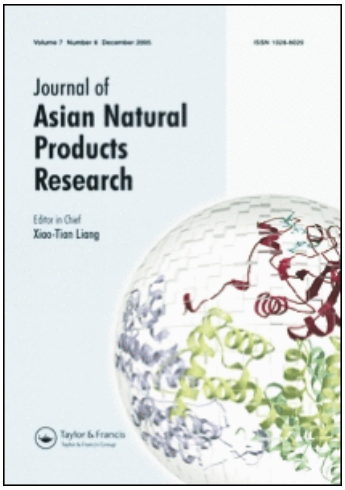

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Elevated levels of DNA repair enzymes and antioxidative enzymes by ( )-catechin in murine microglia cells after oxidative stress

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To cite this Article Huang, Qing , Wu, Li-Jun , Tashiro, Shin-Ichi , Onodera, Satoshi and Ikejima, Takashi(2006) 'Elevated levels of DNA repair enzymes and antioxidative enzymes by ( )-catechin in murine microglia cells after oxidative stress', Journal of Asian Natural Products Research, 8: 1, 61 — 71
To link to this Article: DOI: 10.1080/10286020500209087
URL: http://dx.doi.org/10.1080/10286020500209087

Journal of Asian Natural Products Research,
Vol. 8, No. 1–2, January–March 2006, 61–71

Elevated levels of DNA repair enzymes and antioxidative enzymes by (+)-catechin in murine microglia cells after oxidative stress

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(+)-Catechin possesses a broad range of pharmacological properties, including antioxidative effect. However, little is reported on the mechanism by which (+)-catechin protects microglia cells from DNA damage by oxidative stress. In this study, TUNEL assay and DNA electrophorysis indicated that (+)-catechin markedly blocked DNA fragmentation and apoptosis of microglia cells by tBHP exposure. A potent antioxidative effect of (+)-catechin was confirmed by comparison with a putative antioxidant agent, *N*-acetylcysteine at the lower doses. Furthermore, the increased intracellular ROS by tBHP exposure were scavenged by elevated activities of catalase (CAT) and superoxide dismutase (SOD) after (+)-catechin treament. (+)-Catechin partially inhibited the activation of caspase-3, thereby both cleavage of poly (ADP-ribose) polymerase (PARP) and degradation of inhibitor of caspase-activated DNase (ICAD) were effectively abolished. In addition, the expression of PARP for repair of impaired DNA was significantly increased by (+)-catechin treatment. Taken together, these data suggest that protective effects of (+)-catechin against oxidative DNA damage of microglia cells is exerted by the increased expression of DNA repair enzyme PARP and antioxidant enzyme activities.

Keywords: (+)-Catechin; DNA repair; Caspase-3; PARP; ICAD; Mircroglia

1. Introduction

(+)-Catechin (figure 1), a polyphenolic flavanoid-derived compound, is widely contained in many kinds of Chinese herbs, such as *Acacia catechu L.*, cistus species [1,2]. Remarkably, it also exists in a variety of foods and drinks, such as apple, kiwi fruit, and tea [3–5]. In its various pharmacological and physiological effects, the antioxidant activity has been well documented [6–8]. In addition, its neuroprotective effect [9] and chemoprevention [10] were also reported in recent researches.

A growing body of evidences has suggested that abundant generation of reactive oxygen species (ROS) is one of the important causes of neurodegenerative

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Journal of Asian Natural Products Research
ISSN 1028-6020 print/ISSN 1477-2213 online © 2006 Taylor & Francis
http://www.tandf.co.uk/journals
DOI: 10.1080/10286020500209087

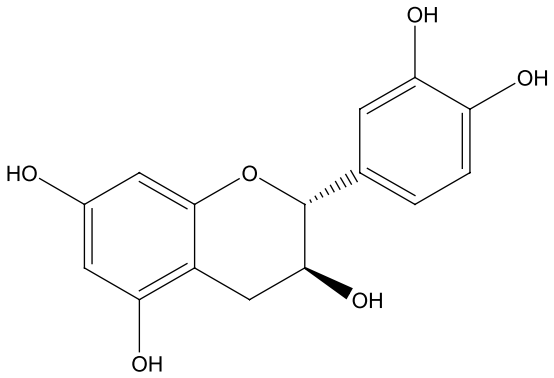

Figure 1. Structure of (+)-catechin.

diseases [11–13], such as Parkinson's disease (PD) and Alzheimer's disease (AD). As we known, excessive generation of ROS impaired cellular membrane, organella, and DNA, subsequently caused cell apoptosis or necrosis, if it was not removed effectively. Apoptosis is characterized by a loss of cell volume, plasma membrane blebbing, and orderly DNA fragmentation. Accumulating evidences have suggested that oxidative stress may cause cell apoptosis via activated caspase family and/or mitochondrial pathway [14,15].

