Research report

Protective effect of green tea extract on ischemia/reperfusion-induced brain injury in Mongolian gerbils

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Abstract

Free radical-induced oxidative damages of macromolecules and cell death are important factors in the pathogenesis of ischemia/reperfusion brain injury. In the present study, an investigation as to whether green tea extract reduces ischemia/reperfusion-induced brain injury in Mongolian gerbils was conducted. The effect of green tea on the ischemia/reperfusion-induced production of hydrogen peroxide, lipid peroxidation and oxidative DNA damage (formation of 8-hydroxydeoxyguanosine), and cell death in addition to locomotor activity was studied. Two doses (0.5 or 2\%) of green tea extract were added into the drinking water and to be accessed by animals ad libitum for 3 weeks prior to the induction of ischemia. A global ischemia was induced by the bilateral occlusion of the common carotid arteries for 5 min. Reperfusion was achieved by releasing the occlusion and restoring blood circulation for 48 h. The infarction volumes were $112 \pm 31$ mm\(^3\) and $76 \pm 11$ mm\(^3\) in the 0.5 and 2\% green tea pretreated animals compared to $189 \pm 12$ mm\(^3\) in the ischemia/reperfusion animals. Green tea extract also reduced the levels of ischemia/reperfusion-induced hydrogen peroxide (from $1470 \pm 170$ to $1034 \pm 46$ and $555 \pm 30$ nmole/mg protein), lipid peroxidation products (from $1410 \pm 210$ to $930 \pm 40$ and $330 \pm 20$ nmole/mg protein) and 8-oxodG (from $3.9 \pm 0.1$ to $2.8 \pm 0.3$ and $1.9 \pm 0.3$ ng/mg DNA, $\times 10^{-3}$) by pretreatment of 0.5 or 2\% green tea for 3 weeks, respectively. Moreover, green tea also reduced the number of ischemia/reperfusion-induced apoptotic cells (from $59 \pm 12$ to $37 \pm 8$, $15 \pm 11$ apoptotic cells/high power field in the striatum region) and locomotor activity (from $15140 \pm 2940$ to $3900 \pm 600$ and $4100 \pm 1200$). This study therefore suggests that green tea may be a useful agent for the prevention of cerebral ischemia damage. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Green Tea extract; Ischemia/reperfusion; Oxidative damage; Gerbil

1. Introduction

It is generally believed that ischemia/reperfusion-mediated brain injury results, at least in part, from the oxidation of cellular macromolecules [21]. Because of the brain’s high consumption of oxygen, high concentration of polyunsaturated fatty acid and transition metals, and low concentration of antioxidants, it is vulnerable to ischemia/reperfusion-induced reactive oxygen species which cause oxidative damage to lipids and brain DNA. The formation of lipids and DNA oxidation products e.g. malondialdehyde and 8-hydroxydeoxyguanosine have been found to cause cellular dysfunction or/and cell death, which leads to further impairment of the central nervous system [7].

The formation of these oxidation products in the human brain increases with age and ischemia/reperfusion animal brain [15,24]. It is therefore of interest to study the effects of antioxidants, free radical scavengers or trapping agents for use as potential cerebroprotective agents of various brain injuries including ischemia/reperfusion-mediated brain injury.

Green tea has been reported to have antioxidant properties [19]. Green tea reduces iron-induced lipid peroxidation in brain homogenates as well as in cultured C6 astrocytes and lung cells [13,14,27,11]. In addition, green tea has also been shown to reduce the formation of the spin-adducts of hydroxyl radicals and hydroxyl radical-induced DNA strand breakage in vitro [6] in addition to UV radiation-induced oxidative DNA damage in calf thymus DNA [23]. Moreover, green tea has been found to have inhibitory effects on the UVB light-induced skin [20].

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and chemical-induced lung tumorigenesis [25]. There is also considerable epidemiological evidence suggesting that the consumption of green tea lowers the risk of heart disease as well as several types of cancer incidences as a result of these antioxidant mechanisms [1].

In the present study, the protective effect that green tea extract has on ischemia/reperfusion-induced brain injury was examined. In particular, the ischemia/reperfusion-induced production of hydrogen peroxide, lipid peroxidation and oxidative DNA damage (formation of 8-hydroxydeoxyguanosine), and cell death as well as locomotor activity on the Mongolian gerbil were focused.

