ORIGINAL ARTICLE

Is boiled food spice curcumin still biologically active? An experimental exploration

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Abstract

Background: As the major active component of turmeric (*Curcuma longa*), curcumin is widely used as a spice and food coloring agent, and also possesses multiple biological activities and therapeutic potential for neurodegenerative diseases. To answer the paradox between curcumin's biological activities and poor systemic bioavailability, we proposed that degradation products of curcumin may make important contributions to its biological activities, which needs to be verified. In addition, curcumin is usually heated or boiled used as a spice, it is necessary to explore whether boiled curcumin, which degrades readily, is still biologically active.
Methods: Thus, in the present study we investigated the protective effects of curcumin and boiled curcumin mixture on H_2O_2-induced oxidative damage in PC12 cells, a widely used model for neurons.
Results: Results showed that in spite of high degradation rates, boiled curcumin mixture still possessed similar protective activities like parent curcumin, and could effectively rescue PC12 cells against H_2O_2-induced damage, via decreasing production of reactive oxygen species and malondialdehyde, reducing caspase-3 and caspase-9 activities. Moreover, curcumin's degradation products including ferulic acid, vanillin and vanillic acid could also improve PC12 cells survival rate.
Conclusion: Our findings indicated that boiled curcumin mixtures still possessed protective activity for PC12 cells, and supported the contribution of degradation products to biological activities of curcumin.

Keywords: *curcumin; degradation; oxidative damage; apoptosis; PC12 cells*

Introduction

Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione) is a natural polyphenolic compound isolated from the spice turmeric (*Curcuma longa*). It is not only widely used as a food coloring pigment and as a preservative but also has been used as an herbal medicine in many Asian countries for thousands of years (1, 2). Accumulating evidence has indicated that curcumin displays a broad spectrum of biological and pharmacological activities, including antioxidant, antiproliferative, anticarcinogenic, anti-inflammatory, antibacterial, and antiviral activities (3–9). Curcumin has been suggested to be a promising natural agent for neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) (10–13).

Although curcumin exhibits various biological activities, curcumin possesses poor systemic bioavailability, which makes its pharmacology elusive. In view of the low stability of curcumin we previously proposed that the degradation products of curcumin may make important contributions to its biological activities, which should be further verified (14–16). In addition, when curcumin is used as a spice (such as in curry) it is often heated or boiled, and the heat-treated curcumin is largely degraded. This raises an interesting question, whether boiled curcumin still possesses biological activities. Therefore, we attempted to provide clues for the above questions through investigating the neuroprotective activities of curcumin, a boiled curcumin mixture, and its degradation products. As oxidative damage to neurons is widely accepted to play an important role in the pathogenesis of neurodegenerative diseases, H_2O_2-induced oxidative damage in rat pheochromocytoma (PC12) cells, a widely used model for neurons, was employed to evaluate the protective effect of parent curcumin, boiled curcumin mixture, and its degradation products. The results indicated that boiled curcumin could effectively rescue PC12 cells against H_2O_2-induced

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death through decreasing the production of reactive oxygen species (ROS) and malondialdehyde (MDA), reducing caspase-3 and caspase-9 activities, which helped elucidate the pharmacology of curcumin.

Results

Cell viability

Cell viability of PC12 cells treated with parent curcumin, a boiled curcumin mixture, and three degradation products of curcumin was investigated. First, cytotoxic doses of H_2O_2, curcumin, boiled curcumin mixture Cur-B1, boiled curcumin mixture Cur-B2, ferulic acid, vanillin,

and vanillic acid were screened. The results indicated that PC12 cell viability was significantly inhibited in the range of 0–1000 μM H_2O_2 alone for 24 h incubation and the IC_{50} value was 499.92 ± 90.83 μM (Fig. 1a). Thus, 500 μM H_2O_2 was regarded as an oxidative damage model *in vitro* for the subsequent experiments. The results also showed that exposing PC12 cells to parent curcumin, Cur-B1, Cur-B2, ferulic acid, vanillic acid, and vanillin for 24 h alone did not reduce cell viability compared with the 0.2% dimethyl sulfoxide (DMSO)-treated control group (Fig. 1b and c).

