

# Crocetin Prevents Amyloid $\beta_{1-42}$ -Induced Cell Death in Murine Hippocampal Cells

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## ABSTRACT

Crocetin is an aglycon of carotenoid extracted by saffron stigmas (*Crocus sativus L.*) and known to have a potent anti-oxidative effect. Amyloid  $\beta$  ( $A\beta$ ), hallmark of Alzheimer's disease, is reported to have neurotoxicity partly via oxidative stress. In this study, we investigated the effect of crocetin on hippocampal HT22 cell death induced by  $A\beta_{1-42}$ . Furthermore, to clarify the mechanism underlying the protective effects of crocetin against  $A\beta_{1-42}$ -induced cell death, we measured reactive oxygen species (ROS) production by CM-H<sub>2</sub>DCFDA kit assay. Crocetin at 1 - 10  $\mu$ M protected HT22 cells against  $A\beta_{1-42}$ -induced neuronal cell death and decreased ROS production increased by  $A\beta_{1-42}$ . These results that crocetin has the potent neuroprotective effect against  $A\beta_{1-42}$ -induced cytotoxicity in hippocampal cells by attenuating oxidative stress, suggest that crocetin may provide a useful therapeutic strategy against  $A\beta$ -related disorders.

## KEYWORDS

Alzheimer's Disease; Amyloid  $\beta_{1-42}$ ; Crocetin; HT22; Oxidative Stress

## 1. Introduction

Alzheimer's disease (AD) is a common neurodegenerative disorder, and amyloid  $\beta$  ( $A\beta$ ) has been considered to have a critical role in the pathogenesis of AD [1]. AD is characterized by cognitive dysfunction and neuronal loss and these changes had been reported to occur as the result of various events, such as oxidative stress, neuroinflammation, and cholinergic deficits [2-4]. It is well known that the oxidative stress is involved in the mechanism of  $A\beta$ -induced neurotoxicity [5].

Crocetin, the aglycone of crocin carotenoids, is found in the saffron stigmas (*Crocus sativus L.*) and gardenia fruit (*Gardenia jasminoides Ellis*) [6,7] and they had been used for treatment of some diseases as traditional medicine [8]. Furthermore, crocetin has various pharmacological effects, such as anti-cancer [9], hepatoprotective

[10], anti-oxidative [11], anti-inflammatory [8], and neuroprotective effects [12]. Crocetin has been reported to inhibit  $A\beta$  fibrillization and stabilize  $A\beta$  oligomers [13]. We recently reported that crocetin prevents retinal damage induced by H<sub>2</sub>O<sub>2</sub>, tunicamycin, and *N*-methyl-*D*-aspartate (NMDA) [14,15]; saffron extracts have been reported to be effective on memory deficit on mild AD patient [16,17]. However, there was no report on protective effects of crocetin in hippocampal neuronal cells having been examined.

In the present study, therefore, we investigated the effects of crocetin against  $A\beta_{1-42}$ -induced cell death in HT22 mouse derived hippocampal cells.

## 2. Material and Methods

### 2.1. Materials

$A\beta_{1-42}$  peptide (Abcam, Cambridge, UK) was dissolved in dimethyl sulfoxide (DMSO), then diluted in phosphate

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buffered saline (PBS; pH 7.4) according to manufacturer's data sheets. Oligomers/fibrils of  $A\beta_{1-42}$  was achieved by incubation of 2 mM stock solution at 37°C for 2 h. Croctetin was obtained from Riken Vitamin Co., Ltd. (Tokyo, Japan).

## 2.2. Cell Cultures

Mouse hippocampal HT22 cells were gifted by Yoko Hirata Ph.D. (Gifu University, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (D-MEM; Nacalai tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), and 100  $\mu$ g/mL streptomycin (Meiji Seika) in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. Cells were passaged by trypsinization every 2 or 3 days, and maintained in a 10 cm dish (BD Biosciences, Franklin Lakes, NJ, USA).

