Mol. Nutr. Food Res. 2005, *49*, 443 – 451 DOI 10.1002/mnfr.200500019



# Dietary grape supplement ameliorates cerebral ischemia-induced neuronal death in gerbils

**Qun Wang[1], Agnes Simonyi[1], Wenlu Li[2], Bryan A. Sisk[1], Rebecca L. Miller[2], Ruth S. MacDonald[3], Dennis E. Lubahn[1], Grace Y. Sun[1] and Albert Y. Sun[2]**

[1]Department of Biochemistry, University of Missouri, Columbia, MO, USA
[2]Department of Medical Pharmacology and Physiology, University of Missouri, Columbia, MO, USA
[3]Department of Food Sciences, University of Missouri, Columbia, MO, USA

Oxidative damage has been implicated as one of the leading causes for neuronal cell death in a number of neurodegenerative diseases including stroke. Many vegetables and fruits are enriched in polyphenolic compounds known to exhibit antioxidant properties. This study is to investigate whether dietary supplement with grape powder (GP) may offer protection against neuronal damage due to global cerebral ischemia induced to Mongolian gerbils by occlusion of the common carotid arteries, a model known to cause delayed neuronal death (DND) in the hippocampal CA1 area. Gerbils were fed either a control diet (AIN76a) or a control diet supplemented with low (5.0 g/kg diet) or high (50 g/kg diet) levels of GP for two months. Four days after ischemia/reperfusion (I/R), the extent of DND, glial cell activation, nuclear DNA oxidation, and apoptotic terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) reaction in hippocampal CA1 region were assessed. Ischemia-induced extensive DND in the CA1 region was accompanied by oxidative and fragmented DNA damage and a marked increase in reactive astrocytes and microglial cells. Dietary GP supplementation significantly protected neurons against I/R-induced DND, DNA damage, and apoptosis as well as attenuated glial cell activation. These results demonstrate that due to the antioxidant properties of polyphenols in GP, nutritional diets supplemented with grape can protect the brain against ischemic damage. The neuroprotective effects of GP supplement may have wide implication in the future for prevention/protection against other neurodegenerative damage.

**Keywords:** DNA oxidation / Grape powder / Ischemia/reperfusion / Neuronal apoptosis / Reactive astrocytes / Reactive microglia

Received: January 1, 2005; revised: February 11, 2005; accepted: February 17, 2005

## 1 Introduction

Increased oxidative stress has been implicated as the leading cause for neuronal cell death in many neurodegenerative disorders including stroke [1]. Due to the high consumption of oxygen, high levels of polyunsaturated fatty acids in neural cell membranes and the relatively low levels of antioxidants, the brain is particularly vulnerable to oxidative insults. Increased production of reactive oxygen species (ROS) is known to cause oxidative damage to lipids, proteins, and DNA [2, 3]. In global cerebral ischemia/reperfusion (I/R), increased production of ROS has been regarded an important factor underlying delayed neuron death (DND), especially the pyramidal neurons in the hippocampal CA1 area [4 – 7].

Many vegetables and fruits are enriched in polyphenolic compounds known to exhibit antioxidant properties [8 – 10]. Epidemiology studies also indicate that moderate wine consumption is linked to a lower incidence of cardiovascular disease, the so-called "French paradox". This main benefit is attributed to the polyphenols in the red wine and grapes [11, 12]. In recent years, understanding the "French paradox" has stimulated new research interest to investigate whether polyphenolic antioxidants may offer protective effects beyond the cardiovascular system, in particular, the central nervous system [13, 14].

**Correspondence:** Dr. Albert Y. Sun, Department of Medical Pharmacology and Physiology, M526 Medical Sciences Bldg, University of Missouri-Columbia, Columbia, MO 65212, USA
**E-mail:** Suna@health.missouri.edu
**Fax:** +1-573-884-4558

**Abbreviations: ANOVA**, analysis of variance; **CCA**, common carotid arteries; **DAB**, 3,3V-diaminobenzidine tetrachloride; **DAPI**, 4′,6-diamidine-2′-phenylindole; **DND**, delayed neuronal death; **GFAP**, glial fibrillary acidic protein; **GP**, grape powder; **I/R**, ischemia/reperfusion; **8-OHdG**, 8-hydroxyl-deoxyguanosine; **ROS**, reactive oxygen species; **TUNEL**, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

Our previous studies have demonstrated the antioxidant effect of resveratrol against oxidative damage imposed on PC-12 cells [15–17]. We have also demonstrated that dietary supplementation of polyphenols extracted from grape skin and seeds can ameliorate oxidative insult to brain synaptic membranes and hepatic morphology due to chronic ethanol consumption [18, 19]. Using the same paradigm, COX-2 mRNA expression in the brain was increased after chronic ethanol administration and this change was ameliorated upon supplementing grape polyphenols to the ethanol diet [20].

The underlying role of oxidative stress-mediated neuronal damage after stroke has raised attention to testing possible beneficial effects of dietary antioxidants, especially those from plant source [21, 22]. Our earlier studies indicated the protective effects of resveratrol, a polyphenol enriched in grape skin and red wine, to protect against DND induced by global ischemia in gerbils [7]. Resveratrol also protected against neuronal damage induced by kainate excitotoxicity in rats [23, 24]. A study by Cui *et al*. [25] indicated that consumption of grape extract can significantly reduce injury due to myocardial ischemia**.** Therefore, a major goal for the present study is to examine whether dietary supplementation of grape powder (GP) can offer protection against neuronal damage due to cerebral ischemia.

## 2 Materials and methods

### 2.1 Grape diet preparation

The freeze-dried GP used for this study was obtained from the California Table Grape Commission (Fresno, CA, USA). The composition of the GP has been described previously [11]. Approximately 100 g of fresh grapes yielded 18.2 g of the GP with the following chemical composition based on 100 g of GP: 4 g protein, 90 g sugar, and 6 g fiber. Control and GP diets were prepared using AIN-76a powder as the basal diet. For the control and GP supplemented diets (5.0 and 50 g/kg), total caloric levels were adjusted with corn starch for each group (Table 1).