Our results indicated that pretreatment of PC12 cells with parent curcumin, Cur-B1, and Cur-B2 could reverse H_2O_2-induced cytotoxicity, respectively. As shown
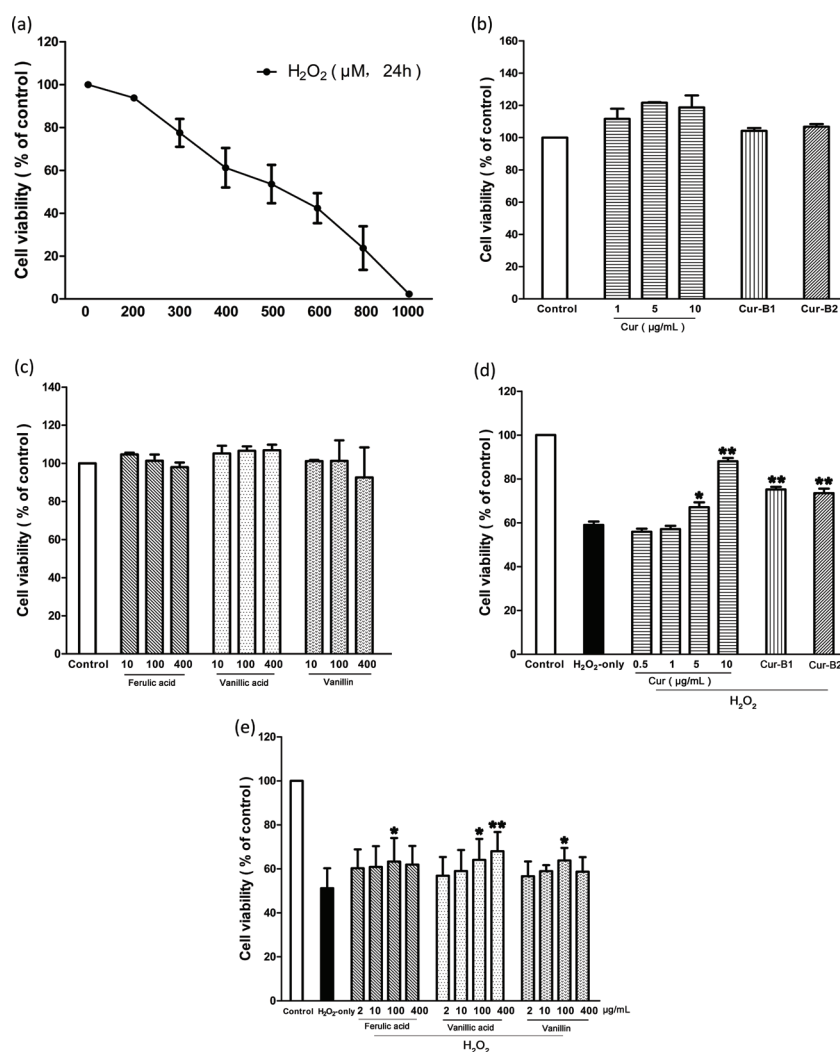
Fig. 1. Cell viability detected by MTT assay. PC12 cells were treated with curcumin (0.5–10 μg/mL), boiled curcumin mixture Cur-B1 (360 μg/mL), boiled curcumin mixture Cur-B2 (360 μg/mL), ferulic acid (2–400 μg/mL), vanillic acid (2–400 μg/mL) and vanillin (2–400 μg/mL) for 0.5 h prior to exposure to 500 μM H_2O_2. The cytotoxic doses of H_2O_2 (a), curcumin, Cur-B1, Cur-B2 (b), and the selected degradation products of curcumin (c) were determined. The protective effect of curcumin, Cur-B1, and Cur-B2 (d) and the selected degradation products of curcumin (e) on H_2O_2 induced cytotoxicity in PC12 cells. Data were displayed as mean ± SD value from three separate experiments. Bars with different features manifested statistical difference at $p < 0.05$. *$p < 0.05$, different from H_2O_2-only group; **$p < 0.01$, significantly different from H_2O_2-only group.

in Fig. 1d, when PC12 cells were pretreated with parent curcumin, Cur-B1, and Cur-B2, the cell survival rates significantly increased from $59.01 \pm 2.69\%$ (H_2O_2 only) to $88.08 \pm 2.58\%$, $75.18 \pm 2.11\%$, and $73.56 \pm 3.47\%$, respectively. Moreover, with pretreatment of PC12 cells with ferulic acid, vanillic acid, and vanillin, the PC12 cell survival rates significantly increased from $51.25 \pm 9.07\%$ (H_2O_2 only) to $63.33 \pm 10.69\%$, $68.06 \pm 8.70\%$, and $63.86 \pm 5.66\%$, respectively (Fig. 1e).