## 2.3. Cell Death Assay

HT22 cells were seeded at  $1 \times 10^3$  cells per well into 96-well plates (BD Biosciences), then incubated for 24 h at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The entire medium was then replaced with fresh medium containing 1% FBS. Then, 2  $\mu$ M of  $A\beta$ , with or without 0.1 - 10  $\mu$ M of croctetin, and *N*-acetyl-L-cysteine (NAC; Wako, Osaka, Japan) were added and incubated for 48 h at 37°C. NAC, which is well known to have a potent anti-oxidant effect, was used as a positive control in this study. Croctetin and NAC were dissolved in PBS containing 1% DMSO. After 46 h incubation, the medium was replaced with fresh medium containing 1% FBS, then the cells viability was measured by using CCK-8 kit (Dojindo, Kumamoto, Japan).

Nuclear staining assays were carried out after 48 h incubation. Cell death was assessed by combination staining with Hoechst 33342 (Molecular Probes, Eugene, OR) and propidium iodide (PI; Molecular Probes). Images were collected by using an inverted epifluorescence microscope (IX70; Olympus. Co., Tokyo, Japan). The number of cells per condition was counted in a blind manner by a single observer (Y.Y.) with the aid of image-processing software (Image-J, version 1.33f; National Institutes of Health, Bethesda, MD, USA).

In the investigation of H<sub>2</sub>O<sub>2</sub> stress, HT22 cells were seeded at  $3 \times 10^3$  cells per well. The entire medium was replaced with fresh medium containing 1% FBS after 24 h incubating. H<sub>2</sub>O<sub>2</sub> were added 1 h after pretreatment with or without 0.1 - 10  $\mu$ M of croctetin, and NAC. Then, nuclear staining assays were carried out after 24 h incubation.

## 2.4. CM-H<sub>2</sub>DCFDA Kit Assay

The end of the culture period, HT22 cells were exposed

to 10  $\mu$ M 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H<sub>2</sub>DCFDA) (Invitrogen Life Technologies, Carlsbad, CA, USA) for 1 h. Fluorescence was measured by using a Varioskan flash 2.4 microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at the excitation/emission wavelengths of 485/535 nm. The levels of reactive oxygen species (ROS) were calculated as percent increases compared with the control, and the control was normalized to 100% of the basal level.

## 2.5. Statistical Analyses

Data were presented as means  $\pm$  S.E.M. Statistical comparisons were made using a two-tailed *t*-test or one-way ANOVA followed by Dunnett's test, *P* < 0.05 being considered to indicate a statistical significance.

## 3. Result

### 3.1. Croctetin Protects HT22 Cells against H<sub>2</sub>O<sub>2</sub>-Induced Cell Death

At first, we evaluated the effect of croctetin against H<sub>2</sub>O<sub>2</sub>-induced cell death by using combination staining with Hoechst 33342 (indication of alive and dead cells) and PI (indication of dead cells) (**Figure 1(b)**). Compared with the control group, H<sub>2</sub>O<sub>2</sub> (200  $\mu$ M) significantly increased the percentage of dead cells. Treatment with croctetin at 3  $\mu$ M protected HT22 cells against H<sub>2</sub>O<sub>2</sub>-induced cell death in a concentration-dependent manner (**Figure 1(c)**).

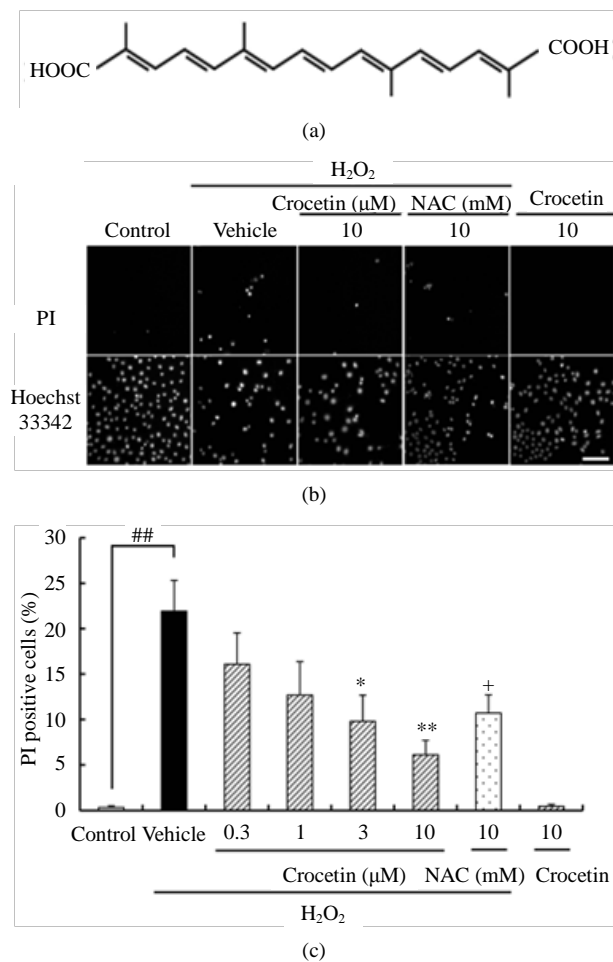
### 3.2. Croctetin Protects HT22 Cells against $A\beta_{1-42}$ -Induced Cell Death