Among masses of apoptotic signaling pathways, caspase pathway is the most documented one. In this family, caspase-3 plays a crucial role in cell apoptosis, and it is activated from an inactive precursor by upstream caspases. Activated caspase-3 acts as an apoptotic executioner to cleave its substrates including ICAD. Then cleaved ICAD leads to activation of a latent cytosolic endonuclease, CAD, which cleaves DNA into oligonucleosomal fragments. PARP is another pivotal downstream signaling molecule of caspase-3. Importantly, PARP participates in the repair of DNA damage in response to the exposure of ROS, UV irradiation, and alkylating agents [16–18].

Microglia, a kind of cells of the macrophage lineage in the central nervous system (CNS), protects neurons from the damage by bacteria or toxins. Abnormal apoptosis of microglia cells could result in dysfunction of immunity in CNS [19]. In this study, we investigated whether (+)-catechin can prevent murine microglia cells from *tert*-butylhydroperoxide (tBHP)-induced apoptosis, and whether the oxidative DNA damage can be repaired by (+)-catechin treatment *in vitro*.

2. Results and discussion

2.1 Inhibition of cell growth by the exposure of tBHP

In many studies [20,21], tBHP was utilized as an oxidant agent to induce the inhibition of cell proliferation and to cause apoptosis. In this study, murine microglia cells were exposed to different concentrations of tBHP ranging from 0.1 to 0.6 mmol L^{-1} for 24 h, and then the growth inhibiting effect was measured by MTT method. The results showed that tBHP induced cell death in a dose-dependent manner (figure 2).

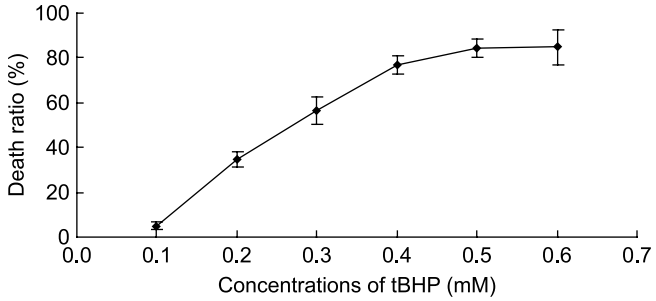

Figure 2. Effect of tBHP-induced microglia cell death. 1×10^4 cells were treated with different concentrations of tBHP ranging from 0.1 to 0.6 mmol L^{-1} for 24 h. Cell death ratio was analyzed by MTT method. n = 3, Mean ± S.D.

2.2 Protective effect of (+)-catechin on DNA fragmentation by oxidative stress

To test the protective effects of (+)-catechin on tBHP-induced apoptotic phenomenon, including DNA fragmentation and chromatin condensation, DNA from cells treated as indicated in experimental section was extracted and detected by 2% agarose gel electrophoresis. (+)-Catechin completely inhibited the nucleosome-sized DNA fragmetation, shown as ladder pattern (figure 3A). We also observed the tBHP-exposed cells showed apoptosis characteristic nuclear fragmentation. However, treatment of (+)-catechin completely protected cells from morphological changes by tBHP (figure 3B). Therefore, (+)-catechin effectively blocked oxidative DNA impairment.