High-performance liquid chromatography with ultraviolet detection (HPLC/UV) analysis of boiled curcumin mixture
HPLC analysis was performed to estimate the residual content of parent curcumin in boiled curcumin mixture Cur-B1 and boiled curcumin mixture Cur-B2 to illuminate the role of degradation products in the protective effects against H_2O_2-induced oxidative damage in PC12 cells. The results indicated that the residual concentrations of curcumin in Cur-B1 and Cur-B2 were 0.66 ± 0.04 µg/mL and 0.25 ± 0.03 µg/mL, respectively (Fig. 2a). However, as suggested by the cell viability assay results, 1 µg/mL parent curcumin alone could not protect PC12 cells against H_2O_2-induced death. Thus, the degradation products made important contributions to the observed protective effects of Cur-B1 and Cur-B2. In addition, the biologically active degradation products, including vanillic acid, ferulic acid, and vanillin, were identified in Cur-B1 and Cur-B2 (Fig. 2b and c), which was consistent with previous studies (17, 18).

Cell morphology and apoptosis
As shown in Fig. 3a, PC12 cells in the H_2O_2-only group flocked together and exhibited typical apoptosis characteristics. However, when PC12 cells were pretreated with parent curcumin, boiled curcumin mixture Cur-B1, and boiled curcumin mixture Cur-B2, a majority of PC12 cells displayed normal cell morphology in each case. The protective effects of boiled curcumin mixture Cur-B1 and boiled curcumin mixture Cur-B2 were similar to those of parent curcumin.

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining by flow cytometry was performed to estimate the apoptosis rate of PC12 cells. As shown in Fig. 3b, the apoptosis proportion of control group was $10.00 \pm 1.76\%$, and it rose to $27.95 \pm 6.71\%$ after exposure to H_2O_2 alone for 12 h. However, this phenomenon could be reversed by pretreatment of curcumin, Cur-B1, or Cur-B2, in which case the apoptosis proportions decreased to $12.64 \pm 2.18\%$, $14.44 \pm 1.32\%$, and $13.52 \pm 1.55\%$, respectively. The results suggested that curcumin, Cur-B1, and Cur-B2 could protect PC12 cells against H_2O_2-induced apoptosis.

