Epigallocatechin Gallate Is the Most Effective Catechin Against Antioxidant Stress via Hydrogen Peroxide and Radical Scavenging Activity

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Hydrogen peroxide-induced neuronal oxidative stress is a serious threat to the nervous system. Catechins and related compounds are effective radical scavengers that protect against nerve cell damage.

Material/Methods: Here, we investigated the antioxidant property of various catechins in protecting against hydrogen peroxide, as well as their radical-scavenging activity.

Result: We found that catechins treatment effectively protected HT22 cells against H_2O_2-induced cell viability by decreasing and attenuating reactive oxidative species production in different proportions. In addition, all tested catechins performed radical scavenging activity, and partially removed the free radicals. Among all investigated catechins, epigallocatechin gallate was the most effective against ROS production and had the strongest radical-scavenging activity. These results suggest that beneficial effects were strongly related with structure of catechins, mainly because of the hydroxyl and galloyl groups.

Conclusions: In conclusion, epigallocatechin gallate is the most effective antioxidant polyphenol against hydrogen peroxide and radical-scavenging activity.

MeSH Keywords: Antioxidants • Catechin • Hydrogen Peroxide

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/911175

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Background

Oxidative stress, which is an imbalance between reactive oxygen species (ROS) and reactive nitrogen species (RNS), results in damage to DNA and oxidation proteins inside cells [1–3]. When the ROS is generated, oxidative stress usually leads to disruptions of function in normal mechanisms and cellular signaling [4–6]. Hydrogen peroxide (H2O2), one of the major types of ROS, regulates cell apoptosis and autophagy, and even causes central nervous system (CNS) [7–9] damage. ROS is essential for the intracellular signaling involved in normal activities in the CNS. Nevertheless, excessive ROS accumulation can result in cellular oxidative damage [10, 11]. One solution to this problem is antioxidant compounds present in natural sources [12]. These compounds effectively reduce ROS in cells and are consequently useful for treating human diseases, including atherosclerosis, inflammatory injuries, cardiovascular diseases, cancer, and even neurodegenerative diseases [13–16].

In living aerobic organisms, an integrated antioxidant system plays the first effective role in blocking harmful effects, in which multiple enzymatic and nonenzymatic antioxidants are involved [17,18]. In addition, antioxidant testing of natural compounds has made attracted increasing attention to their use against ROS. Catechins, main polyphenols in many food, can be direct antioxidants by scavenging reactive oxygen species [19–21]. It is comprised mainly by (+)-catechin(C), (–)-epicatechin (EC), (–)-gallocatechin (GC), (–)-epigallocatechin (EGC), (+)-catechin gallate (CG), (–)-epicatechin gallate (ECG), (–)-gallocatechin gallate (GCG) and (–)-epigallocatechin gallate (EGCG) [22]. EGCG is the most abundant polyphenol in green tea and it has been shown that a single molecule, such as EGCG, can affect diverse physiological activities and be involved in the inhibition of carcinogenesis or tumor growth *in vivo* and *in vitro* [23–25].

Interestingly, the common structure of catechins contains the diphenylpropane skeleton, and they have a saturated heterocyclic ring. These features make it lack electron delocalization between ring A and B, leading to stabilization of the phenoxyl. The delocalization enhances the antioxidant activity of these compounds [26]. Thus, the potent radical scavenging activity of catechins is due to multiple phenoxy groups. Previous studies have shown that some catechins affect DPPH radical scavenging activity, and further prove that it is pH-dependent [27,28]. However, the protective effect of catechins against ROS production has not been systemically analyzed. In addition, it is unclear how the radical scavenging activity of catechins is affected by use of various radical substrates.

In this study, we utilized the mouse hippocampal neuronal cell line HT22 as the model and investigated the structure and cytotoxicity of different catechins. The cytotoxicity was found to be concentration-dependent, and no cytotoxicity was seen when supplied with low-concentration catechins. The neural cells were more susceptible to catechins that share more phenoxy groups with their structure. EGCG had the strongest ability to attenuate H_2O_2-induced oxidative stress and thus is also the most effective at scavenging free radicals by multiple substrates. These natural compounds may be clinically useful by modulating oxidative stress-related neurodegenerative diseases in humans.