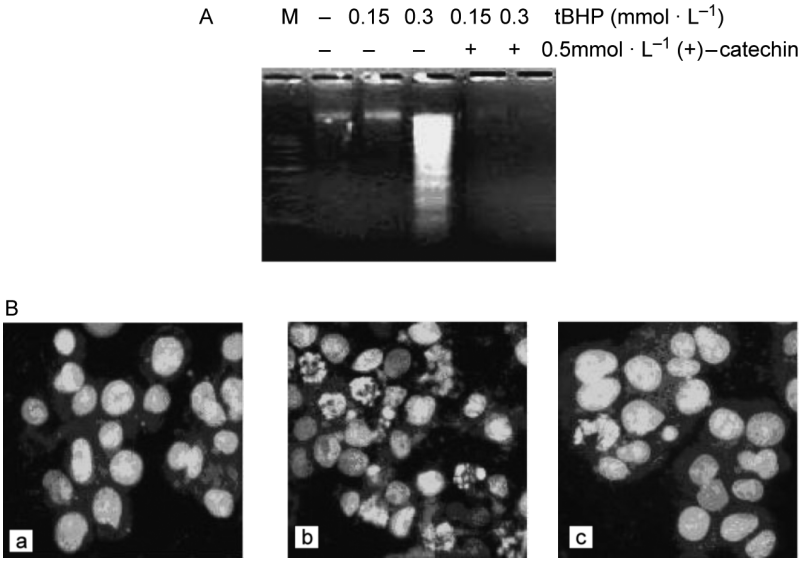

Figure 3. Protective effect of (+)-catechin on N9 cell nuclei and DNA fragmentation by tBHP exposure. A: N9 cells were treated with or without 0.5 mmol L^{-1} (+)-catechin after exposed to 0.15 and 0.3 mmol L^{-1} tBHP for 1 h, respectively. After 12 h, the cells were collected and DNA fragments were analyzed by 2% agarose gel electrophoresis. Hae-III-digested phage ϕx-174 DNA fragments were used as molecular markers (M). B: Nuclear changes were observed by using Hoechst 33258 staining after indicated treatment for 12 h (magnification × 200). N9 cells were incubated in medium alone (a). Cells were exposed to 0.3 mmol L^{-1} tBHP for 12 h without (b) and with (c) 0.5 mmol L^{-1} (+)-catechin treatment.

2.3 Inhibitory effect of (+)-catechin on tBHP-induced apoptosis by TUNEL assay

To further determine the number of DNA fragmented nuclei, TUNEL assay was carried out. In medium control group, the ratio of TUNEL-positive cell was 5.3 ± 0.8%. After exposure to 0.3 mmol L^{-1} tBHP for 12 h, apoptotic cell numbers increased to 39.4 ± 3.7%. However, the ratio of apoptotic cells significantly decreased to 8.9 ± 2.1% after treatment with 0.5 mmol L^{-1} (+)-catechin (table 1). The changes of the percentage of TUNEL-positive cells in the three groups indicated that (+)-catechin availably inhibited tBHP-induced DNA damage and cell apoptosis.

2.4 Scavenging effects of (+)-catechin on intracellular ROS by tBHP

To investigate the effect of (+)-catechin on the generation of intracellular ROS, cells were exposed to 0.3 mmol L^{-1} tBHP for 1 h, and then treated with 0.5 mmol L^{-1} (+)-catechin for 12 h. Intracellular ROS was detected by fluorescent microscopy using a peroxide-sensitive fluorescent probe, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). In the presence of intracellular ROS, DCFH-DA is oxidized to fluorescent 2′,7′-dichlorodihydrofluorescein (DCF), which is easily detected by using fluorescent microscopy [22]. The results of fluorescent intensity analysis indicated that the generation of intracellular ROS increased in the tBHP exposure group, but (+)-catechin completely cleaned the excessive of intracellular ROS by tBHP exposure (figure 4). As expected, (+)-catechin revealed a strong ROS scavenging effect.

2.5 Comparison of protective effects of (+)-catechin and *N*-acetylcysteine (NAC) on microglia cells after oxidative stress

NAC has many pharmacological functions such as anti-inflammatory activity, anti-angiogenetic function, immunological activity, and inhibition to progression of malignancy. It also can treat the acetaminophen isntoxication, and provide protection of liver from toxic damage by alcohol or CCl_4 [23,24]. In addition, it exerts a strong antioxidant activity, especially towards DNA damage in response to oxidative stress [25]. NAC presents regulative effect on DNA repair, inhibition of genotoxicity, and modulation of gene expression [26]. To evaluate the antioxidative effect of (+)-catechin, we compared the survival ratio of (+)-catechin treatment group with that of NAC treatment group at 24, 36, 48 h. As the results shown, the survival ratio achieved 64.63 ± 5.85% after treatment with 1 mmol L^{-1} (+)-catechin at 24 h, but it is only 26.64 ± 4.27% after treatment with similar concentration of NAC at the same time. We also found that the percentages of survival cells in (+)-catechin treatment group (figure 5a) were far more than those in NAC treatment group

Table 1. Quantitive analysis of TUNEL-positive N9 cells.