Intracellular ROS levels
The inhibitory effects of parent curcumin, boiled curcumin mixture Cur-B1, and boiled curcumin mixture Cur-B2 on H_2O_2-induced enhancement of intracellular ROS in PC12 cells were estimated by 2',7'-dichlorofluorescin diacetate (DCFH-DA) probe. As shown
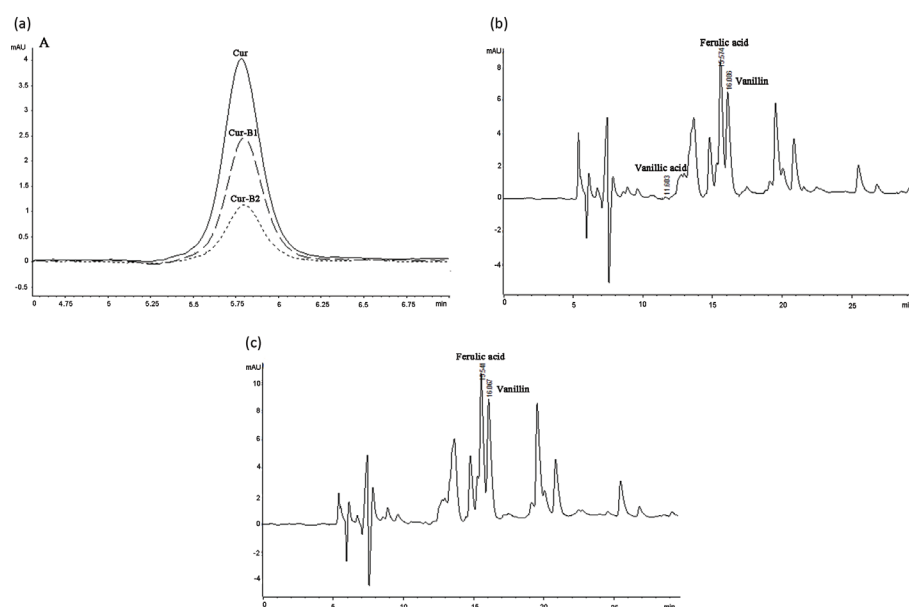
Fig. 2. Chromatogram of standard curcumin, boiled curcumin mixture Cur-B1, and boiled curcumin mixture Cur-B2. The selected degradation products of curcumin (ferulic acid, vanillic acid, and vanillin) were identified by HPLC/UV. Overlapping picture of 1 µg/mL standard curcumin, 360 µg/mL Cur-B1, and 360 µg/mL Cur-B2 at 430 nm (a); 360 µg/mL Cur-B1 at 280 nm (b); 360 µg/mL Cur-B2 at 280 nm (c).
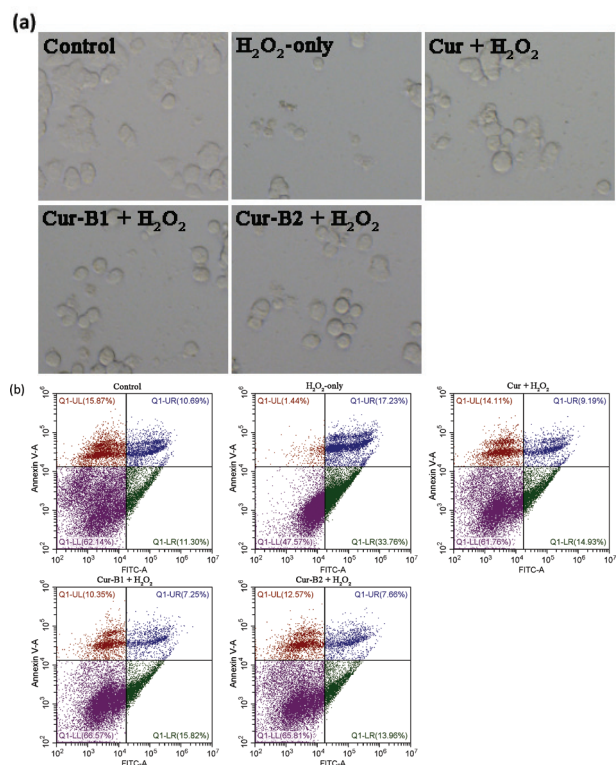
Fig. 3. Inhibitory effects of curcumin, boiled curcumin mixture Cur-B1, and boiled curcumin mixture Cur-B2 on H_2O_2-induced PC12 cell apoptosis. PC12 cells were treated with 10 µg/mL curcumin, 360 µg/mL Cur-B1, and 360 µg/mL Cur-B2 for 0.5 h prior to exposure to 500 µM H_2O_2. Then, PC12 cells were observed by a phase contrast microscope (×200) (a). PC12 cells were stained with a double dye of annexin V-FITC/PI after treatment with curcumin or boiled curcumin mixture and the apoptosis rates of each group were detected by flow cytometer (b).

in Fig. 4a, the intracellular ROS level of PC12 cells exposed to H_2O_2 alone for 4 h increased markedly to 231.24 ± 60.00%. However, with pretreatment of PC12 cells with curcumin, Cur-B1, and Cur-B2, the intracellular ROS levels decreased to 141.94 ± 28.65%, 148.98 ± 30.87%, and 144.69 ± 21.41%, respectively. Thus, Cur-B1 and Cur-B2 could effectively attenuate ROS generation increase caused by H_2O_2. The results were in agreement with representative images of DCFH-DA dyeing for PC12 cells as shown in Fig. 4b.

MDA levels
When PC12 cells were exposed to H_2O_2 alone for 12 h the intracellular MDA generation increased to 159.16 ± 14.79% compared with the control group as shown in Fig. 5. Pretreatment of curcumin, boiled curcumin mixture Cur-B1, and boiled curcumin mixture Cur-B2 could effectively suppress enhancement of MDA levels, and the values decreased to 125.09 ± 15.42%, 116.28 ± 7.21%, and 122.01 ± 11.41%, respectively.