**Table 1.** Composition of the experimental diets (g/100 g)

| Ingredient | Control (g) | Low grape (g) | High grape (g) |
|---|---|---|---|
| Choline bitartrate | 0.25 | 0.25 | 0.25 |
| DL-Methionine | 0.3 | 0.3 | 0.3 |
| Vitamin mix (AIN76a) | 1 | 1 | 1 |
| Salt mix (AIN93) | 3.5 | 3.5 | 3.5 |
| Grape powder | 0 | 0.5 | 5 |
| Corn starch | 39.75 | 39.25 | 34.75 |
| Dyetrose | 13.2 | 13.2 | 13.2 |
| Sucrose | 10 | 10 | 10 |
| Alphacell | 5 | 5 | 5 |
| Casein | 20 | 20 | 20 |
| Safflower oil | 2 | 2 | 2 |
| Corn oil | 5 | 5 | 5 |
| Total | 100 | 100 | 100 |

Low-GP = 5.0 g/kg and high-GP = 50 g/kg. Substituted corn starch for extracts to maintain isocaloric: grape powder has 3.75 kcal/g.

### 2.2 Animals

Adult male Mongolian gerbils (60–80 g body wt) (Charles River, Wilmington, MA, USA) were housed in the Small Animal Facilities, Medical Sciences Building, University of Missouri, Columbia, MO. Gerbils (three per cage) were given free access to water and lab chow, and were maintained at 22 $\pm$ 2°C with a constant humidity and a 12 : 12 h light : dark cycle. Initially, a pilot experiment was carried out to assess the intake of the AIN-76a diet. Results indicated an average consumption of 15 g of AIN-76a diet for each gerbil and body weight gain for gerbils consuming the AIN-76a diet was not different from those given the regular lab chow diet (Purina). Gerbils were fed the AIN76a diet with or without supplementation of a low (5.0 g/kg diet) and a high (50 g/kg diet) level of GP for 2 months. Gerbils given the AIN-76a diet supplemented with GP did not differ in body weight gain as compared to those given the control diet (data not shown). After dietary treatment, gerbils were divided into six groups, namely, sham (fed with control diet, $n = 6$), sham + low GP ($n = 6$), sham + high GP ($n = 6$), ischemia (fed with control diet, $n = 10$), ischemia + low GP ($n = 11$) and ischemia + high GP ($n = 11$).

### 2.3 Induction of global forebrain ischemia

I/R was performed according to the procedure as described by Wang *et al*. [7]. Briefly, both common carotid arteries (CCAs) were clamped with aneurysm clips for 5 min and recirculation was achieved by removing the aneurysm clips. The sham-operated group underwent the same procedures without occlusion of CCAs. The surgical protocol for induction of ischemia has been approved by the University of Missouri-Columbia Animal Care and Use Committee (Protocol #1741). Experiments were carried out according to the guidelines set forth by the NIH Guide for the Care and Use of Laboratory Animals. After ischemia, gerbils were continued with the same diet until sacrifice at 4 days after ischemic treatment. During the ischemic treatment, the presence of communicating arteries in gerbils was detected by monitoring the decrease in regional cerebral blood flow before and after clamping the bilateral CCA using a laser doppler blood flow monitor (MBF3D; Moor Instruments, Axminster, Devon, UK). Gerbils showing a

 

decrease in cerebral blood flow of less than 80% after ischemia were excluded from subsequent analyses [7].

### 2.4 Preparation of brain samples

Four days after ischemia, gerbils were transcardially perfused with 30 mL heparinized saline and then 100 mL 4% paraformaldehyde in 0.05 mol/L PBS (pH 7.4). The brains were then post-fixed in the same fixative for 3 days. Brain tissues were embedded in paraffin for histochemical and immunocytochemical examinations. Six μm thick coronal sections were cut at the dorsal hippocampal area.

### 2.5 Histochemical and immunohistochemical staining for neurons, astrocytes, and microglial cells

Brain sections were stained with cresyl violet for detection neurons and immunohistochemical staining of glial fribillary acidic protein (GFAP), a marker for astrocytes according to protocol described by Wang *et al*. [7]. Microglial cells in brain sections were identified using peroxidase-labeled isolectin B4 (Sigma, St. Louis, MO, USA) according to the protocol described by Streit [26]**.** Briefly, deparaffinized sections were placed in PBS containing 0.1 mM each of $CaCl_2$, $MgCl_2$, $MnCl_2$, and 0.1% Triton X-100 for 30 min. The sections were then incubated overnight at 4°C with peroxidase-labeled isolectin $B_4$ that was diluted to 20 μg/mL in PBS containing the divalent cations and 0.1% Triton X-100. After washing with PBS, the peroxidase reaction was carried out by incubating the slides with freshly prepared 0.5 mg/mL 3,3V-diaminobenzidine tetrachloride (DAB) and 0.03% $H_2O_2$ for 5 min and then post-stained with 0.05% cresyl violet for 10 min. After dehydration in graded ethanol, sections were mounted with Permount and examined under Nikon Eclipse E600 microscope equipped with Nikon digital still camera and DXM1200 software (Nikon, Melville, NY, USA).

### 2.6 DAPI staining to assess nuclear DNA damage

The fluorescent dye, 4′,6-diamidine-2′-phenylindole (DAPI) (Roche Molecular Biochemicals, Mannheim, Germany), which intercalates specifically into the adenine-thymidine base pairs of DNA, was used to identify nuclear DNA in the cells [27]**.** The deparaffinized and hydrated sections were immersed with 0.1 μg/mL DAPI in PBS for 20 min at room temperature and then examined using the same microscope described above. The DAPI staining in aqueous solution shows an absorbance maximum at 340 nm and an emission maximum at 448 nm.