Material and Methods

Cell culture

HT-22, the mouse hippocampal neuronal cell line, was purchased from Fuxiang Biotech (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS (v/v), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. The medium was replaced every 2 days. When cell density reached about 70%, cells were exposed to the indicated catechin derivative compounds for 3 h.

Materials

Eight tea catechin derivatives – (+)-catechin(C), (–)-epicatechin (EC), (–)-gallocatechin (GC), (–)-epigallocatechin (EGC), (+)-catechin gallate (CG), (–)-epicatechin gallate (ECG), (–)-gallocatechin gallate (GCG), and (–)-epigallocatechin gallate (EGCG) – were purchased from Sigma (St. Louis, MO, USA). The compounds, purified using the HPLC system, were of analytical grade. We purchased 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), and all other chemicals from Sigma Aldrich (St. Louis, MO, USA) and all were of analytical grade.

Cell viability assay

Cell viability was measured using the Cell Counting Assay Kit-8 (CCK-8) according to the manufacturer's instructions. Briefly, 100 μL of HT22 cells were seeded into 96-well plates containing 10 000 cells. HT22 cells were routinely cultured until density reached 70%, and then supplied with different concentrations of compounds for 3 h. We added 10 μL of CCK-8 solution and incubated it for another 3 h. The absorbance at 450 nm was determined using a microplate reader. Similarly, when appropriate, H_2O_2 were added for additional 45 min before adding CCK-8 solution. Four biological repeats were done. Results are represented as the percentage of the control group.

Antioxidant properties

To determine of antioxidant activity, DPPH, ABTS+, and FRAP assays were used. DPPH radical scavenging activity was assessed as described before [29]. Four biological repeats were done for each. Briefly, DPPH stock solution was fresh prepared and dissolved in methanol with the concentration of 0.1 mM. We added 4 ml of DPPH into samples of different compounds in methanol and then shaken vigorously in the dark for 30 min. Control samples with DPPH were used without any compound added. The absorbance at 517 nm was determined. The percent quenching of DPPH was calculated based on the observed decrease in absorbance of the radicals. The radical scavenging activity was calculated by the following formula: % DPPH scavenging=[(Acontrol-Asample)×100/Acontrol].

The ABTS+ radical scavenging activity method was used [30]. ABTS+ stock solution was dissolved in water, with a final concentration at 7 mM. Supplied potassium persulfate with ABTS+ stock solution and made potassium persulfate final 2.45 mM. The mixture was kept in dark at room temperature overnight before use. The ABTS+ radical solution was diluted with ethanol to an absorbance of 0.70 at 734 nm. 1 ml of the diluted solution was added to 10 ml sample, mix briefly. The results were recorded with absorbance after 5 min. The inhibition was calculated by the following equation: % ABTS+scavenging=[(Acontrol-Asample)×100/Acontrol]

The FRAP assay was done with modifications [31]. FRAP reagent is a mixture that consist of 10 mM TPTZ in 40 mM HCl, 20 mM ferric chloride and 300 mM acetate buffer (pH 3.6) in the ratio of 1: 1: 10 (v/v/v). Incubate150 µL FRAP and 5 µL sample for 10 min in 96-well plate. The absorbance at 593 nm was determined by use of a microplate reader. Results are expressed as relative percentage of absorbance at 400 µM EGCG.

Flow cytometry

Reactive oxygen species (ROS) production was measured using 2,7-dichlorofluorescein diacetate (DCFDA) fluorescence dye as described previously [32,33]. HT22 cells were supplied with 1 µM DCFDA at 37°C for 30 min in the dark, then cells were passed through a 40-µM cell strainer before loading for flow cytometry (FACS Caliber; Becton-Dickinson). At least 10 000 cells were quantified and analyzed using CellQuest software according to the manufacturer's instructions.