Caspase-3 and caspase-9 activities
The effects of boiled curcumin mixture Cur-B1 and boiled curcumin mixture Cur-B2 on caspase-3 and caspase-9 activities in H_2O_2-induced PC12 cells were explored and compared with parent curcumin. As shown in Fig. 6, when PC12 cells were exposed to H_2O_2 alone for 12 h, the intracellular caspase-3 activity increased to 142.11 ± 22.73%. However, pretreatment with parent curcumin, Cur-B1, and Cur-B2 could effectively suppress enhancement of caspase-3 activity, and the value decreased to 107.51 ± 14.31%, 114.24 ± 8.02%, and 102.95 ± 5.51%, respectively. Similar results were obtained for caspase-9 activity and the corresponding activity decreased from 134.83 ± 5.68% for the H_2O_2 group to 103.94 ± 6.81% for the parent curcumin group, 101.69 ± 5.33% for Cur-B1, and 105.12 ± 9.21% for Cur-B2.

Discussion

Curcumin is one of the most intensively studied natural compounds for its wide range of biological and pharmacological activities, as well as its potential therapeutic applications for many diseases. However, curcumin has very poor systemic bioavailability resulting from its very low water solubility, instability, rapid metabolism, and poor absorption as indicated in preclinical and clinical studies (2, 19–23). Many studies demonstrated that the levels of curcumin and its metabolites were rather low in serum and tissue levels even with high doses *in vivo* (2, 19–23). Thus, how curcumin executes the observed biological effects with the perquisite of poor systemic bioavailability remains elusive. On the basis of the low stability of curcumin, we previously proposed that the degradation products of curcumin could play an important role in executing the biological activities of curcumin (14–16). In addition, curcumin is considered to be the major active component of the curry spice turmeric, which is often heated or boiled during cooking. It is interesting to explore whether the boiled curcumin, while largely degraded, is still biologically active. The present study was designed to investigate the protective effects of curcumin, a boiled curcumin mixture, and its selected degradation products on H_2O_2-induced oxidative damage in PC12 cells. Consistent with previous findings it was found that parent curcumin can protect PC12 cells from H_2O_2-induced damage (24, 25). Despite the high degradation rates after boiling, the boiled curcumin mixture still exhibited strong protective activities against H_2O_2-induced damage in PC12 cells, and its activity was similar to that of parent curcumin. The observed higher concentrations of boiled curcumin mixture than parent curcumin in exerting the protective effects should arise in part from the large content of phosphate buffer saline (PBS) in the mixture. Thus, the current study suggested that the boiled curcumin mixture was still biologically active in protecting PC12 cells against

(a)