### 2.7 Detection of oxidized DNA by 8-OhdG immunohistochemistry

Formation of 8-hydroxyl-deoxyguanosine (8-OHdG) is regarded a hallmark of oxidative DNA damage [28, 29]**.** A time course study by Hwang *et al*. [30] showed an increase in immunoreactivity of 8-OHdG in the gerbil hippocampal CA1 region peaking at 12 h and 4 days after ischemic insult. In this study, brain samples obtained 4 days after I/R were used. Briefly, deparaffinized brain sections were first incubated in 0.3% $H_2O_2$ in PBS for 30 min, followed by incubation with 150 μg/mL RNase A for 1 h at 37°C to exclude interference effects of oxidative RNA products. After rinsing with PBS (5 min, 3 ×), the sections were incubated in 50 mM sodium hydroxide (NaOH) in 40% ethanol for 10 min in order to denature DNA. After washing with PBS (5 min, 3 ×), the sections were incubated in 0.5% bovine serum album (BSA) and 10% normal goat serum (NGS) in PBS for 30 min. The sections were then incubated with mouse anti-8-OHdG antibody (diluted at 5 μg/mL; OxisResearch, Portland, OR, USA) in 1% NGS, 0.3% Triton X-100 in PBS at 4°C overnight, followed by incubating with goat anti-mouse immunoglobulin G (IgG) labeled with horseradish peroxidase (HRP) (dilution 1: 200; Sigma, St. Louis, MO, USA) in 1% NGS, 0.3% Triton X-100 in PBS at room temperature for 2 h. After washing with PBS, the peroxidase reaction was carried out by incubating the slides with freshly prepared 0.5 mg/mL DAB (Sigma) and 0.03% $H_2O_2$ for 5 min. After dehydration in graded ethanol, the sections were mounted with Permount and examined under above microscope.

### 2.8 TUNEL staining to identify fragmented DNA

For detecting apoptotic cell death, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining was performed using an *in situ* cell death detection kit (Roche Molecular Biochemicals). The kit contains terminal deoxynucleotidyl transferase, which catalyzes polymerization of fluorescent dUTP to free 3′-OH DNA ends in a template-independent manner. TUNEL-positive cells were identified by fluorescence of incorporated dUTP. Briefly, deparaffinized and rehydrated sections were incubated with 20 μg/mL proteinase K in 0.01 M Tris-HCl (pH 7.4) for 30 min at room temperature. After washing, the sections were permeabilized in a solution containing 0.1% Triton-X 100 and 0.1% sodium citrate for 10 min at room temperature. The sections were incubated in the TUNEL-reaction mixture. The reaction was stopped by transferring the sections to termination buffer for 15 min at room temperature. After washing, slides were examined under the same microscope described above using excitation wavelength 450 – 500 nm and detection using a 515–565 nm filter.

 

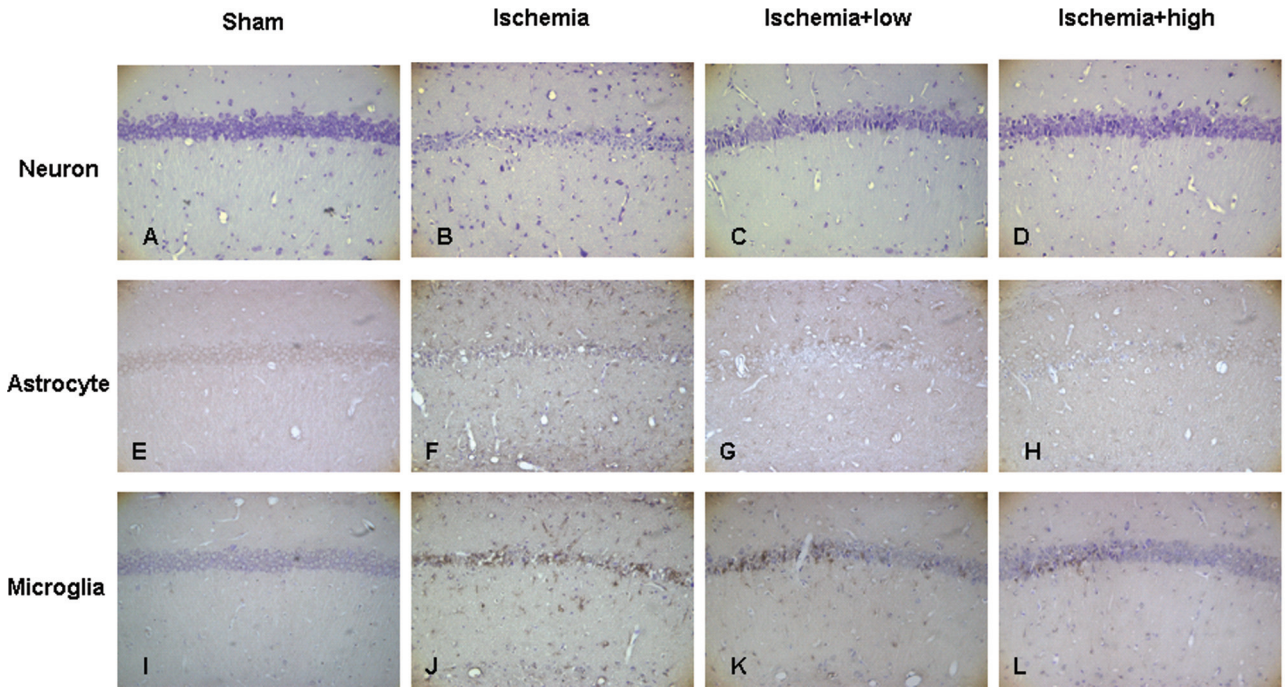


**Figure 1.** Protection of dietary GP on neuronal survivability, astrocytic and microglial activation after transient ischemic insult. Neurons (cresyl violet), astrocytes (GFAP), and microglial cells (isolectin-B4) were detected in the hippocampal CA1 subfield of gerbils at 4 days after a 5 min CCA occlusion. For each staining type, comparisons were made among sham, ischemia, and ischemia with low (5.0 g/kg) or high dosage (50 g/kg) of GP, which were orally supplemented for 2 months before ischemia. After ischemia, gerbils were continued with the same diet until decapitation at 4 days after surgery. (A, B, C, D) Cresyl violet staining; (E, F, G, H) GFAP staining; (I, J, K, L) isolectin-B4 staining. (A, E, I) Sham-operated control; (B, F, J) Ischemia/reperfusion; (C, G, K) Ischemia + low GP; (D, H, L) ischemia + high GP. Magnification, 200 × .