Statistical

Data are presented as mean ± standard deviation (SD). The t test was used to evaluate significance of changes, with $p < 0.05$ considered significant.

Results

### Comparative structure and cell viability analyses on HT22 cells

Catechins are a group of natural compounds that share an identical basic chemical structure. They are connected by aromatic rings with several hydroxy groups [34]. Catechins can be classified into 2 groups: epistructured and nonepistructured ones. The most representative epistructured catechins include EGCG, EGC, ECG, and EC, and GCG, GC, CG, and C are the most representative nonepistructured catechins (Figure 1A).

Although catechins consist of highly similar chemist structure and groups, their biological activity might be different. To determine the cytotoxicity on mouse hippocampal neuronal HT22 cells, the cell viability assay was performed for all 8 typical catechins. The cells were supplemented with 400 µM as the highest concentration, followed by 2-fold serial dilution. No cytotoxicity was found in the compounds, including C and EC. Partial inhibition was observed in the other 6 compounds in which 200 µM or higher concentrations of compounds were used. The highest concentration of EGCG at 400 µM reduced the cell viability to almost 20%, which was significantly lower compared with others (Figure 1B). The IC50 of those compounds is listed in Table 1. These results indicate the HT22 cell cytotoxicity by catechins occurs in a concentration-dependent manner, and EGCG was the most susceptible of all tested catechins.

Protective Effect of catechins against H_2O_2-induced oxidative stress.

The cell viability of HT22 cells was not affected when individual catechins at the concentration of 100 µM were tested. Therefore, 100 µM catechins was selected as the safe concentration to HT22 cytotoxicity and used in subsequent oxidative stress experiments. The protective effect of catechins were assessed by adding H_2O_2 for an additional 45 min before the cell viability assay.