Next, we evaluated the effect of croctetin against  $A\beta_{1-42}$ -induced cell death by using nuclear staining (**Figure 2(a)**).  $A\beta_{1-42}$  treatment at 0.2 to 20  $\mu$ M induced neuronal cell death in a concentration-dependent manner (data not shown). Compared with the control group,  $A\beta_{1-42}$  at 2  $\mu$ M significantly increased the percentage of dead cells. Treatment with croctetin at 1 to 10  $\mu$ M protected HT22 cells against  $A\beta_{1-42}$ -induced cell death (**Figure 2(b)**).

We also evaluated the effect of croctetin on cell viability on HT22 cells, by using CCK-8. Compared with vehicle control,  $A\beta_{1-42}$  at 2  $\mu$ M significantly decreased the cell viability rate. Treatment with croctetin at 10  $\mu$ M significantly reversed HT22 cells against  $A\beta_{1-42}$ -induced decrease cell viability (**Figure 2(c)**).

### 3.3. Effect of Croctetin for ROS Production of HT22 Cells at $A\beta_{1-42}$ -Induced Cell Death

To clarify the mechanism underlying the protective effects of croctetin against  $A\beta_{1-42}$  (2  $\mu$ M)-induced cell death,

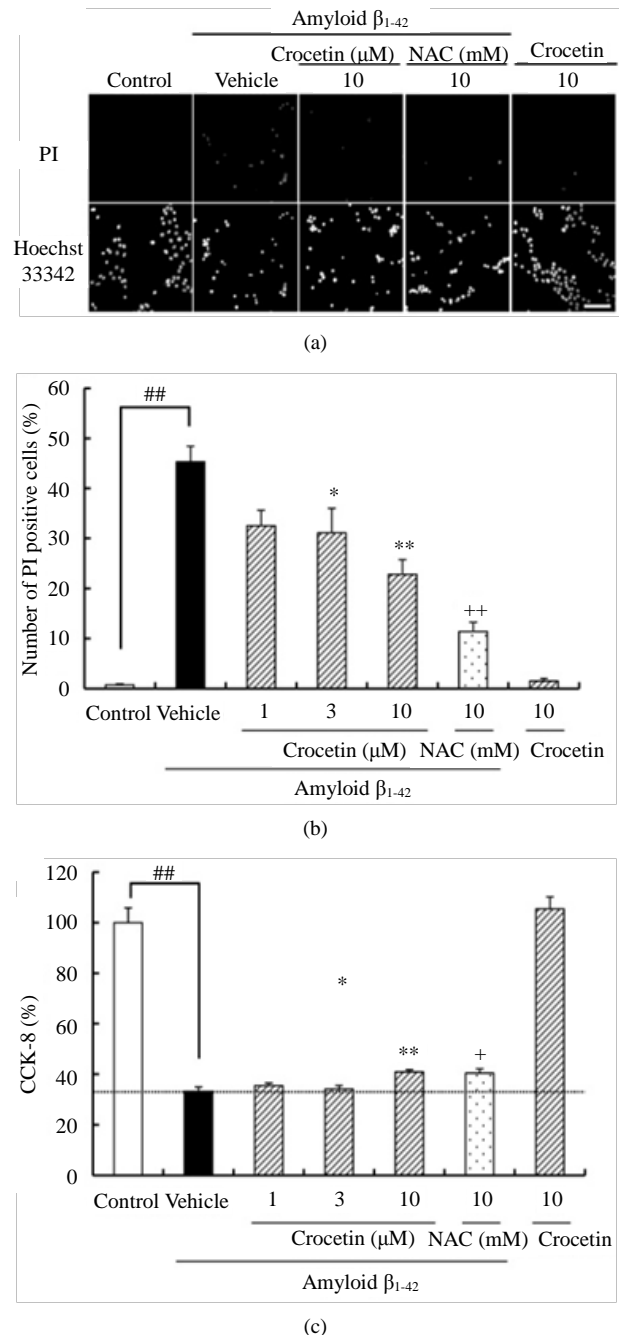


**Figure 1.** Crocin protects HT22 cells against  $H_2O_2$ -induced cell death. (a) The chemical structure of crocin. (b) Representative fluorescence micrographs of Hoechst 33342 (blue) and propidium iodide (PI: red) in HT22 cells. Cells were treated with vehicle, with or without crocin, followed by  $H_2O_2$  (200  $\mu$ M) or vehicle for 24 h. (c) The number of Hoechst 33342 or propidium iodide (PI) positive cells were counted, and the cell death rate was expressed as the percentage of PI-positive cells to Hoechst 33342-positive cells. ## $P$  < 0.01 versus control (Student's  $t$ -test). \* $P$  < 0.05; \*\* $P$  < 0.01 versus vehicle (Dunnett's test). + $P$  < 0.05 versus vehicle (Student's  $t$ -test). Each column and bar represent mean  $\pm$  S.E.M. (n = 6). Scale bar shows 100  $\mu$ m.