### 2.9 Quantification of data and statistical analysis

Neuronal damage was quantified by counting the number of live neurons in the middle of the CA1 area in both sides of the CA1 regions (320 μm length) under microscopy (magnification 400 × ) by using the Bioquant Image Analysis System (Bioquant True Color Windows 95 Software Version 2.50; Nashville, TN, USA) attached to a Nikon Eclipse E600 microscope equipped with Nikon digital still camera. Areas for counting GFAP- and isolectin-B4-positive cells (100 μm × 320 μm) included an adjacent area above the middle CA1 region. For counting, one slide from each brain (cutting through the hippocampal region) was used and assessment was made from both sides of the brain. Quantitative analysis of DAPI, 8-OHdG immunoreactivity, and TUNEL-positive staining were performed in the same area of the hippocampus (100 μm × 320 μm). Immunoreactivity was assessed based on four grading scores according to the staining intensity as described by Niwa *et al*. [31]: grade 0, no damage or staining; grade 1, weak damage or staining within one-third of neuron; grade 2, moderate damage or staining within two-third of neuron; grade 3, strong damage or staining within the entire neuron. Data were subjected to a two-way analysis of variance (ANOVA) followed by Bonferroni's post-tests using the Graph Pad Prism program Version 4.0 (Graph Pad Software Inc., San Diego, CA, USA). A $p$-value < 0.05 was considered statistically significant.

## 3 Results

### 3.1 GP dietary supplement offered neuroprotection against I/R-induced DND

Four days after 5 min of CCA occlusion, extensive DND was observed in the hippocampal CA1 area (Fig. 1B) as compared to sham controls (Fig. 1A). Both low and high doses of GP supplement decreased DND in the hippocampal CA1 area as comparing Fig. 1C and Fig. 1D with Fig. 1B. Two-way ANOVA revealed a significant interaction between ischemia and GP ($p$ = 0.0469). The main effect of ischemia was significant ($p$ < 0.0001), and the main effect of GP was marginally significant ($p$ = 0.052). Bonferroni's post-tests showed a significant effect of high GP ($p$ < 0.001) and low GP ($p$ < 0.01) but there were no significant differences between low and high GP ($p$ > 0.05) on ischemia (Fig. 2A).

 

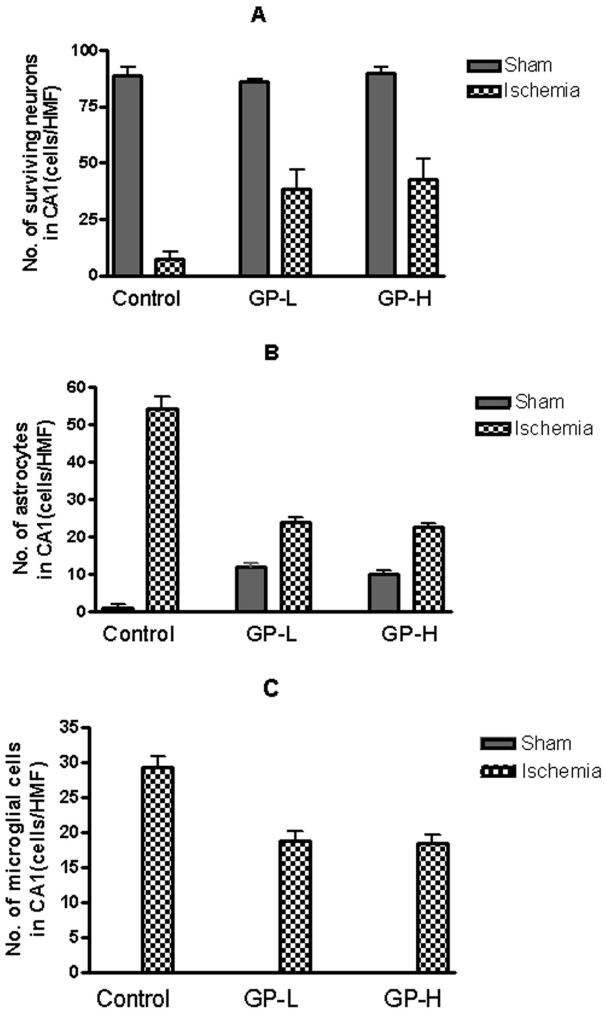


**Figure 2.** Quantification of surviving neurons, astrocytes, and microglial cells after ischemic insult. Histograms depict the number of (A) neurons, (B) astrocytes, and (C) microglial cells in the hippocampal CA1 area. Sham ($n$ = 6), sham + low GP ($n$ = 6), sham + high GP ($n$ = 6), ischemia ($n$ = 10), ischemia + low GP ($n$ = 11), and ischemia + high GP ($n$ = 11). Data represent means ± SEM. There are significant interactions for surviving neurons ($p$ = 0.0469) and activation of astrocytes ($p$ < 0.0001) and microglial cells ($p$ = 0.0001) between GP and ischemia. Bonferroni's post-tests showed a significant effect of high GP on neurons, astrocytes, and microglial cells (all $p$ < 0.001) and low GP on them ($p$ < 0.01 for neurons and $p$ < 0.001 for astrocytes and microglial cells) but there were no significant differences of these three parameters between low and high GP ($p$ > 0.05) on ischemia.

### 3.2 GP dietary supplement diminished I/R-induced glial cell activation

Immunohistochemical staining of GFAP showed that astrocytes with small cell bodies and fine cytoplasmic processes were distributed around the hippocampal CA1 area. Only few GFAP-positive astrocytes were found in the sham control groups (Fig. 1E). Animals subjected to I/R showed an increase in GFAP-positive astrocytes, which are present in multiple hippocampal layers (Fig. 1F). Ischemia induced to gerbils supplemented with both low and high doses of GP showed less reactive astrocytes as compared to the ischemic control group (Fig. 1G *vs*. Fig. 1F; Fig. 1H *vs*. Fig. 1F).