Levels of H_2O_2 cell damage were determined after addition of H_2O_2 up to 800 µM in the absence of catechins, and the cell viability was reduced to 31% (Figure 2A). We further performed the assay to determine how the individual catechins protect HT22 against H_2O_2-induced oxidative stress. Supplemented with different catechins, followed by treatment with H_2O_2, the cell viability was expressed by heat map and in plot graph (Figure 2A, 2B). Pretreatment with different concentrations of catechins did not significantly affect the cell viability. Most of catechins, especially EGCG, protect against H_2O_2-induced damage. When 100 µM EGCG was used, followed by 800 µM H_2O_2, the cell viability was rescued to up to 60%. However, C and EC do little to protect the HT22 cells from damage caused
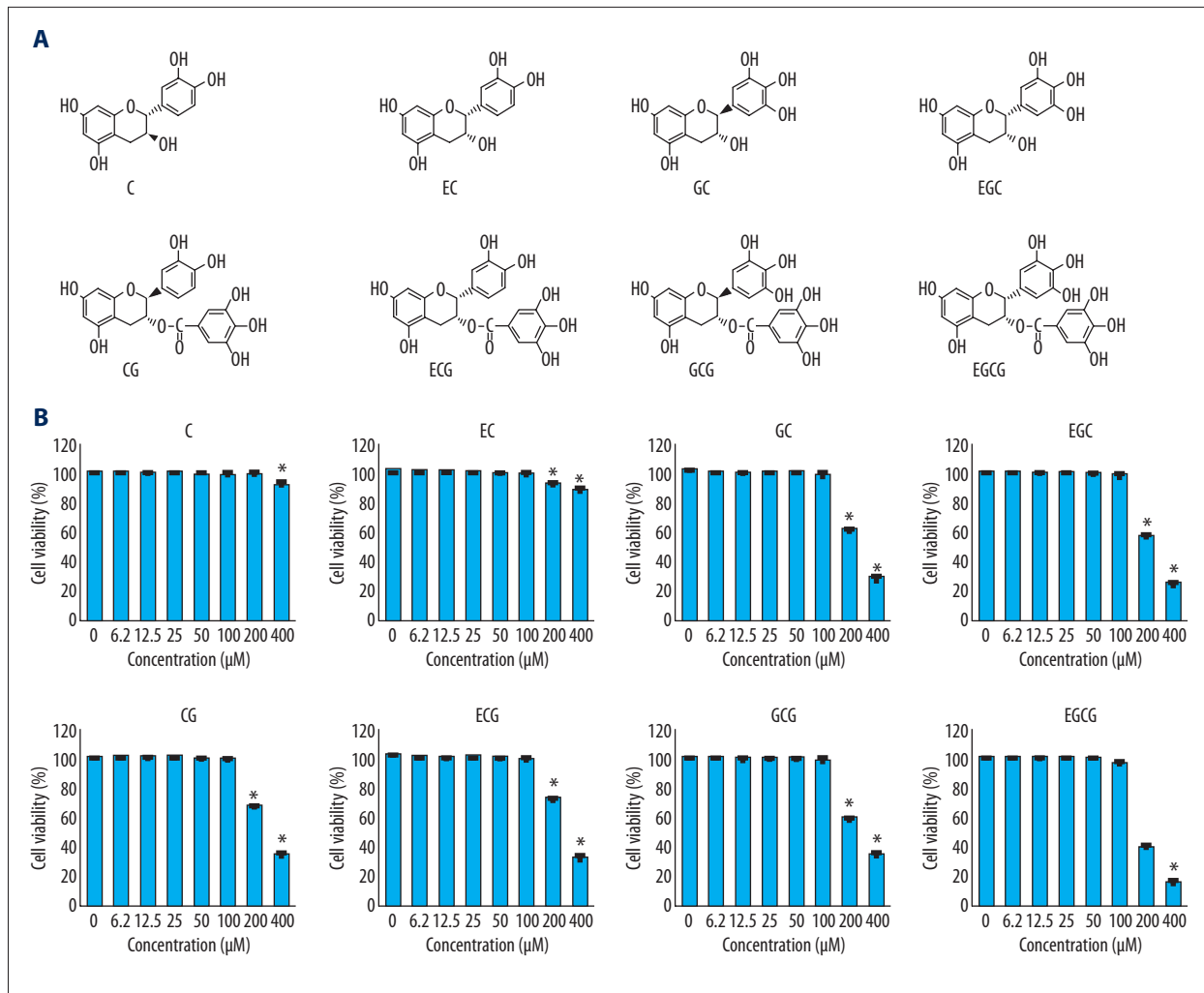
Figure 1. Effects of green tea polyphenols on HT22 cell viability. (**A**) Chemical structures of investigated compounds C, EC, GC, EGC, CG, ECG, GCG, and EGCG. (**B**) Compounds C, EC, GC, EGC, CG, ECG, GCG, and EGCG, were added for 24 h at the indicated concentrations. Cell viability (% control) was measured using CCK-8 assay, as detailed in Methods. Values are expressed as the means ±S.D. (n=5). Statistically significant differences are indicated by asterisks as follows; * P<0.05, ** P<0.01, and *** P<0.001 (compared with DMSO-treated cells at each time point) using a two-tailed t test (n=4).

Table 1. IC50 of various catechins.

IC50 (µM)	Compound							
	C	EC	GC	EGC	CG	ECG	GCG	EGCG
Mean ±SD	>400	>400	195.2±1.5	190.6±2.2	201.3±3.1	218.0±4.4	183.8±1.4	170.3±2.0

by H_2O_2. Taken together, these results suggest that EGCG has a strong protective effect on H_2O_2-induced cell stress in a concentration-dependent manner.

Catechins reduce H_2O_2-induced ROS production.