2. Materials and methods

2.1. Animals

The female Mongolian gerbils used were maintained in accordance with the National Institute of Toxicological Research of the Korea Food and Drug Administration guideline for the care and use of laboratory animals. The female Mongolian gerbils (body weight 50–60 g) were housed 4–5 per cage and maintained at 22±2°C with a constant humidity for at least 1 week prior to the commencement of the experiments. All the animals were allowed free access to food and water before and after ischemia surgery. The 2 doses (0.5 or 2%) of green tea extract were added into the drinking water and were accessed by animals for 3 weeks ad libitum before the induction of ischemia. The method of green tea extraction and compounds in the extracts are described elsewhere [8]. Briefly, dried green tea leaves were extracted with 70% ethanol at 90–95°C for 6 h. The extract was then fractionated by column chromatography using Amberite XAD-7 (mean pore diameter, 90 A). The total catechin content of the extract was determined by UV spectroscopy, and the composition was determined using HPLC.

2.2. Ischemic surgery

The acclimatized Mongolian gerbils were anesthetized with a gas mixture of 2% isoflurane, 75% N₂O and 25% O₂. The bilateral common carotid arteries were occluded using sugita aneurysm clips for 5 min. During the occlusion and postoperative period, the gerbils were kept on thermostat-control warming plates in order to maintain body temperature at 37°C. Following the occlusion, the clips were removed to restore the blood flow. The same surgical operated animals without carotid ligation were served as sham control animals.

2.3. Morphometric determination of infarct volume

For detection of the ischemia infarction area of the brain, the cross-sectional infarction area on the surfaces of each brain slice was defined by the 2,3,5-triphenyltetrazolium chloride (TTC) staining method. After 48 h reperfusion, the gerbils received an intracardiac perfusion of 0.9% buffered saline. The brain was then removed, and cut into 2-mm serial slices starting 1 mm from frontal pole. The coronal slices were then immersed in a 2% phosphate-buffered solution for 50 min at 37°C. After TTC staining, the slices were fixed in 10% phosphate-buffered formalin and the infarction area was then determined by an image analyzer using the leicaQwin programme (Leica Microsystems Image Solution Ltd., Cambridge, UK). The infarct area (mm²) from each thicken-brain slice was determined, and the infarct volume (mm³) was calculated from sum of the slice areas (7 slices in all)×thickness (2 mm).

2.4. Determination of hydrogen peroxide level, lipid peroxidation and 8-hydroxydeoxyguanosine (8-oxodG)

The level of hydrogen peroxide in the whole brain tissue was measured using the BIOXYTECH H₂O₂-560 assay kit (OXIS International Inc., USA). The formation of malonaldehyde and 4-hydroxynonenal, as lipid peroxidation products in the whole brain homogenate was also determined using a lipid peroxidation assay kit (OXIS International Inc., Ohio, USA) according to the methods described in the manufacturer’s protocol. For the determination of 8-oxodG, isolated DNA from whole brain homogenate was purified and digested as described previously [5]. The digested DNA was then analyzed by the ELISA assay kit (Japan Institute for the Control of Aging, Nikken Foods Co., Japan) to detect 8-oxodG formation.

2.5. Detection of apoptotic cells

The terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) method was used for the detection of apoptotic bodies. The brains were fixed with 4% phosphate-buffered paraformaldehyde (pH 7.4), and stored in 4% phosphate-buffered paraformaldehyde (pH 7.4). After 24 h, they were transferred into a 20% phosphate-buffered sucrose solution and cut into 10 μm cryostat coronal slices starting between 7 and 9 mm from the frontal pole. The tissue sections were fixed with 4% phosphate-buffered paraformaldehyde (pH 7.4) for 30 min at room temperature, and then incubated with a blocking solution (3% H₂O₂ in water) for 5 min at room temperature. This was followed with immersion in a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. 50 μl of the TUNEL mixture (Boehringer Mannheim, USA) was deposited on the tissue sections and incubated in a humidified chamber for 60 min at 37°C. A 50 μl Converter-POD solution (horse-radish peroxidase, Boehringer Mannheim) was then added and then further incubated for 30 min at 37°C. Subsequently, a 100 μl DAB (3,3′-diaminobenzidine
tetra-chloride) substrate solution was added onto tissue sections for 10 min at room temperature. After each step, the tissue sections were rinsed twice with a phosphate-buffered saline solution (PBS, pH 7.4). The apoptotic bodies were identified under microscope (×200) as containing brown colored nuclei. The quantity of apoptotic bodies were expressed as the average number of apoptotic cells per high power field (visible apoptotic cells/HPF).

