suggested that folic acid’s action of suppressing ROS levels, telomere attrition, and telomeric DNA oxidation, while increasing telomerase activity, might account for the vitamin’s protection against NSC apoptosis.

Our previous study found that folic acid deficiency worsened neural cell injury in the hippocampus following ischemia/reperfusion injury [28]. Folic acid supplementation stimulates NSCs proliferation [12, 27] and neuronal differentiation [13]. We also found folic acid inhibited apoptosis in astrocytes in vitro and endothelial cells in vivo and in vitro. The protective effects may be due to folic acid decreased oxidative stress [8, 9]. Moreover, folic acid supplementation delayed age-related neurodegeneration and cognitive decline in SAMP8 mice, and alleviating telomere attrition could serve as one influential factor in the process [17]. For the depth of the previous study, the effect of folic acid on apoptosis and telomeric attrition in primary NSCs were discussed in this study.

This study has a limitation on the small size of rat NSCs telomere length detected by Southern blot. Maybe this small size could be explained by aging-induced telomere attrition. In an animal study, in both sham-operated SD rats and sinoaortic denervated SD rats, the TRF length of the kidneys was shorter in older rats than that in younger rats [45]. We previously reported that telomere length in brain astrocytes of 10-month-old SAMP8 mice was shorter than that of 4-month-old SAMP8 mice, and telomere length of primary astrocyte from generation 5 was shorter than cells...
from generation 1 [46]. Furthermore, a previous study showed that there was an attrition of cell telomeres due to the culture. It was reported that telomere length was gradually decreased following cell passage of human umbilical vein endothelial cell [47]. Combined with our results, we suspect that small size of telomere length of NSCs may be due to attrition of telomeres by the cell culture.

Conclusion

In conclusion, inhibition of oxidative damage–induced telomere attrition in NSCs may be a potential mechanism of inhibiting NSCs apoptosis by folic acid. The results suggested that folic acid supplementation might be a therapeutic strategy to people with neurodegenerative disease by preventing NSC apoptosis.

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Data Availability All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval The Tianjin Medical University Animal Ethics Committee approved all experimental protocols in this study.

Consent to Participate Not applicable to this study.

Consent for Publication Not applicable to this study.

Conflict of Interest The authors declare no competing interests.

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