Reactive oxygen species (ROS) are chemically reactive chemical species containing oxygen, which is strongly associated with environmental stress, and H_2O_2 is a representative inducer [35]. Next, we performed flow cytometry to quantify ROS production by using 2,7-dichlorofluorescein diacetate (DCFDA) (Figure 3). We added 100 µM of individual catechins to HT22 cells at 70–80% confluency or 12 h, then 800 µM H_2O_2 was supplied for 45 min. ROS levels increased dramatically. The DCFDA-positive cells comprised 78.63%, compared to 0.87% with the control groups in which no H_2O_2 was used. The proportion of
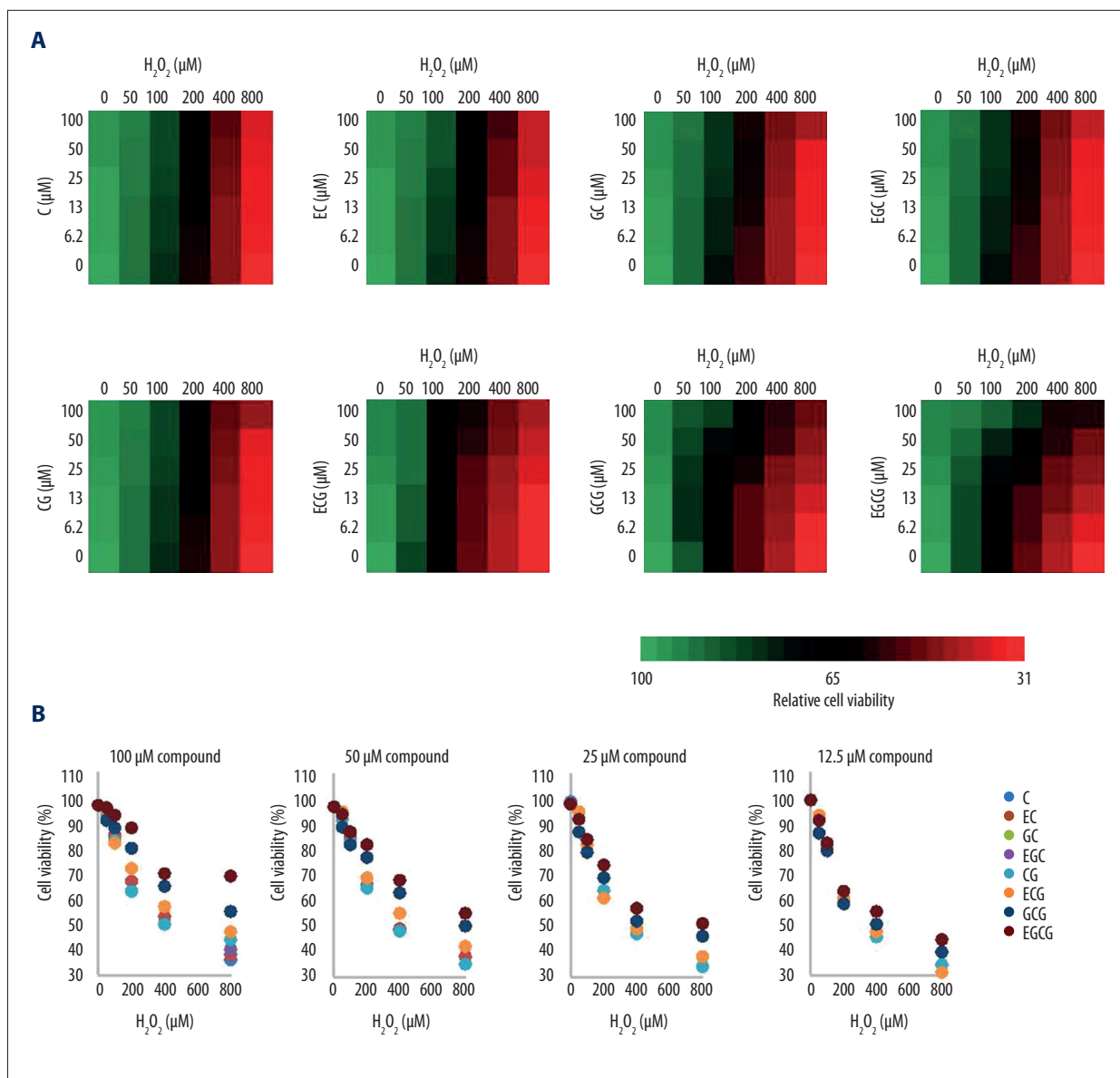
Figure 2. The protective abilities of catechins on H$_2$O$_2$ induced decreased HT22 cell viability. (**A**) Cell viability assay results in heat map. (**B**) Cell viability assay results in plot figure.