Two-way ANOVA of astrocytes revealed a significant interaction between ischemia and GP ($p$ < 0.0001). The main effects of ischemia and GP were significant (both $p$ < 0.0001). Bonferroni's post-tests showed a significant effect of high GP ($p$ < 0.001) and low GP ($p$ < 0.001) but there were no significant differences between low and high GP ($p$ > 0.05) on ischemia (Fig. 2B).

Using isolectin B4 as a marker, very few microglial cells were found in the sham control groups (with or without GP diets) (Fig. 1I). Ischemia induced a substantial increase in microglial cells, in particular in the hippocampal CA1 region, where pyramidal neuron death was apparent (Fig. 1J). Ischemia induced to gerbils supplemented with both low and high doses resulted in a less microglial proliferation as compared to the ischemic control group (Fig. 1K *vs*. Fig. 1J; Fig. 1L *vs*. Fig. 1J). Two-way ANOVA of microglial cells revealed a significant interaction between ischemia and GP ($p$ = 0.0001). The main effect of ischemia was significant ($p$ < 0.0001), and the main effect of GP was significant ($p$ < 0.0001). Bonferroni's post-tests showed a significant effect of high GP ($p$ < 0.001) and low GP ($p$ < 0.001) but there were no significant differences between low and high GP ($p$ > 0.05) on ischemia (Fig. 2C).

### 3.3 GP dietary supplementation attenuated I/R-induced DNA oxidation and fragmentation

DAPI staining was used to observe alterations in nuclear morphology after ischemic insult. In the brain sections from sham controls, pyramidal neurons in the hippocampal CA1 region showed large round nuclei with clear nucleolus (Fig. 3A). However, 4 days after I/R, most of the nuclei of CA1 pyramidal neurons became fragmented and showed irregular and condensed shape (Fig. 3B). These changes were inhibited by supplementation with both low and high GP diets (Fig 3B. *vs*. Fig. 3C; Fig. 3B *vs*. Fig. 3D). Oxidation of DNA was assessed by immunohistochemistry of 8-OHdG in the hippocampal CA1 region. In the sham control group, weak 8-OHdG immunoreactivity was observed (Fig. 3E). Four days after I/R, 8-OHdG immunoreactivity was elevated in the CA1 region of the ischemic group (Fig. 3F) and these increases were diminished by supplementation with both low- and high-GP diets (Fig. 3F *vs*. Fig. 3G; Fig. 3F *vs*. Fig. 3H). In the sham-operated groups, very few TUNEL-positive cells were observed in the hippocampal CA1 region (Fig. 3I). However, TUNEL-positive cells increased 4 days after I/R (Fig. 3J). Supplementation

 

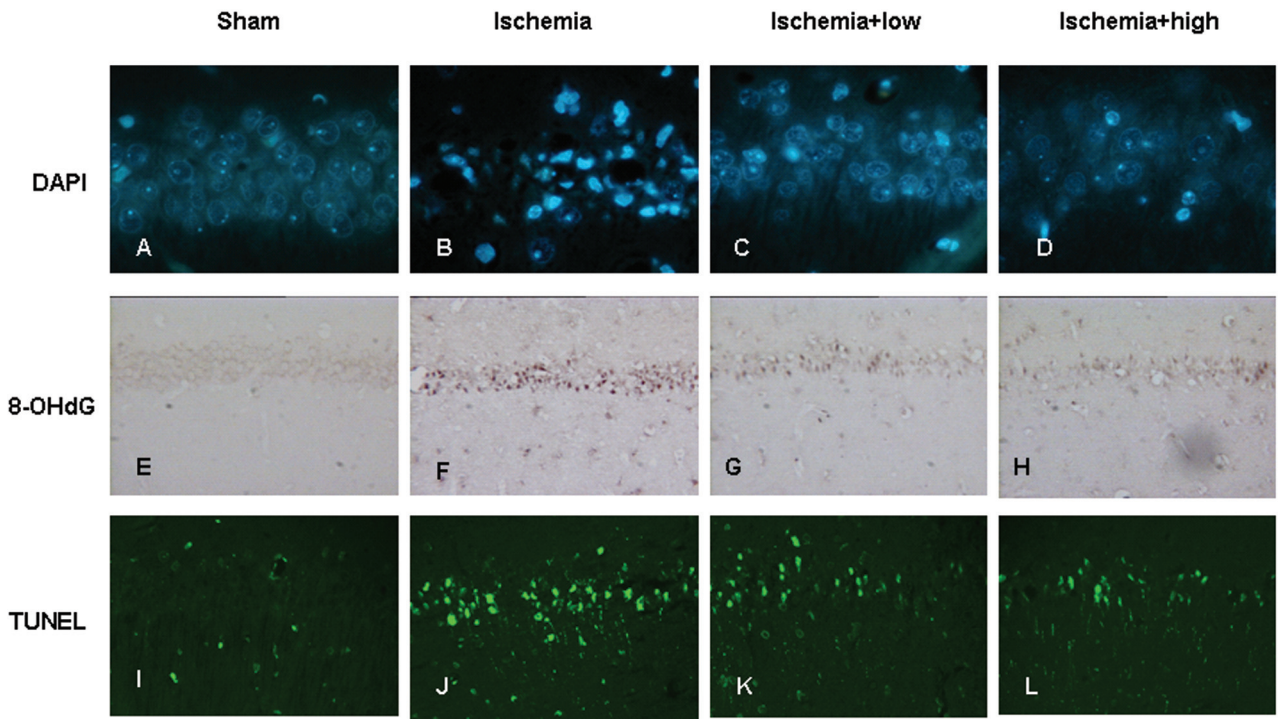


**Figure 3.** Effects of dietary GP on DNA oxidation and fragmentation after transient ischemic insult. Nuclear staining (DAPI), oxidative DNA (8-OHdG), and fragmented DNA (TUNEL) staining in the hippocampal CA1 subfield of gerbils at 4 days after a 5 min CCA occlusion. For each staining type, comparisons were made among sham, ischemia, and ischemia with low (5.0 g/kg) or high dosage (50 g/kg) of GP, which were orally supplementation for 2 months before ischemia. After ischemia, gerbils were continued with the same diet until decapitation at 4 days after surgery. (A, B, C, D) DAPI staining; (E, F, G, H) 8-OHdG staining; (I, J, K, L) TUNEL staining. (A, E, I) sham-operated control; (B, F, J) ischemia/reperfusion; (C, G, K) ischemia + low GP; (D, H, L) ischemia + high. Magnification, 600 × for DAPI and 400 × for oxidized and fragmentated DNA.

with both low- and high-GP diets reduced the number and intensity of TUNEL-positive cells as compared to those in the ischemic brain (Fig. 3J *vs*. Fig. 3K; Fig. 3J *vs*. Fig. 3L).

