

Preventive effects of crocin on neuronal damages induced by D-galactose through AGEs and oxidative stress in human neuroblastoma cells (SH-SY5Y)

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Key Words

D-galactose, Crocin, Saffron, Aging, Reactive oxygen species, Advanced glycation end products

Abstract

Objective: D-galactose (D-gal) is well-known agent to induce aging process. In the present study, we selected crocin, the main constituent of *Crocus sativus* L. (saffron), against D-gal- induced cytotoxicity in human neuroblastoma SH-SY5Y cells.

Methods: Pretreated cells with crocin (25-500 μ M, 24 h) were exposed to D-gal (25-400 mM, 48 h). The MTT assay was used for determination cell viability. Dichlorofluorescein diacetate assay (DCF-DA) and senescence associated β -galactosidase staining assay (SA- β -gal) were used to evaluate the generation of reactive oxygen species and beta-galactosidase as an aging marker, respectively. Also advanced glycation end products (AGEs) expression which is known as the main mechanism of age-related diseases was measured by western blot analysis.

Results: The findings of our study showed that treatment of cells with D-gal (25-400 mM) for 48h decreased cell viability concentration dependency. Reactive ox-

xygen species (ROS) levels which are known as main factors in age-related diseases increased from $100 \pm 8\%$ in control group to $132 \pm 22\%$ in D-gal (200 mM) treated cells for 48h. The cytotoxic effects of D-gal decreased with 24h crocin pretreatment of cells. The cell viability at concentrations of 100 μ M, 200 μ M and 500 μ M increased and ROS production decreased at concentrations of 200 and 500 μ M to $111.5 \pm 6\%$ and $108 \pm 5\%$, respectively. Also lysosomal biomarker of aging and carboxymethyl lysine (CML) expression as an AGE protein, significantly increased in D-gal 200 mM group after 48h incubation compare to control group. Pretreatment of SHSY-5Y cells with crocin (500 μ M) before adding D-gal significantly reduced aging marker and CML formation.

Conclusion: Treatment of SH-SY5Y cells with crocin before adding of D-gal restored aging effects of D-gal concentration dependency. These findings indicate that crocin has potent anti- aging effects through inhibition of AGEs and ROS production.

1. Introduction

Aging is a progressive and irreversible phenomenon with cell and organ damages that enhances aging diseases such as Alzheimer. About 40% of over 60 years old suffer from varying degrees of cognitive dysfunctions and movement disorders [1].

The production of free radicals and advanced glyca-

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tion end products (AGEs) are the main mechanisms of aging pathogenesis through development of protein damages. The non-enzymatic reaction between reducing sugars and amino groups of proteins is called glycation or Maillard reaction producing Schiff bases which switch to Amadori products. AGEs that are generated from oxidation of unstable Amadori products and its irreversible cross-linked to tissues increase free radicals production rate 50 times more than unglycated molecules [2]. The most reported AGEs in the studies are N ϵ -(carboxymethyl)lysine (CML), N ϵ -(carboxyethyl)lysine (CEL), hydroimidazolones (HIs), pentosidine and pyrroline. CML, the abundant AGE in the heated foods, is most selected as an AGE molecule in laboratory investigations [3]. AGEs accumulation in tissue and elevated levels of ROS can induce molecular (DNA, protein and phospholipids) damages and disturb cellular function [4].

D-galactose (D-gal) is a simple sugar that is found in some foods and can be made by the body. Generally, it is metabolized completely in normal level by D-galactokinase and galactose-1-phosphate uridylyltransferase. However, an elevation level of D-gal induces oxidative stress and excessive D-gal. D-gal is oxidized into aldehydes and hydrogen peroxide and also it causes galactitol accumulation in cells which reacts with amines of amino acids in proteins to form glycation products [5]. Therefore, D-gal increases ROS production. Oxidative stress is one of the main mechanism of neurodegeneration that leads to other disorder such as stroke, multi-infarct dementia, Alzheimer's, Parkinson's and Huntington's disease [6, 7]. Regarding to important role of oxidative stress in aging phenomena, it seems that, this process can be postponed with exogenous antioxidants consumption. [8]. There are several evidences that plants as natural anti-oxidants can improve brain aging and cognitive dysfunction [9].