DCFDA-positive cells was almost identical when C, EC, or GC were added, and those selected catechins could not reduce ROS production. In contrast, the other 5 catechins partially reduced ROS production. It is not surprise that EGCG has the strongest ability to protect the cells from oxidative stress, in which the ROS production was reduced to 5.43%. Our results indicate that EGCG is one of the strongest catechins against H$_2$O$_2$-induced ROS production.

Radical scavenging activity assay

Free radicals are important for biochemical processes. These reactive species play significant roles in oxidative stress-related

diseases [36]. Therefore, we evaluated radical scavenging activity of selected catechins *in vitro*. DPPH, ABTS+, and FRAP assays were used. Various concentrations of catechins were determined, including 6.2, 25, 100, and 400 µM of each compound. The DPPH assay results decreased in absorbance of the DPPH, which is due to the radical scavenging abilities of the catechins (Table 2). The ability of different catechins to scavenge DPPH radical increased when higher-concentrations compounds are used. When 400 µM of catechins was used, the radical scavenging abilities of the compound decreased in the following order: EGCG>ECG>GCG>CG>EGC>GC>EC>C. The DPPH scavenging rate of EGCG was 77.2%, while the DPPH scavenging rate of C was only 32.3%. A similar result of antioxidant activity
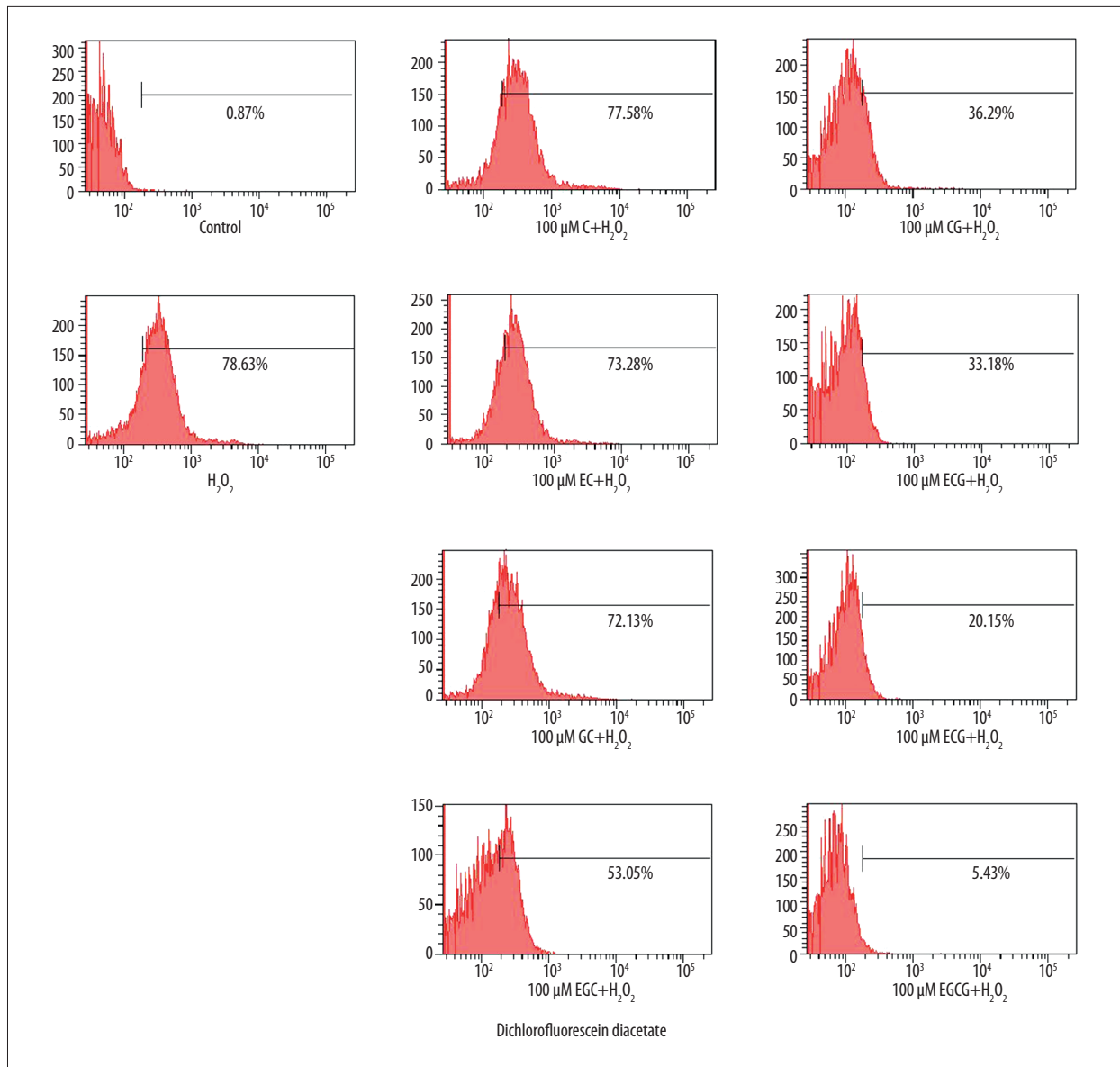
Figure 3. Catechins protect against H_2O_2-induced ROS production in HT22 cells. ROS production was measured using fluorometric probe DCFDA by flow cytometry.