2.6. Measurement of locomotor activity

The locomotor activity was determined by the distances traveled by the animals for 1 h (counts/cm) in the locomotor activity apparatus (OPTI-Varimex®, Columbus Instrument, Ohio, USA). Spontaneous locomotor activity was monitored by a single break (defined as an activity) in the photocell light beam emitted from 15 sites in the photocell chamber.

2.7. Statistics

The data was expressed as the mean±standard error. The data was analyzed with a one-way analysis of the variance followed by either Dunnett’s or Bonferroni’s method as a post hoc test. Differences were considered significant at P<0.05.

3. Results

3.1. Ischemia/reperfusion-induced brain infarction

The effect of the occlusion and reperfusion time on brain infarct was examined initially. Different degrees of ischemia infarction volume were created by varying duration of occlusion and reperfusion (data not shown). The highest infarct volume (total infarction volume was 189±12 mm³) was induced in the animals after 5 min-ischemia/48 h-reperfusion. Treatment with green tea for 3 weeks prior to the induction of ischemia attenuated the ischemia/reperfusion-induced brain infarction (112±31 mm³ in the 0.5% green tea pretreated animals and 76±11 mm³ in the 2% green tea pretreated animals, Fig. 1).

3.2. Hydrogen peroxide, lipid peroxidation product and 8-oxodG levels

Since the TTC results showed that most parts of the brain were damaged by 5 min-ischemia/48 h-reperfusion, the whole brain was used for the biochemical assays. Fig. 2A shows the hydrogen peroxide level. The hydrogen peroxide level increased in the ischemia/reperfusion animals (from 1040±20 to 1470±170 nmole/mg protein). The increase in the hydrogen peroxide level was reduced by green tea pretreatment. The values were found to be 1034±46 nmole/mg protein and 555±30 nmole/mg protein in brain of animals pretreated by 0.5 and 2% green tea for 3 weeks, respectively. Consistent with the hydrogen peroxide level, the level of lipid peroxidation products (malonaldehyde and 4-hydroxynonenal) in the animals had also increased as a result of ischemia/reperfusion (from 1020±60 to 1410±210 nmole/mg protein). The animals pretreated with green tea had substantially reduced lipid peroxidation products especially the ones that were administered the 2% extract. The malonaldehyde and 4-hydroxynonenal levels were 930±40 and 330±20 nmole/mg protein in brains of animals pretreated with 0.5 and 2% green tea for 3 weeks, respectively (Fig. 2B). The 8-oxodG level had also increased in brains of ischemia/reperfusion animals (3.9±0.1 ng/µg DNA, ×10⁻²) compared to the control animals (2.1±0.7 ng/µg DNA, ×10⁻²). These values were reduced to 2.8±0.3 ng/µg DNA, ×10⁻², 1.9±0.3 ng/µg DNA, ×10⁻² as a result of pretreatment with 0.5 or 2% green tea for 3 weeks, respectively (Fig. 2C).

3.3. Formation of apoptotic cells

In order to determine whether green tea ingestion had the effect of reducing the level of oxidative damage to macromolecules and hence prevent neuronal death, the level of apoptotic cell formation was measured. In the preliminary study, it was found that the number of surviving neurons was greatly reduced in the striatum and cortex as well as the hippocampus [16]. Therefore, neuronal cell death in striatum and cortex regions was detected to observe whether cell death could influence neuronal cell loss. In the ischemia/reperfusion group, there was a clear increase in the number of apoptotic cells in the striatum of the brain. In this region, only occasional apoptotic cells (2–3 cells/HPF) were detected in the sham-operated group (Fig. 3). In the striatum region of the brains of animals pretreated by 0.5 and 2% green tea, the number of apoptotic cells (59±11) by ischemia/reperfusion was reduced to 37±8 and 15±11, respectively. A similar increase in the number of apoptotic cells by ischemia/reperfusion and inhibitory effect of green tea was also found in the cortex region (Fig. 3).