Groups	*Apoptotic cells (%) (TUNEL positive)*
Medium control	5.3 ± 0.8
0.3 mM tBHP	39.4 ± 3.7**
0.3 mM tBHP +0.5 mM (+)-catechin	8.9 ± 2.1##

The cells were treated with or without 0.5 mmol L^{-1} (+)-catechin for 12 h after exposure to 0.3 mmol L^{-1} tBHP for 1 h. The results are representative of three independent experiments. All data were presented as Mean ± S.D. and considered statistically significant at $^{**}p < 0.01$ *vs* medium control group, $^{\#\#}p < 0.01$ *vs* 0.3 mmol L^{-1} tBHP exposure group.

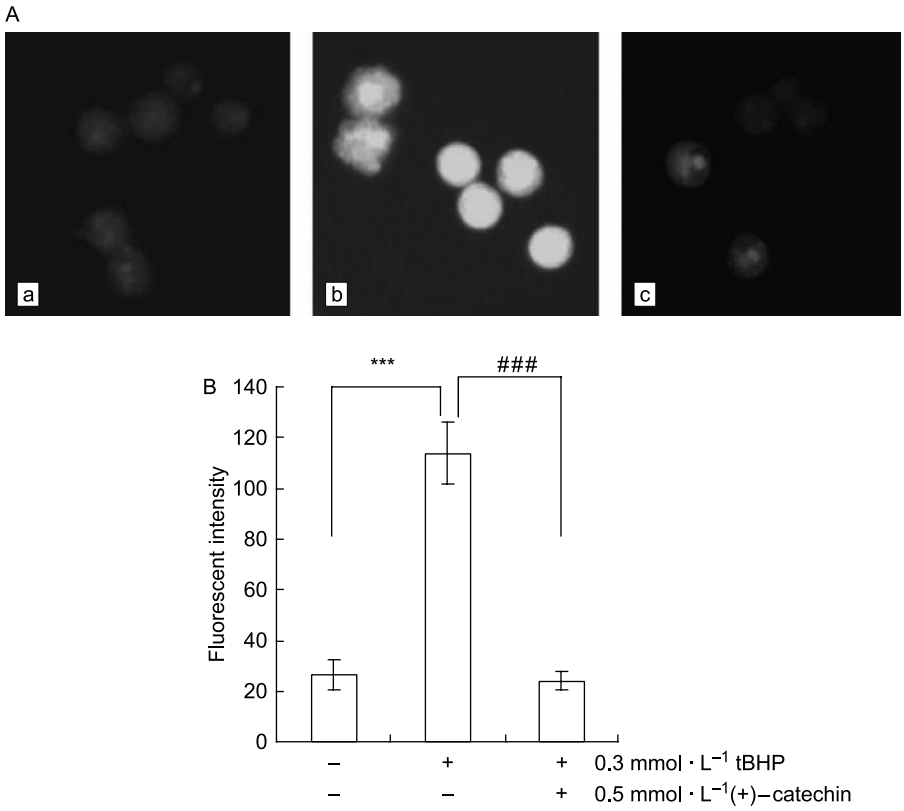

Figure 4. The effect of (+)-catechin scavenging intracellular ROS. A: The generation of ROS was detected by using a peroxide-sensitive fluorescent probe DCFH-DA. (a) medium control group. (b) 0.3 mmol L^{-1} tBHP-exposed group. (c) 0.5 mmol L^{-1} (+)-catechin treated group, which preincubated in 0.3 mmol L^{-1} tBHP for 1 h. B: The fluorescent intensities were analyzed by using Meta Imaging Series 4.5 Software. The results were representative of three independent experiments. All data were presented as Mean ± S.D. $^{***}p < 0.001$ *vs* medium control group, $^{\#\#\#}p < 0.001$ *vs* tBHP-exposed group.