was also found by ABTS+ assay (Table 3). When 400 µM EGCG was used, the results showed the best ability of scavenging ABTS+, and the results decreased to 90.2%, while the same concentration of C inhibited ABTS+ to only 38.2%. The results were also consistent when FRAR assay were used, which indicated EGCG is the most effective compound for radical scavenging activity (Table 4). These results are due in part to the higher number of polyphenols in EGCG and suggest that EGCG is the most active compound.

Discussion

Catechins are a group of polyphenols products, mainly existing in natural plants. They exhibit antiinflammatory, microvascular, anticarcinogenic, and antioxidant properties [37–40]. Recently, more attention has been focussed on dietary components that play essential roles in neurodegenerative disease, especially those involving polyphenols. Many studies have focused on the beneficial effect of EGCG in inhibition of ROS production, but few studies systematically investigated all representative catechins regarding H_2O_2-induced nerve cell damage. In the present study, we established a H_2O_2-induced cell model by using HT22 as the target cell. We reported the partial inhibition effects of

Table 2. DPPH scavenging rate at different concentrations.

Concentration (μM)	Compound							
	C	EC	GC	EGC	CG	ECG	GCG	EGCG
6.2	5.4±0.1	6.6±2.1	6.9±2.8	7.3±2.2	6.8±1.1	13.1±1.2	13.5±1.5	13.3±2.0
25	11.2±1.1	13.4±2.7	15.5±2.8	17.5±2.5[a]	19.3±2.4[a]	28.1±2.2[a]	27.1±2.4[a]	28.2±2.4[a]
100	21.3±2.7[b]	31.2±2.6[b]	45.3±6.1[b]	43.4±2.7[b]	41.8±2.2[b]	48.3±2.3[b]	44.5±2.6[b]	50.3±4.1[b]
400	32.3±5.1[c]	40.3±4.7	52.3±5.7	53.3±4.1[c]	62.8±3.5[c]	73.3±2.9[c]	68.2±3.4[c]	77.2±4.3[c]

Data are expressed as mean ±SD of n=3; [a–c] values in the same column followed by a different letter represent a significant difference at $p<0.05$.

Table 3. ABTS+ scavenging rate at different concentrations.