3.4. Locomotor activity

The value of locomotor activity (traveled distance, cm) was found to be 300±26 soon after ischemic surgery. However, the activity gradually increased after reperfusion and peaked at 6 h (28 000±1900). The level was sustained at the 15 000 level for as long as 2 days. Therefore, the locomotor activity was compared 1 and 3 days after ischemia. There was a 2–3-fold increase in the ischemia/reperfusion animals (15 140±2940) compared with the sham-operated animals (4950±3570) 1 day post-ischemia. One day after ischemia, the locomotor activity had significantly decreased in animals pretreated with green tea.
Fig. 1. Brain infarction after 5 min-ischemia/48 h-reperfusion caused by the occlusion of the bilateral common carotid artery in Mongolian gerbils. The brain infarction was detected in brain slices cut at 6 mm away from the frontal pole by the 2,3,5-triphenyltetrazolium chloride staining methods described in Materials and methods. 0.5 and 2% green tea extracts (GTE) were given to the animals for 3 weeks before induction of ischemia. A; sham control, B; 5 min-ischemia/48 h-reperfusion, C; 5 min-ischemia/48 h-reperfusion after 0.5% GTE pretreatment for 3 weeks, D; 5 min-ischemia/48 h-reperfusion after 2% GTE treatment for 3 weeks. E; Infarction volumes (n=6). *; significant difference from ischemia/reperfusion group (P<0.05), # significant difference from 0.5% green tea group.

extract (Fig. 4). The values were 3900±600 and 4100±1200 in the animals pretreated by 0.5 and 2% green tea for 3 weeks, respectively. After 3 days, the locomotor activity in the ischemia/reperfusion animals was still 2~3-fold higher (3840±1731) than in the sham control (1840±50). Pretreatment with green tea extract did not induce any further decrease in the ischemia/reperfusion-induced increase of locomotor activity (Fig. 4).
of cerebral ischemia has been widely studied [16,4]. In this investigation, it was found that pretreatment with green tea extract had inhibitory effects on the ischemia/reperfusion-induced increase of hydrogen peroxide, lipid peroxidation products and 8-oxodG (a form of oxidative DNA damage) levels. Moreover, pretreatment with green tea attenuated ischemia/reperfusion-induced brain infarction and cell death.

Both the modification of enzymes involving the free radical generating procedure and treatment of free radical scavengers have been used by many investigators to ameliorate brain injury in animals following focal or global cerebral ischemia. Chan et al. [3] reported that increased endogenous superoxide dismutase activity in transgenic mice resulted in the alteration of the antioxidant system that favored cytoprotection of the brain against ischemia injury. Dimethyl sulfoxide (DMSO) and ethanol, having an ability to scavenge free radicals were also found to reduce 5-min ischemia/5-day reperfusion-evoked neuronal cell death in the gerbil hippocampus [18]. Melatonin protected ischemia/reperfusion-induced oxidative lipid and DNA damage by scavenging free radicals [22]. The effect of green tea on the hydrogen peroxide level in the brain was first examined since hydrogen peroxide is one of the major reactive oxygen species present after ischemia/reperfusion-induced oxidative brain injury. The hydrogen peroxide level had increased about 30% after ischemia/reperfusion. This increased level was prevented in green tea extract pretreated animals. This suggests that green tea extract acts as a scavenger of existing hydrogen peroxide, as seen in the other in vitro and in vivo systems [12,6,23]. The hydrogen peroxide levels found in the 2% green tea extract pretreated group was far lower than that found in the sham control group. This inhibitory effect of green tea extract on the hydrogen peroxide level partly results from the preventive effect on hydrogen peroxide production via increasing enzyme activity or expression of the enzyme. In fact, it was reported that pretreatment with green tea extract increased the level of catalase activity in the liver, lung and kidney [12,9,26].

The next stage was to determine whether the production of hydrogen peroxide subsequently resulted in oxidative damage to the lipids and DNA (lipid peroxidation and formation of 8-oxodG) and whether green tea pretreatment could inhibit such damage. Consistent with the hydrogen peroxide level, the lipid peroxidation products (malonaldehyde and 4-hydroxynonenal) level had increased as a result of ischemia/reperfusion. However, this increase was inhibited by pretreatment with 0.5 or 2% green tea extract for 3 weeks. The 8-oxodG level had also increased approximately 2-fold in the ischemia/reperfusion animals compared to the control animals and this increase was completely inhibited by pretreatment with green tea extract (0.5 or 2%). Unlike the inhibitory effect on lipid peroxidation, green tea did not further inhibit the endogenous