(figure 5b) at the concentrations ranging from 0.063 to 1 mmol L^{-1} at the indicated times. Therefore, (+)-catechin has the more efficacious inhibition on cellular oxidative stress at the lower doses.

2.6 Changes of the activities of SOD and CAT

There are some intracellular enzymes, such as SOD, CAT, and glutathione peroxidase (GPx), which protect cells from the impairment by ROS. Superoxide ($O_2^{\cdot -}$) is detoxified by SOD through converting two molecules of $O_2^{\cdot -}$ to yield one molecule each of O_2 and H_2O. CAT and GPx work stimultaneously with glutathione to reduce hydrogen peroxide and ultimately produce water. All these enzymes repair oxidized DNA, degrade oxidized protein, and destroy oxidized lipid [27,28]. In this study, we determined the activities of the antioxidant enzymes SOD and CAT. As the results shown in table 2, the activities of both SOD and CAT significantly decreased in the 0.3 mmol L^{-1} tBHP exposed group at 12 h. After treatment with 0.5 mmol L^{-1} (+)-catechin, the level of CAT activity was markedly upregulated, and the activity of SOD increased slightly. Therefore, the increased activities of CAT and SOD in

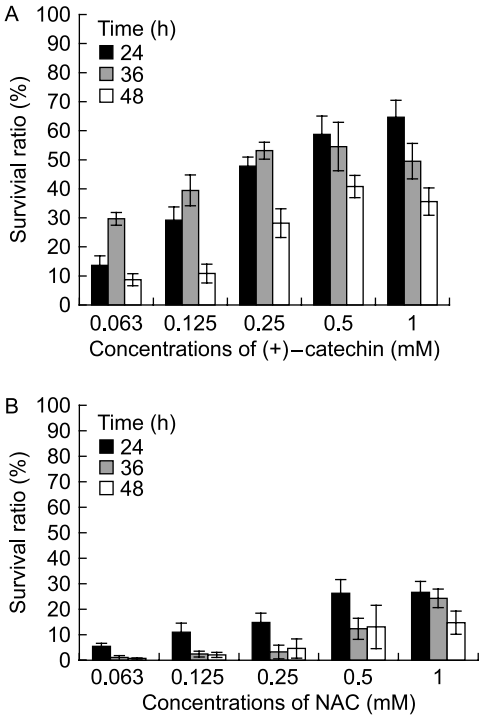

Figure 5. Comparison of antioxidant effects of (+)-catechin and NAC. N9 cells were exposed to 0.3 mmol L^{-1} tBHP for 1 h, and then were treated with different concentrations of (+)-catechin (a) and NAC (b) for 24, 36, 48 h, respectively. Survival ratio was analyzed by MTT method. n = 3, Mean ± S.D.

(+)-catechin treated cells may play crucial roles in preventing microglia cells from impairment in response to the exogenous oxidants.

2.7 Involvement of caspase-3 in tBHP-induced N9 cell apoptosis

In various cell types undergoing apoptosis, caspase-3 constitutes the major pool of executor caspase. To investigate whether caspase-3 was involved in cytotoxicity of tBHP, the expression of procaspase-3 protein was detected by western blot analysis. As the result shown in figure 6, the expression of procaspase-3 began to decline after tBHP exposure for 3 h, and it significantly decreased at 9 h. These change revealed that procaspase-3 was cleaved and activated. In addition, PARP, one of the substrates of activated caspase-3, was cleaved into 85 kDa at 6 h. The results indicated that N9 cell apoptosis was attributed to activated caspase-3, which caused PARP cleavage.

Table 2. Activities of SOD and CAT.

Groups	*SOD (kNU g^{-1} protein)*	*CAT (kU g^{-1} protein)*
Medium control	32.15 ± 2.38	9.62 ± 0.39
0.3 mM tBHP	21.43 ± 1.07**	5.31 ± 0.47**
0.3 mM tBHP +0.5 mM (+)-catechin	25.86 ± 3.41$^{\#}$	11.24 ± 0.98$^{\#\#}$

N9 cells preincubated in 0.3 mmol L^{-1} tBHP for 1 h, and then were treated without or with 0.5 mmol L^{-1} (+)-catechin for 10 h. Data were considered statistical significant at $^{**}p < 0.01$ *vs* medium control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs* 0.3 mmol L^{-1} tBHP exposure group. n = 3, Mean ± S.D.