Concentration (μM)	Compound							
	C	EC	GC	EGC	CG	ECG	GCG	EGCG
6.2	5.1±0.8	6.6±2.5	6.4±2.4	7.3±2.3	9.8±1.2	10.5±1.6	15.5±2.1	17.2±2.1
25	21.2±1.2[a]	23.5±1.7[a]	25.3±2.5[a]	27.5±2.8[a]	29.3±3.8[a]	42.1±3.5[a]	47.1±2.8[a]	48.5±2.8[a]
100	29.3±2.5[b]	41.8±2.2[b]	45.8±4.2[b]	50.4±2.9[b]	47.7±2.5[b]	58.3±5.3[b]	64.5±2.6[b]	66.1±2.8[b]
400	38.2±3.5[c]	43.9±4.5	62.4±5.8[c]	62.3±5.2[c]	67.8±5.7[c]	79.3±2.8[c]	82.2±3.8[c]	90.2±3.1[c]

Data are expressed as mean ±SD of n=3; [a–c] values in the same column followed by a different letter represent a significant difference at $p<0.05$.

Table 4. FRAP scavenging rate at different concentrations.

Concentration (μM)	Compound							
	C	EC	GC	EGC	CG	ECG	GCG	EGCG
6.2	15.8±2.8	18.6±2.0	23.8±2.1	27.8±2.8	38.7±1.8	42.3±3.3	53.5±2.8	53.7±2.4
25	21.8±2.3	25.4±1.7	32.5±2.5	35.7±3.1[a]	49.9±2.8[a]	58.1±2.2[a]	77.1±2.8[a]	78.2±2.8[a]
100	31.5±2.9[b]	38.8±5.6[b]	48.3±6.4[b]	52.6±2.4[b]	61.2±2.8[b]	78.3±6.3[b]	81.2±2.8[b]	92.3±3.7[b]
400	42.5±5.9[c]	42.2±2.8	52.7±4.7	63.8±4.1[c]	75.7±2.1[c]	89.1±5.9	95.5±3.9[c]	100±3.1

Data are expressed as mean ±SD of n=3; [a–c] values in the same column followed by a different letter represent a significant difference at $p<0.05$.

all catechins polyphenols and free radical scavenging activity. The present study demonstrated that EGCG or its derivative can protect neurons against oxidative stress-induced neurodegeneration. EGCG is the most effective polyphenols against H_2O_2-induced HT22 cell stress and exhibits a strong ability to reduce ROS production and radical scavenging.

The structural difference between these catechins is the number of hydroxyl groups on the B-ring and the presence of a galloyl group, which makes cell viability of each catechin different (Figure 1A, 1B). The present study revealed the important structural element contributing to the inhibition of decreasing cell viability by H_2O_2 (Figure 2A, 2B). It is further proved by flow cytometry that those polyphenols attenuated the ROS production, of which EGCG has the strongest effect. These results and observations also strongly indicate that the galloyl group is essential during antioxidation. In addition, structure-related activity analyses of catechins identified the substructures that contribute to biological functions of catechins. EGCG, containing both a galloyl group and a B-ring linked with a pyrogallol structure, exhibited greater activities than EGC or ECG, which have only partial structure (either pyrogallol structure or gallate group) [41,42].

In the present study, DPPH, ABTS+, and FRAP assays were used to investigate the radical scavenging activity of catechins. Different oxidants result in the formation of different oxidation products with catechins. We found that the antioxidant action of catechins not only depends on the oxidant used, but also depends on the structures (Figure 1A, Tables 1–3). Our results may help in evaluating the antioxidant functions of catechins in biological systems.

Conclusions

Catechins, especially EGCG, have been widely used in clinical trials as potential modulators [43,44]. Preclinical and clinical studies have shown that oxidative stress associated with the inflammatory response is one of the main determinants of secondary induced brain damage. Additionally, neutrophils and macrophages are integrated in the inflammatory induced response and H_2O_2-induced oxidative stress [45–47]. There is urgent need to identify novel strategies to reduce the oxidative stress and its secondary damage due to the inflammatory cascade. Thus, these compounds might serve as a unique therapy against oxidative stress-related neurodegenerative disease.

Conflicts of interest

None.

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