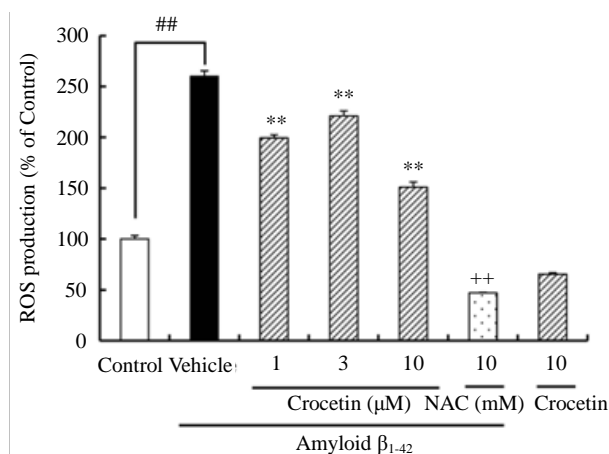
we investigated the ROS production by CM-H2DCFDA kit assay. Treatment with  $A\beta_{1-42}$  at 2  $\mu$ M significantly increased the ROS production, whereas treatment with crocin at 1 to 10  $\mu$ M decreased the ROS production induced by  $A\beta_{1-42}$  (Figure 3). NAC at 10 mM was used as a positive control, and reduced the  $A\beta_{1-42}$ -induced increase in ROS by about 80%.

#### 4. Discussion

In the present study, we investigated the neuroprotective effects of crocin against  $A\beta_{1-42}$ -induced neurotoxicity in



**Figure 2.** Crocin protects HT22 cells against  $A\beta_{1-42}$ -induced cell death. (a) Representative fluorescence micrographs of Hoechst 33342 (blue) and propidium iodide (PI: red) in HT22 cells. Cells were treated with vehicle, with or without crocin, followed by  $A\beta_{1-42}$  (2  $\mu$ M) or vehicle for 48 h. (b) The number of Hoechst 33342 or PI positive cells were counted, and the cell death rate was expressed as the percentage of PI-positive cells to Hoechst 33342-positive cells. (c) Cell viability was measured by Cell Counting Kit-8 (CCK-8) assay, with absorbance recorded at 492 nm. ## $P$  < 0.01 versus control (Student's  $t$ -test). \* $P$  < 0.05; \*\* $P$  < 0.01 versus vehicle (Dunnett's test). + $P$  < 0.05; ++ $P$  < 0.01 versus vehicle (Student's  $t$ -test). Each column and bar represent mean  $\pm$  S.E.M. (n = 6). Scale bar shows 100  $\mu$ m.