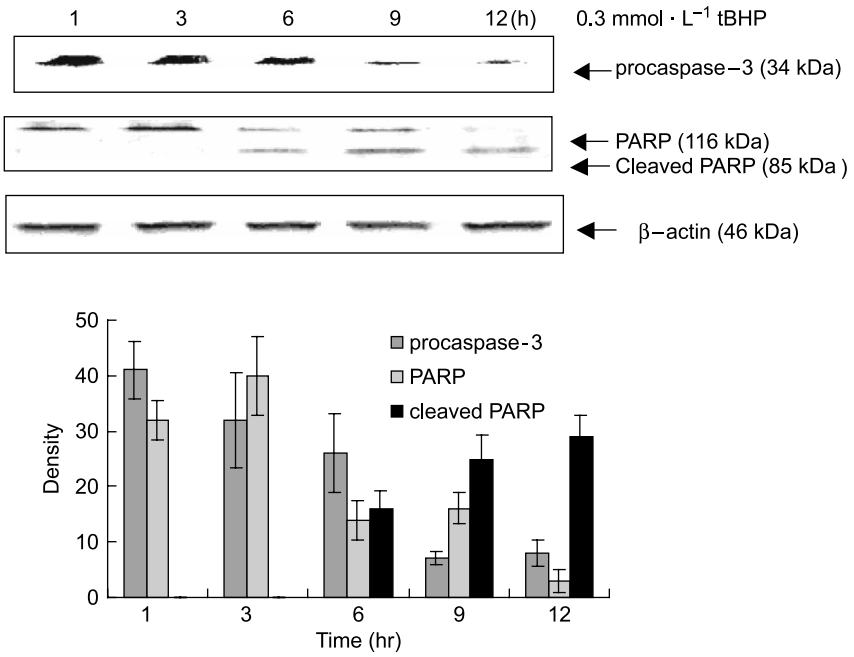

Figure 6. Activation of caspase-3 by the exposure of 0.3 mmol L^{-1} tBHP on microglia cells. Western blot analysis was carried out to detect the changes of procaspase-3 and PARP in different time points. Data shown are shown Means ± S.D. and were obtained from three independent experiments carried out in triplicate. Density of each band was quantitated by NIH image 1.63 software, and expressed in the lower column diagram.

2.8 Involvement of PARP on the repair of oxidative N9 cell DNA by (+)-catechin treatment

PARP protein plays an important role in DNA repair. It contains three functional domains: a DNA-binding domain (DBD) located at the amino terminus; a central automodification domain that acts as an acceptor for poly (ADP-ribose); and a catalytic domain located at the carboxyl terminus. While single-strand breaks arise in DNA by the effects of ROS or DNA-damaging agents, the DBD rapidly recongnizes and binds to the strand breaks. PARP participates in the strand-break repair process, either by affecting the higher-order chromatin structure or by protecting unrejoined strand breaks or helping to recruit repair enzymes [29]. In this study, (+)-catechin markedly abolished the digestion of PARP and significantly increased the expression of PARP to protect murine microglia cells from DNA damage by tBHP exposure (figure 7, upper panel). DNA fragmentation, an inreversible DNA damage, is dependent on the released CAD from its inhibitor ICAD, which is cleaved by caspase-3. (+)-Catechin blocked the activition of caspase-3, subsequently decreased the degradation of ICAD that inhibited DNA fragmentation by the release of CAD (figure 7, middle and lower panels).

In conclusion, (+)-catechin possesses potent antioxidant effect and DNA repair effect on microglia cells. In one hand, it enhanced the activities of antioxidant enzymes CAT and SOD to scavenge intracellular ROS and repair oxidized DNA. In the other hand, it inhibited the activation of caspase-3, which causes cell apoptosis and degrades PARP and ICAD, and significantly increased the expression of PARP to repair DNA lesion. Therefore, (+)-catechin may be a neuroprotective agent against microglia cell injury by ROS.