Two-way ANOVA revealed significant interactions for nuclear damage ($p = 0.0026$), oxidative DNA ($p = 0.0177$), and apoptosis ($p = 0.0025$) between GP and ischemia. The main effects of ischemia on these parameters ($p < 0.0001$ for all) and GP on them (nuclear damage: $p = 0.001$; oxidized DNA: $p = 0.0084$, and apoptosis: $p = 0.0135$) were also significant (Figs. 4A, B, and C). Bonferroni's post-tests showed a significant effect of high GP on nuclear damage, oxidized DNA and apoptosis (all $p < 0.001$) and low GP on them (all $p < 0.001$) but there were no significant differences for nuclear damage, oxidative DNA, and apoptosis between low and high GP (all $p > 0.05$) on ischemia (Figs. 4A, B, and C).

## 4 Discussion

The Mongolian gerbil has been widely used for studies to investigate neuronal damage due to global cerebral ischemia induced by transient occlusion of both common carotid arteries [7, 32]. Four days after a 5 min ischemic treatment, substantial DND can be observed in the hippocampal CA1 area [6]. This ischemic insult triggers the increased production of ROS, which in turn causes cellular damage through oxidation of macromolecules including lipids, proteins, and DNA as well as alterations of the redox-sensitive signaling pathways [2, 33–35]. In this study, I/R-induced an increase in 8-OHdG immunoreactivity and TUNEL-positive cells in the hippocampal CA1 area, indicating oxidative damage to nuclear DNA and neuronal apoptosis [36]. Results from previous reports have indicated that the DND in the hippocampus following transient global ischemia is attributed largely to the apoptotic type [5, 6, 37, 38]. Our results further demonstrate that oxidative stress is involved in the I/R-mediated apoptotic cell death mechanisms. Furthermore, results in this study indicate that dietary GP supplement can ameliorate both oxidative and apoptotic neuronal death induced by I/R to the gerbils.

Besides DND, I/R also caused extensive activation of astrocytes and microglial cells, which are abundant in the CA1 layer with prominent dying neurons. In this study, gerbils supplemented with GP showed less glial cell activation after

 

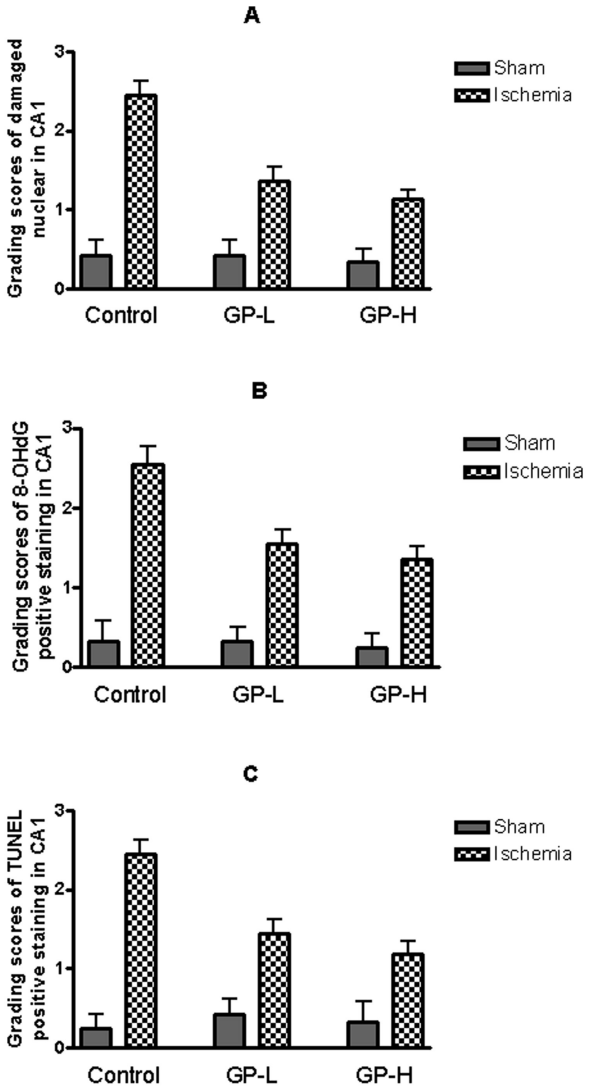


**Figure 4.** Quantification of nuclear damage, DNA oxidation, and fragmentation after ischemia insult. Histograms depicting grading scores of nuclear damage (DAPI, A), oxidative DNA (8-OHdG, B), and fragmented DNA (TUNEL, C) in hippocampal CA1 area. Sham ($n$ = 6), sham + low GP ($n$ = 6), sham+-high GP ($n$ = 6), ischemia ($n$ = 10), ischemia + low GP ($n$ = 11) and ischemia + high GP ($n$ = 11). Data represent means $\pm$ SEM. There are significant interactions for nuclear damage ($p$ = 0.0026), oxidative DNA ($p$ = 0.0177), and apoptosis ($p$ = 0.0025) between GP and ischemia. Bonferroni's post-tests showed a significant effect of high GP on nuclear damage, oxidized DNA, and apoptosis (all $p < 0.001$) and low GP on them (all $p < 0.001$) but there were no significant differences for nuclear damage, oxidative DNA, and apoptosis between low and high GP (all $p > 0.05$) on ischemia.

I/R as compared with the group without GP supplementation. Astrocytes are known to play an important role in maintaining the proper environment in the brain and are crucial for the survival of neurons [39, 40]. However, although *in vitro* studies have indicated the involvement of reactive gliosis in oxidative and inflammatory responses [39], our results show that GP supplement not only protects neurons from I/R-induced DND, but also diminishes glial cell activation.