(b)
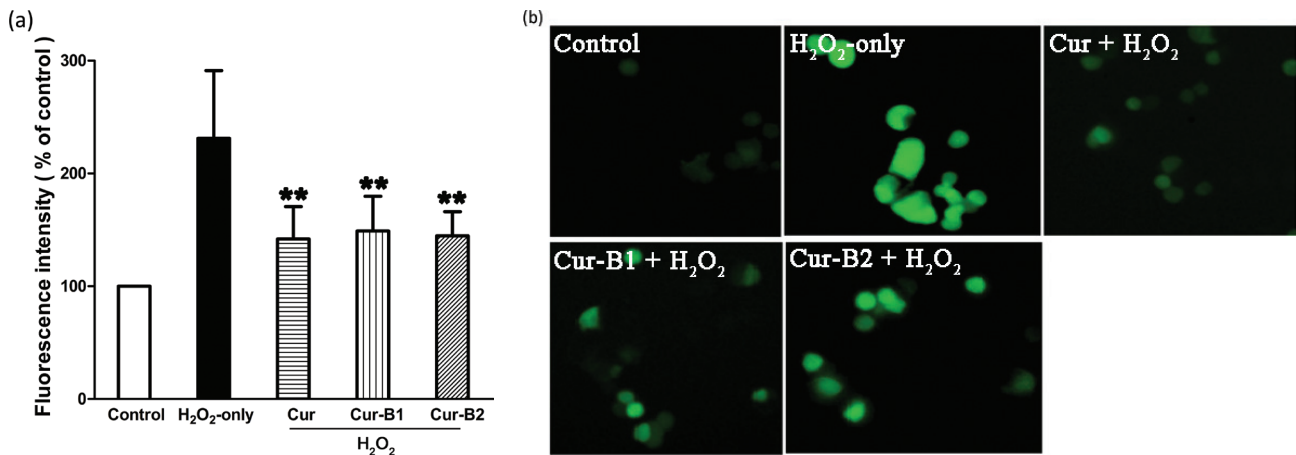
Fig. 4. Inhibitory effects of curcumin, boiled curcumin mixture Cur-B1, and boiled curcumin mixture Cur-B2 on H_2O_2-induced intracellular ROS enhancement in PC12 cells. PC12 cells were treated with 10 μg/mL curcumin, 360 μg/mL Cur-B1, and 360 μg/mL Cur-B2 for 0.5 h prior to exposure to 500 μM H_2O_2. DCFH-DA fluorescence intensity analysis was shown as mean ± SD values from three separate experiments through a fluorescence microplate reader (a). Bars with different features manifested statistical difference at $p < 0.05$. *$p < 0.05$, different from the H_2O_2-only group; **$p < 0.01$, significantly different from the H_2O_2-only group. Representative images of DCFH-DA staining in PC12 cells were observed by fluorescent microscopy (b).
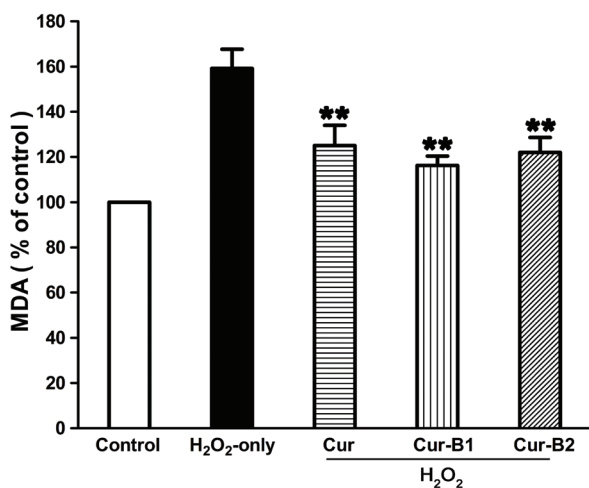
Fig. 5. Suppressing effects of curcumin, boiled curcumin mixture Cur-B1, and boiled curcumin mixture Cur-B2 on H_2O_2-induced enhancement of MDA level in PC12 cells. PC12 cells were treated with 10 μg/mL curcumin, 360 μg/mL Cur-B1, and 360 μg/mL Cur-B2 for 0.5 h prior to exposure to 500 μM H_2O_2. Data were manifested as mean ± SD values from three independent experiments. Bars with different characteristics show statistical difference at $p < 0.05$. *$p < 0.05$, different from H_2O_2-only group; **$p < 0.01$, significantly different from H_2O_2-only group.

oxidative damage and supported the theory that the degradation products of curcumin make important contributions to the biological activities of curcumin.

The observed biological activities of the boiled curcumin mixture may result from the similar biological activities of curcumin's degradation products. It was demonstrated that curcumin degraded readily under high temperature or pH, and despite the varying degradation products used, most of them possessed the phenolic hydroxyl group (26–28). Thus, many identified degradation products, such as ferulic acid, vanillin, and vanillic acid, possess antioxidant potentials (26–28). In agreement with our present findings, many studies reported that ferulic acid and vanillin exhibited protective effects on oxidative damage in PC12 cells (29–31). Moreover, we previously reported that curcumin's degradation product mixture also possessed biological activities for AD-associated therapeutic targets, including inhibiting amyloid-β peptide fibril formation, acetylcholinesterase, and β-amyloid precursor cleavage enzymes (32). In combination with the present findings of the protective effects of the boiled curcumin mixture on PC12 cells, we proposed that boiled curcumin mixture also possessed multiple biological activities.