**Figure 3.** Effect of crocetin for ROS production of HT22 cells at  $A\beta_{1-42}$ -induced cell death. ROS production was measured by CM- $H_2$ DCFDA kit assay at 48 h. Cells were presented with vehicle, with or without crocetin, followed by  $A\beta$  (2  $\mu$ M) or vehicle for 48 h. ## $P$  < 0.01 versus control (Student's *t*-test). \* $P$  < 0.05; \*\* $P$  < 0.01 versus vehicle (Dunnett's test). ++ $P$  < 0.01 versus vehicle (Student's *t*-test). Each column and bar represent mean  $\pm$  S.E.M. (n = 6).

murine HT22 hippocampal cells. Addition of  $A\beta_{1-42}$  peptide to HT22 cells induced cell death as well as a previous report [18]. Crocetin has been known to have potent anti-oxidant [19], anti-cancer [9], and anti-inflammation activities [8]. A number of researches show that oxidative stress involves in AD pathogenesis [20]. Hence, crocetin, a potent antioxidant, is considered to have potential of therapy for AD pathogenesis. As we predicted, crocetin showed neuroprotective effects against  $H_2O_2$ - and  $A\beta_{1-42}$ -induced neuronal cell death.

Furthermore, crocetin at concentrations of 1 to 10  $\mu$ M showed significantly the protective effect against  $A\beta_{1-42}$ -induced neurotoxicity. In rat, crocetin distributed in plasma at 0.14 mM and in brain at about 40  $\mu$ M by oral administration of crocetin (at a dose of 100 mg/kg) and reduced oxidative stress in brain [19]. In our previous paper, the mean peak of serum concentration of crocetin was 0.2  $\mu$ g/ml (about 0.6 mM: calculated by the body weight) when healthy volunteers were administrated crocetin at a single oral dose 15 mg [21].

As we used in this study, crocetin showed a neuroprotective effect at 1 to 10  $\mu$ M which is lower than that in the brain *in vivo*. These results suggest that crocetin may have the effect against  $A\beta_{1-42}$ -induced neuronal cell death in the concentrations distributed in human blood.

Free radical and oxidative stress-induced neuronal cell death has been implicated in various neurological disorders, such as Parkinson's disease and AD [22,23]. The AD-associated  $A\beta$  accumulating in central nerve system (CNS) plaques of AD patients' brains induces the generation of oxygen-free radicals [24]. Hippocampal physiological function relates to memory formation and cogni-

tion system [25]. Neuronal cell death on hippocampus occurs in AD patient, and this results in cognitive dysfunction [26]. As the hippocampus is a major target for a neuronal degeneration in the brains of patients with AD, HT22 hippocampal neuronal cells used in the present study were useful.

As the mechanism underlying protective effect of crocetin, oxidative stress by ROS is considered the main pathway. A number of researches tend them attention to oxidative stress. However, the other mechanisms had been reported in partly. In previous reports, the protective effects of crocetin against various stresses in retina related to inhibition activity for caspase-3 and caspase-9 [14,15]. These results suggest that crocetin also inhibits caspase-3 and caspase-9 expression to protect the brain against cells apoptosis.

Crocetin or crocin (crocetin di-gentiobiose ester) has been reported to have the protective effects against various cognitive impairment models induced by ethanol, streptozotcin, scopolamine, aging, captured stress, or brain ischemia *in vivo* [27-31]. Taken together, these findings suggest that crocetin may be useful to prevent AD.

In conclusion, the present findings indicate that crocetin has neuroprotective properties against  $A\beta_{1-42}$ -induced cytotoxicity in murine HT22 hippocampal neuronal cells, by attenuating oxidative stress.

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## Abbreviations

$A\beta$ : Amyloid  $\beta$ ;

AD: Alzheimer's Disease;

CCK-8: Cell Counting Kit-8;

CNS: Central Nerve System;

D-MEM: Dulbecco's Modified Eagle's Medium;

DMSO: Dimethylsulfoxide;

FBS: Fetal Bovine Saline;

NAC: *N*-Acetyl-L-Cysteine;

NMDA: *N*-Methyl-D-Aspartate;

PBS: Phosphate Buffered Saline;

PI: Propidium Iodide;

ROS: Reactive Oxygen Species.