Due to the recognition that polyphenols from fruits, vegetables, and beverages have demonstrated health benefits, there is increasing interest to under their bioavailability [21, 41]. Although much attention has been devoted to resveratrol in grape and red wine [42], it is also recognized that other polyphenols, such as quercetin, catechins, flavonols, and proanthocyanidins, are present in grapes. While resveratrol is enriched in grape skin and proanthocyanidin is enriched in the seeds [12], both compounds have been shown to offer protective effects against ischemia-induced neuronal damage [7, 30, 43]. However, a study by Shanmuganayagam *et al*. [44] indicated that the combination of grape skin and grape seed extracts could elicit a greater antiplatelet effect as compared to that applied individually [44]. Previous studies from our laboratory demonstrated that dietary supplement of a polyphenol extract from grape (5 mg/100 mL of a standardized liquid diet/d) to rats for 2 months resulted in protection of the brain and liver against oxidative damage due to chronic alcohol administration [18, 19]. Since the polyphenol extract from grape comprises approximately 10% of the total GP, the levels of GP used in this study (*i.e.*, 25 or 250 mg GP/animal/day) are comparable to the amount used in our previous study [18, 19]. In a recent study by Zern *et al.* [45], a much higher dose (10 g/100 g) of the same GP was used in the experiment with Guinea pigs. Apparently, results from the present study indicate that the low dose of GP has already reached maximum protection against the I/R injury. Although the high dose of GP did not increase the protective effects, administration of this level did not produce harmful effects to the gerbils. These results are in general agreement with reports by others that high doses of polyphenols are not harmful [45, 46]. Our previous study had indicated that resveratrol (administered by i.p. injection) can cross the blood brain barrier [7]. Obviously, it is expected that other polyphenols in GP may also cross the blood brain barrier to exert their protective effects. Further studies should include developing new methods for monitoring the major polyphenols and their glycoconjugates in the plasma and in brain.

In summary, our results demonstrate for the first time that GP supplementation in diets can protect the brain against neuronal damage induced by global cerebral ischemia. Since many neurodegenerative diseases have similar oxidative mechanisms, the protective properties of GP may have wide implication in the future for prevention/protection against stroke and neurodegenerative damages.

*The study was supported by DHHS 5P01 ES10535, 1P01 AG18357 from NIH and a grant from the California Table*

 

*Grape Commission. The authors are grateful to Alisa Nettles-Strong for assistance in the preparation of this manuscript.*

## 5 References


[1] Sun, A. Y., Chen, Y. M., Oxidative stress and neurodegenerative disorders. *J. Biomed. Sci*. 1998, *5*, 401–414.

[2] Chan, P. H., Reactive oxygen radicals in signaling and damage in the ischemic brain. *J. Cereb. Blood Flow Metab.* 2001, *21*, 2–14.

[3] Love, S., Oxidative stress in brain ischemia. *Brain Pathol*. 1999, *9*, 119–131.

[4] Graham, S. H., Chen, J., Programmed cell death in cerebral ischemia. *J. Cereb. Blood Flow Metab*. 2001, *21*, 99–109.

[5] Hara, A., Mori, H., Niwa, M., Novel apoptotic evidence for delayed neuronal death in the hippocampal CA1 pyramidal cells after transient ischemia. *Stroke* 2000, *31*, 236–238.

[6] Kirino, T., Delayed neuronal death. *Neuropathology* 2000, *20*, S95–S97.

[7] Wang, Q., Xu, J., Rottinghaus, G. E., *et al.*, Resveratrol protects against global cerebral ischemic injury in gerbils. *Brain Res*. 2002, *958*, 439–447.

[8] Bravo, L., Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* 1998, *56*, 317–333.

[9] Deschamps, V., Barberger-Gateau, P., Peuchant, E., Orgogozo, J. M., Nutritional factors in cerebral aging and dementia: epidemiological arguments for a role of oxidative stress. *Neuroepidemiology* 2001, *20*, 7–15.

[10] Youdim, K. A., Joseph, J. A., A possible emerging role of phytochemicals in improving age-related neurological dysfunctions: a multiplicity of effects. *Free Radic. Biol. Med*. 2001, *30*, 583–594.

[11] Cui, J., Juhasz, B., Tosaki, A., *et al.*, Cardioprotection with grapes. *J. Cardiovasc. Pharmacol*. 2002, *40*, 762–769.

[12] Das, D. K., Sato, M., Ray, P. S., *et al.*, Cardioprotection of red wine: role of polyphenolic antioxidants. *Drugs Exp. Clin. Res*. 1999, *25*, 115–120.

[13] Esposito, E., Rotilio, D., Di Matteo, V., *et al.*, A review of specific dietary antioxidants and the effects on biochemical mechanisms related to neurodegenerative processes. *Neurobiol. Aging* 2002, *23*, 719–735.

[14] Sun, A. Y., Simonyi, A., Sun, G. Y., The "French Paradox" and beyond: neuroprotective effects of polyphenols. *Free Radic. Biol. Med*. 2002, *32*, 314–318.

[15] Chanvitayapongs, S., Draczynska-Lusiak, B., Sun, A. Y., Amelioration of oxidative stress by antioxidants and resveratrol in PC12 cells. *Neuroreport* 1997, *8*, 1499–1502.

[16] Chen, Y., Sun, A. Y., Activation of transcription factor AP-1 by extracellular ATP in PC12 cells. *Neurochem. Res*. 1998, *23*, 543–550.

[17] Draczynska-Lusiak, B., Chen, Y. M., Sun, A. Y., Oxidized lipoproteins activate NF-$\kappa$B binding activity and apoptosis in PC12 cells. *Neuroreport* 1998, *9*, 527–532.

[18] Sun, G. Y., Xia, J., Draczynska-Lusiak, B., *et al.*, Grape polyphenols protect neurodegenerative changes induced by chronic ethanol administration. *Neuroreport* 1999, *10*, 93–96.

[19] Sun, G. Y., Xia, J., Xu, J., *et al.*, Dietary supplementation of grape polyphenols to rats ameliorates chronic ethanol-induced changes in hepatic morphology without altering changes in hepatic lipids. *J. Nutr.* 1999, *129*, 1814–1819.