Conclusion

In summary, our study suggested that curcumin was still active after boiling to protect PC12 cells from H_2O_2-induced damage, via decreasing the production of ROS and MDA and inhibiting the activities of caspase-3 and caspase-9. The findings further supported our hypothesis that the degradation products played an important role in executing the biological activities of curcumin and also provided a plausible interpretation of the paradox between the widely recognized biological activities and *in vivo* poor bioavailability of curcumin.
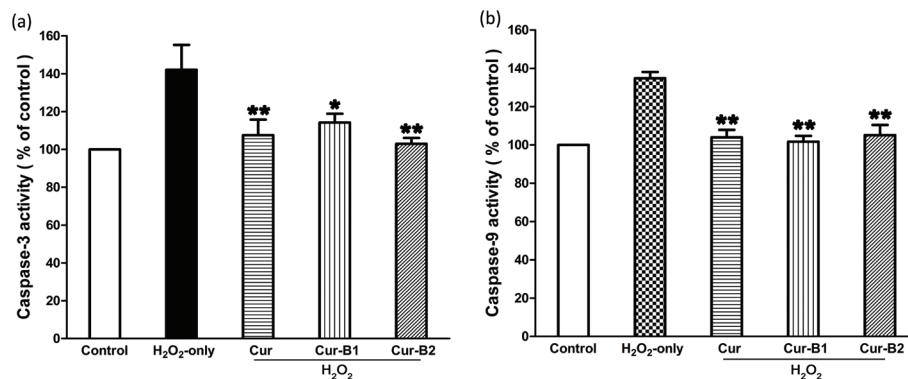
Fig. 6. Suppressing effects of curcumin, boiled curcumin mixture Cur-B1 and boiled curcumin mixture Cur-B2 on H_2O_2-induced caspase-3 (a) and caspase-9 (b) activation in PC12 cells.

Materials and methods

Materials and reagents

Poly-D-lysine hydrobromide, ferulic acid (99%), vanillin (99%), vanillic acid (97%), 3-(4,5-dimethylthial-zal-z-yl)-2,5-diphenylterazolium (MTT), DCFH-DA probes, and Nutrient Mixture F-12 Ham were purchased from the Sigma-Aldrich Shanghai Trading Co. (Shanghai, China). Curcumin (98%) was obtained from the Aladdin Industrial Corporation (Shanghai, China). Fetal bovine serum (FBS) was bought from (Gibco BRL, Grand Island, NY, USA). Horse serum was bought from Solarbio Biological Technology Co., Ltd. (Beijing, China). Hydrogen peroxide (H_2O_2) was obtained from Shuangshuang Chemical Co., Ltd. (Yantai, China). An annexin V-FITC/PI apoptosis detection kit was obtained from Bibo Biological Technology Co., Ltd. (Nanjing, China). A Bradford protein assay kit, caspase-3 activity assay kit, caspase-9 activity assay kit, and cell MDA assay kit were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other reagents used were of analytical grade.

Preparation of boiled curcumin mixture

Our preliminary experiments and previous studies demonstrated that curcumin degraded readily when it was incubated in physiological PBS at high temperature or pH (data not shown) (17, 18). After a systematic exploration we chose the degradation conditions (PBS, pH 7.2 and 8.0, heated to 100°C for 2 h) to obtain two boiled curcumin mixtures. Aliquots of 1.0 mg curcumin powder were added to 1.0 mL of 0.01 M PBS, pH 7.2 and 8.0, and the samples were heated to 100°C. After boiling for 2 h, the supernatant of the mixture was frozen at low temperature (–45°C) by LGJ-10B Vacuum Freeze Dryer (Four-Ring Science Instrument Plant Beijing, Beijing, China) to get the boiled curcumin mixture powders called Cur-B1 (PBS, pH 7.2, heated to 100°C for 2 h) and Cur-B2 (PBS, pH 8.0, heated to 100°C for 2 h) and was redissolved in DMSO.

Cell culture

A rat pheochromocytoma PC12 cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). PC12 cells were grown in Nutrient Mixture F-12 Ham media containing 10% horse serum and 5% FBS at 37°C under an atmosphere of 5% CO_2. To evaluate the protective effect of boiled curcumin on H_2O_2-induced oxidative damage, PC12 cells were pre-incubated with curcumin, boiled curcumin mixture Cur-B1, or boiled curcumin mixture Cur-B2 before adding H_2O_2.