4. Discussion

Reactive oxygen radicals have been implicated in the pathogenesis of ischemia/reperfusion brain injury. The generation of reactive oxygen radicals as well as the free radical-mediated oxidative damage of macromolecules (DNA and lipid), and neuronal cell death in the processes
Fig. 3. Induction of apoptotic cells in ischemia/reperfusion brains of Mongolian gerbils. The Apoptotic cells were detected by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) method as described in Materials and methods. A; sham control brain, B; 5 min-ischemia/48 h-reperfusion brain, C; 5 min-ischemia/48 h-reperfusion brain pretreated with 0.5% green tea extracts for 3 weeks before induction of ischemia, D; 5 min-ischemia/48 h-reperfusion brain of Mongolian gerbils pretreated with 2% green tea extracts for 3 weeks before induction of ischemia, E; the number of apoptotic cells in each regions (n=6). Magnification ×200. (bar=50 μM). *; significant difference from ischemia/reperfusion group (P<0.05), # significant difference from 0.5% green tea group.
8-oxodG level. The reasons for the differential vulnerability of macromolecules to hydrogen peroxide are unclear. One possible explanation is that catalase (induced by green tea extract) can detoxify cytosolic hydrogen peroxide, but not in the nucleus where the DNA resides. However, this causative correlation between hydrogen peroxide production and increase in oxidative damage of macromolecules, in addition to the inhibitory effect of green tea extract, further confirmed the free radical hypothesis in ischemia/reperfusion-induced brain injury.

Evidence that lipid peroxidation product mediates oxidative stress-induced neuronal cell death has been demonstrated [10]. There was also a report showing the coincidence of 8-oxodG formation and cell death during ischemia/reperfusion [24]. Therefore, the inhibitory effect of green tea on cell death was examined. It was previously found that the number of surviving neuronal cells in the brain of ischemia/reperfusion animals significantly decreased in the striatum and cortex region as well as hippocampus [4,18]. In the present study, there was a clear increase in the number of apoptotic cells in the ischemia/reperfusion brain. The number of apoptotic cells as a consequence of ischemia/reperfusion was reduced by green tea pretreatment (values reported in the Results section). A similar protective effect of green tea extract on the increase in the number of apoptotic cells was detected in the cortex region. This attenuation pattern was correlated with the amelioration of the brain infarction (the correlation coefficient values were $r=0.995$ in cortex and $r=0.971$ in striatum, respectively). The infarction volume was $189\pm12\ mm^3$ in the 5 min-ischemia/48 h-reperfusion sham-operated animals, whereas it was $112\pm31\ mm^3$ in the 0.5% green tea and $76\pm11\ mm^3$ in the 2% pretreated animals. This suggests that the protective effect that green tea has on brain injury is related to the inhibitory effect on macromolecule oxidative damage, which leads to a protective effect on the ischemia/reperfusion-induced cell death.

Since ischemia generally results in significant changes in behavior due to brain damage, the effect that green tea extract has on the locomotor activity after an ischemia/reperfusion-induced change was examined. The locomotor activity had increased significantly in the ischemia/reperfusion animals. Pretreatment with green tea extract inhibited the ischemia/reperfusion-induced increase in locomotor activity measured 1 day after ischemia/reperfusion. After 3 days, the increased locomotor activity was not inhibited by green tea extract, and was maintained at a level 2 times that of the control. It has been reported that antioxidants protect ischemia/reperfusion-induced brain damage, and consequently protect behavioral deficits [2,17]. It is possible that the protective effect of green tea on brain injury might contribute to a beneficial effect on the behavioral changes.

In conclusion, these findings clearly demonstrate that green tea has a protective effect on ischemia/reperfusion-induced brain injury and behavior deficits that are at least in part the result of its antioxidant properties. This study suggests that green tea may be a useful agent for the prevention of cerebral ischemia damage. Recently, it was also found that green tea has anti-thrombosis effects in mice and anti-platelet aggregation activity in the human platelet. This may have an important influence on the re-establishment and maintenance of cerebral blood flow post ischemia [8]. The roles and mechanisms of the anti-platelet and thrombosis activities of green tea on ischemia/reperfusion-induced brain injury, and other biological functions of green tea, are currently under investigation.

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References