[20] Simonyi, A., Woods, D., Sun, A. Y., Sun, G. Y., Grape polyphenols inhibit chronic ethanol-induced COX-2 mRNA expression in rat brain. *Alcohol Clin. Exp. Res*. 2002, *26*, 352–357.

[21] Simonyi, A., Wang, Q., Miller, R., *et al.*, Polyphenols in cerebral ischemia: novel targets for neuroprotection. *Mol. Neurobiol*. 2005, in press.

[22] Voko, Z., Hollander, M., Hofman, A., *et al.*, Dietary antioxidants and the risk of ischemic stroke: the Rotterdam Study. *Neurology* 2003, *61*, 1273–1275.

[23] Wang, Q., Yu, S., Simonyi, A., *et al.*, Resveratrol protects against neurotoxicity induced by kainic acid. *Neurochem. Res*. 2004, *29*, 2105–2112.

[24] Wang, Q., Yu, S., Simonyi, A., *et al.*, Kainic acid-mediated excitotoxicity as a model for neurodegeneration. *Mol. Neurobiol*. 2005, in press.

[25] Cui, J., Cordis, G. A., Tosaki, A., *et al.*, Reduction of myocardial ischemia reperfusion injury with regular consumption of grapes. *Ann. N. Y. Acad. Sci*. 2002, *957*, 302–307.

[26] Streit, W. J., An improved staining method for rat microglial cells using the lectin from Griffonia simplicifolia (GSA I-B4). *J. Histochem. Cytochem*. 1990, *38*, 1683–1686.

[27] Hara, A., Niwa, M., Iwai, T., *et al.*, Neuronal apoptosis studied by a sequential TUNEL technique: a method for tract-tracing. *Brain Res. Brain Res. Protoc*. 1999, *4*, 140–146.

[28] Kim, G. W., Lewen, A., Copin, J., *et al.*, The cytosolic antioxidant, copper/zinc superoxide dismutase, attenuates blood-brain barrier disruption and oxidative cellular injury after photothrombotic cortical ischemia in mice. *Neuroscience* 2001, *105*, 1007–1018.

[29] Won, M. H., Kang, T. C., Jeon, G. S., *et al.*, Immunohistochemical detection of oxidative DNA damage induced by ischemia-reperfusion insults in gerbil hippocampus *in vivo*. *Brain Res*. 1999, *836*, 70–78.

[30] Hwang, I. K., Yoo, K. Y., Kim, D. S., *et al.*, Neuroprotective effects of grape seed extract on neuronal injury by inhibiting DNA damage in the gerbil hippocampus after transient forebrain ischemia. *Life Sci*. 2004, *75*, 1989–2001.

[31] Niwa, M., Hara, A., Iwai, T., *et al.*, Relationship between magnitude of hypothermia during ischemia and preventive effect against post-ischemic DNA fragmentation in the gerbil hippocampus. *Brain Res*. 1998, *794*, 338–342.

[32] Ginsberg, M. D., Busto, R., Rodent models of cerebral ischemia. *Stroke* 1989, *20*, 1627–1642.

[33] Hou, S. T., MacManus, J. P., Molecular mechanisms of cerebral ischemia-induced neuronal death. *Int. Rev. Cytol*. 2002, *221*, 93–148.

[34] Juurlink, B. H., Sweeney, M. I., Mechanisms that result in damage during and following cerebral ischemia. *Neurosci. Biobehav. Rev.* 1997, *21*, 121–128.

[35] Yamato, M., Egashira, T., Utsumi, H., Application of *in vivo* ESR spectroscopy to measurement of cerebrovascular ROS generation in stroke. *Free Radic. Biol. Med*. 2003, *35*, 1619–1631.

[36] Nagayama, T., Lan, J., Henshall, D. C., *et al.*, Induction of oxidative DNA damage in the peri-infarct region after permanent focal cerebral ischemia. *J. Neurochem*. 2000, *75*, 1716–1728.


 

[37] Jin, K., Chen, J., Nagayama, T., *et al.*, *In situ* detection of neuronal DNA strand breaks using the Klenow fragment of DNA polymerase I reveals different mechanisms of neuron death after global cerebral ischemia. *J. Neurochem*. 1999, *72*, 1204 – 1214.

[38] Nitatori, T., Sato, N., Waguri, S., *et al.*, Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. *J. Neurosci*. 1995, *15*, 1001 – 1011.

[39] Ridet, J. L., Malhotra, S. K., Privat, A., Gage, F. H., Reactive astrocytes: cellular and molecular cues to biological function. *Trends Neurosci*. 1997, *20*, 570 – 577.

[40] Tacconi, M. T., Neuronal death: is there a role for astrocytes? *Neurochem. Res*. 1998, *23*, 759 – 765.

[41] Scalbert, A., Williamson, G., Dietary intake and bioavailability of polyphenols. *J. Nutr*. 2000, *130*, 2073S – 2085S.

[42] Pervaiz, S., Resveratrol: from grapevines to mammalian biology. *Faseb J*. 2003, *17*, 1975 – 1985.

[43] Huang, S. S., Tsai, M. C., Chih, C. L., *et al.*, Resveratrol reduction of infarct size in Long-Evans rats subjected to focal cerebral ischemia. *Life Sci*. 2001, *69*, 1057 – 1065.

[44] Shanmuganayagam, D., Beahm, M. R., Osman, H. E., *et al.*, Grape seed and grape skin extracts elicit a greater antiplatelet effect when used in combination than when used individually in dogs and humans. *J. Nutr*. 2002, *132*, 3592 – 3598.

[45] Zern, T. L., West, K. L., Fernandez, M. L., Grape polyphenols decrease plasma triglycerides and cholesterol accumulation in the aorta of ovariectomized Guinea pigs. *J. Nutr*. 2003, *133*, 2268 – 2272.

[46] Juan, M. E., Vinardell, M. P., Planas, J. M., The daily oral administration of high doses of trans-resveratrol to rats for 28 days is not harmful. *J. Nutr*. 2002, *132*, 257 – 260.

 