Cell viability

MTT assay was used to detect PC12 cell viability. Briefly, PC12 cells were seeded at a density of 10^4 cells/well in 96-well tissue culture plates with Poly-D-lysine hydrobromide. After attachment PC12 cells were pre-incubated with different concentrations of curcumin (0.5–10 μg/mL), Cur-B1 (360 μg/mL), and Cur-B2 (360 μg/mL), ferulic acid (2–400 μg/mL), vanillin (2–400 μg/mL), or vanillic acid (2–400 μg/mL) for 0.5 h and treated without or with H_2O_2 (500 μM) for an additional 24 h. Then, PC12 cells were incubated with 0.5 mg/mL MTT for 4 h, and DMSO was used to dissolve the blue formazan product. The absorbance reflected cell viability measured at 570 nm by Multiskan GO (Thermo Fisher Scientific, Vantaa, Finland). Cell viability was shown as a percent of the value in the control groups.

HPLC analysis

HPLC analysis was carried out on an Agilent 1100 (Santa Clara, CA, USA) HPLC system, which was equipped with an Agilent UV-DAD G1315B detector and an Agilent G1311A quaternary pump. Curcumin was separated by C18 column (Diamonsil, 5 μm, 4.6 × 250 mm, Dikma, China) monitored with a refillable pre-column (C18, 2.0 × 20 mm, Alltech, Virginia, USA) at 35°C. We chose 430 nm as the detection wavelength and the flow rate was 1.0 mL/min. The residual content of

curcumin in the boiled curcumin mixtures Cur-B1 and Cur-B2 was based on its calibration standard curves. The HPLC/UV method was used to identify the three active components of the boiled curcumin mixture: ferulic acid, vanillin, and vanillic acid. The HPLC system was the same as described above. To separate the three active components we chose 280 nm as the detection wavelength and 0.4 mL/min as the flow rate, maintaining the temperature at 35°C.

Cell morphology and apoptosis

A phase contrast microscope was used to observe the cell morphology. Briefly, PC12 cells were exposed to 10 μg/mL curcumin, 360 μg/mL Cur-B1, or 360 μg/mL Cur-B2 for 0.5 h and co-incubated with 500 μM H_2O_2 for another 12 h. The cellular morphology of the PC12 cells was observed and compared by a phase contrast microscope (×200). Then, PC12 cells were collected and stained with a combination of annexin V-FITC and PI to detect apoptosis by flow cytometer following the manufacturer's instructions for the annexin V-FITC apoptosis detection kit (Beckman Coulter, USA).

Intracellular ROS assay

ROS generation was detected by a fluorometric assay based on the intracellular oxidation of DCFH-DA with 485 nm excitation and 520 nm emission wavelengths. Curcumin or boiled curcumin mixture treatment of PC12 cells was the same as with the preceding apoptosis assay. Then, PC12 cells were dyed with 10 μM DCF-H-DA probes for 30 min in the darkness, mildly bathed four times (employing basal medium without serum), and promptly observed by fluorescent microscopy (Olympus IX73, Tokyo, Japan) or fluorescent Varioskan Flash (Thermo Fisher Scientific Oy). An improved method was referred as detailed in reference to enhance accuracy (33). The intracellular ROS level of control group was regarded as 100% and the levels of other groups were calculated as a percentage of it.

MDA assay

The MDA level of PC12 cells was detected by the Bradford protein assay kit and cell MDA assay kit following the manufacturer's instructions. Curcumin or boiled curcumin mixture treatment of PC12 cells was the same as the previously described apoptosis assay. The MDA levels of the control group were regarded as 100% and other groups were calculated as a percent of it.

Caspase-3 and caspase-9 activity assay

The caspase-3 and caspase-9 activities were estimated by the Bradford protein assay kit, Caspase-3 assay kit, and Caspase-9 assay kit. PC12 cells were given the same treatment as in the preceding apoptosis assay; they were then harvested and their caspase-3 and caspase-9 activities were estimated following the corresponding manufacturer's instructions. The detection wavelength of the chromophore p-nitroanilide generation was 405 nm by MultiskanGo (Thermo Fisher Scientific). The caspase levels of the control group were defined as 100% and the other groups were calculated as a percent of them.

Statistical analysis

Statistical analysis was operated in this study through SPSS (16.0) software. One-way analysis of variance and Tukey's multiple comparison post-test were used to analyze the differences between groups. All values were described at the lowest from three independent experiments and shown as the mean ± SD. Differences were expressed as $p < 0.05$ (*) and significant differences were expressed as $p < 0.01$ (**).

Conflict of interest and funding

Ethical approval

This article does not contain any studies with human participants performed by any of the authors.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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