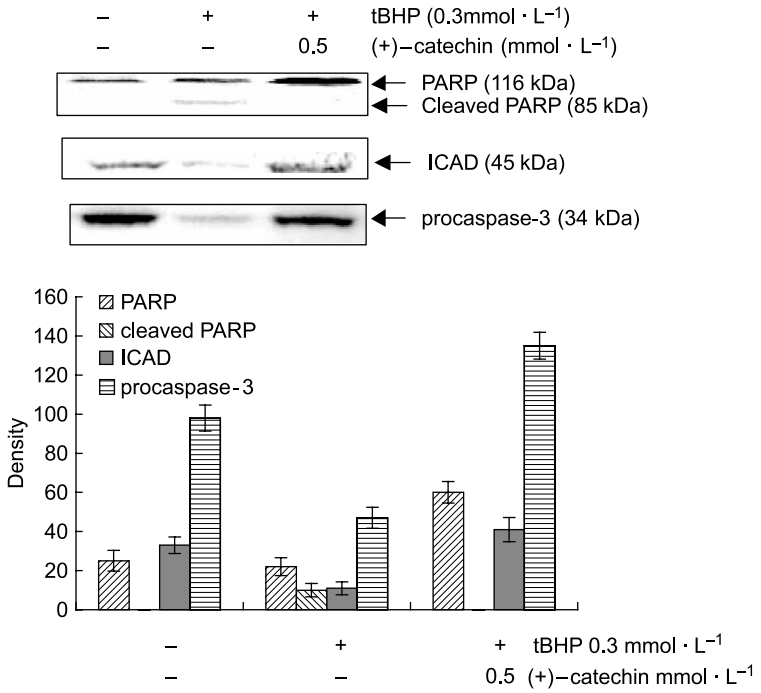

Figure 7. Increased expression of PARP and inhibited activation of caspase-3 by (+)-catechin treatment. After indicated treatment for 12 h, the cells were collected and lysated. The same amount of cell lysate was resolved on 12% SDS-PAGE and immunoblotted with PARP, ICAD, and caspase-3 antibodies. Data shown are shown Means ± S.D. and were obtained from three independent experiments carried out in triplicate. Density of each band was quantitated by NIH image 1.63 software, and expressed in the lower column diagram.

3. Experimental

3.1 Chemicals

(+)-Catechin was obtained from Beijing Institute of Biologic Products (Beijing, China). The purity was measured by HPLC and determined to be above 98%. TACS^{TM}2 TdT-DAB *In Situ* Apoptosis Detection Kit was purchased from Trevigen (Gaithersburg, MD, USA). Propidium iodide (PI), ethidium bromide, proteinase K, *N*-acetylcysteine, 3[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium, RNase A, dithiothreitol (DTT), and 3,3-diaminobenzidine tetrahydrochloride (DAB) were purchased from Sigma (St. Louis, MO, USA). 2′,7′-dichlorodihydrofluorescein diacetate was obtained from Molecular Probes (Eugene, OR, USA.). Iscove's modified dulbecco's medium (IMDM) and horse serum were purchased from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) was from Dalian biological reagent company (Dalian, Liaoning, China). Rabbit polyclonal antibodies against PARP, ICAD, caspase-3, and horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

3.2 Cell culture and viability assay

The murine microglia cell line (N9) was generally provided from Dr Wu Chunfu (Department of Pharmacology, Shenyang Pharmaceutical University, Shenyang, China).

The cells were cultured in IMDM with 5% FBS, 5% horse serum, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. N9 cells were seeded into 96-well plates (NUNC, Denmark) at 1×10^4 cells per well in 100 μL IMDM overnight. After preincubation with indicated concentration of tBHP at given concentrations for 1 h, cells were added by various concentrations of (+)-catechin. Viabilities were measured by MTT assay using a Plate Reader (Bio-Rad, USA).

3.3 Fluorescent staining of nuclei

The nuclei of N9 cells were stained with chromatin dye, Hoechst 33258. Cells were fixed with 3.7% paraformaldehyde for 30 min. After fixation at room temperature, cells were washed twice with PBS, and then incubated with 10 μM Hoechst 33258 in PBS at room temperature for 30 min. After washed, cells were observed at 360 nm under fluorescence microscope (Leica, Nussloch, Germany).

3.4 DNA extraction and fragmentation assay

N9 cells (1×10^6), treated with different doses of tBHP with or without (+)-catechin, were harvested and centrifuged at $150 \times g$ for 5 min, and then washed once with PBS. The cellular pellet was resuspended in lyses buffer (10 mmol L^{-1}, Tris-HCL pH 7.4, 10 mmol L^{-1} EDTA, pH 8.0, 0.5% Triton X-100) on ice for 30 min. The supernatants were incubated with 40 μg L^{-1} proteinase K and 40 μg L^{-1} RNase A at 37°C each for 1 h, respectively. Then 5 mol L^{-1} NaCl and 50% isopropyl alcohol were added to it at −20°C overnight. DNA was separated by 2% agarose gel electrophoresis and stained with 0.1 mg L^{-1} ethidium bromide. After electrophoresis, gels were illuminated with ultraviolet light for examination and photography.

3.5 Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

The TUNEL Assay was used for detection of DNA strand breaks and apoptosis. The detection was carried out according to the instructions of TACS^{TM}2 TdT-DAB In Situ Apoptosis Detection Kit. Briefly, the cells were rinsed once with PBS and fixed in 3.7% buffered formaldehyde at room temperature for 10 min. The fixed sections were pretreated with 10% H_2O_2, and end-labeling was performed with TdT labeling reaction mix for 37°C for 1 h. Nuclei exhibiting DNA fragmentation were visualized by incubation in DAB for 7 min. At last, the section were counterstained with methyl green and observed by light microscopy. The nuclei of apoptotic cell were stained dark brown, and TUNEL-positive N9 cells were determined by randomly counting 100 cells.

3.6 Measure of intracellular ROS

1×10^5 Cells were incubated in 25 mm dishes with coverslips. While treated with (+)-catechin after exposed to tBHP, the coverslips were washed with PBS and stained with 5 μmol L^{-1} DCFH-DA for 20 min at 37°C, and then were washed with PBS. The coverslips were sealed with the liquid containing 50% glycerol, 20 μmol L^{-1} citric acid and 50 mmol L^{-1}

NaH_2PO_4. The photographs were taken by fluorescence microscope (Leica). The excitation wave length at 513 nm was used. The fluorescent intensities of random 50 cells were averaged to indicate the quantities of intracellular ·OH. Data were analyzed by using Meta Imaging Series 4.5 (Universal Imaging Corporation, PA, USA).

3.7 Western blot analysis

Cells were suspended in lysis buffer [50 mmol L^{-1} Tris, 5 mmol L^{-1} edetic acid, 150 mmol L^{-1}NaCl, 0.5% NP-40, 10 μg mL^{-1} leupeptin (Sigma, Mo, USA), 10 μg mL^{-1} aprotinin (Sigma, Mo, USA), 1 mmol L^{-1} PMSF (Sigma, Mo, USA)] on ice for 1 h. Cell lysates were centrifuged at 4,000 × g, at 4°C for 15 min. Protein concentrations in the supernatant were detemined by using Bio-Rad protein assay reagent (Bio-Rad, USA). Total cellular proteins were resolved by 10% SDS-PAGE and blotted onto nitrocellulose membrane. Primary antibodies and diluted concentration used were: PARP (1:500), caspase-3 (1:200), and ICAD (1:300). The secondary antibodies used for these proteins were goat anti-rabbit IgG (1:500), conjugated with horseradish peroxidase (HRP), respectively, and 3,3-diaminobenzidine tetrahydrochloride as the substrate of HRP. Density of each band was quantitated by NIH image 1.63 software (National Institutes of Health, MD, USA)

3.8 Detection of cellular SOD and CAT activities

The cells were washed and collected after indicated treatment. Then, the activities of SOD and CAT were detected by using biochemical methods following the instructions for reagent kits (Nanjing Institute of Jiancheng Biological Engineering, China). Briefly, the assay for SOD was based on its ability to inhibit the oxidation of oxymine by the xanthine-xanthineoxidase system. The red product (nitrite) produced by the oxidation of oxymine had absorbance at 550 nm. One unit of SOD activaty was defined as the amount that reduced the absorbance at 550 nm by 50%. The assay of CAT activity was based on its ability to decomposed H_2O_2. The absorbance of supernatant at 254 nm changed when the H_2O_2 solution was injected into the cuvette. The change of the absorbance reflected the catalase activity.

3.9 Statistical analysis

Statistical analysis was conducted using Student's *t*-test for analysis of significance between the different values. Data were expressed as means ± S.D., and they were considered significance at a $p < 0.05$.

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