Saffron is the colored stigma of the plant *Crocus sativus* L. from Iridaceae family [10]. It is recently used for flavoring and coloring food and in traditional medicine has been used as anodyne, tranquilizer, antidepressant and stomachic [11]. The main active constituents of saffron are picrocrocin (bitter principles), safranal (volatile agents), crocetin and its glycoside crocin (dye materials) [12]. The major effects of saffron extracts and its active constituents are neuroprotective [13], antioxidant [14], anti-cancer [15], anti-diabetic and anti-hyperlipidemic [16], anti-atherosclerotic [17], anti-arthritis [18], and memory enhancement effects [19]. Crocin is a carotenoid pigment which has several health-promoting effects [20]. In a recent study, crocin attenuated acrolein induced tau hyperphosphorylation and malondialdehyde formation in the rat cerebral cortex by modulation mitogen-activated protein kinases (MAPKs) signaling pathway and elevation of glutathione levels [21]. Also, in streptozotocin induced-diabetic rats, administration of crocin exhibited hypoglycemic effects and decreased the level of AGEs formation [22].

Since, the protective effect of crocin against brain aging through D-gal and AGE production has not been studied, so in the present study we used human neuronal cells (SH-SY5Y) to investigate aging mechanisms and protective effects of crocin on oxidative stress and AGEs formation caused by high level of D-gal.

2. Materials and Methods

D-gal (99% purity) and fluorescent probe 2,7-dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma. Senescence beta-galactosidase staining Kit (#9860) was purchased from cell signaling and rabbit polyclonal CML was obtained from Abcam. Polyvinylidene fluoride (PVDF) membrane was provided from Bio-Rad.

Crocin crystals were obtained from saffron stigmas (purchased from Novin Saffron of Ghaen, Khorasan province, Northeast of Iran) by using crystallization method as previously has been described [23].

The SH-SY5Y human neuroblastoma cell line was obtained from Pasteur institute (Tehran, Iran). Cells were cultured in Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient mixture (DMEM-F12) supplemented with 15% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin and maintained at 37 °C with 95% humidified air and 5% CO₂. Cells in culture flasks were harvested for experiments at 80% confluence. Cell viability was determined by using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay that has been described previously [24]. Cells were incubated in 96-well plates with 5000 cell in each well. Different crocin concentrations (25- 500 μ M) were added to SH-SY5Y cells incubated in a plate for 72 h to investigate crocin effects on cell viability. In the separate plates, the cells were incubated with different concentrations of D-gal (25-400 mM) for 24h and 48h to find appropriate treatment period and D-gal concentration. Finally, D-gal at concentration of 200 mM selected and the cells after 24 h pretreatment with crocin (25-500 μ M) were incubated with 200 mM D-gal for 48 h to investigate the protective effects of crocin on D-gal neurotoxicity. After ending each treatment, cells were incubated with MTT solution (final concentration 0.5 mg/mL) for 2 h at 37°C. Then, the medium was removed and the purple formazan crystals were dissolved in 150 μ l dimethylsulfoxide (DMSO). Absorbance was measured at 545 nm (630 nm as a reference) in an ELISA reader (Start Fax- 2100, UK).

The dichlorofluorescein diacetate assay was used to evaluate the generation of reactive oxygen species [25]. H2DCF-DA readily crosses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to nonfluorescent H2DCF, which is oxidized in the presence of ROS to highly fluorescent DCF (2',7'-dichlorofluorescein). The DCF fluorescence intensity is equal to the amount of intracellular ROS. In brief, the SH-SY5Y cells were exposed to crocin (25-500 μ M) in a 96 well culture plate for 24h, then 200 mM D-gal was added to each well and the exposure time was 48 h. After washing of the cells with PBS solution, the cells were incubated with 20 μ M DCF-DA for 30 min at 37 °C in the dark. Bio Tek Synergy H4 plate reader at excitation and emission wavelengths of 488 nm and 527 nm, respectively measured intensity fluorescence of oxidized dichlorofluorescein.

Senescence associated β -galactosidase activity assay has been defined at pH: 6.0 to investigate expression of lysosomal β -galactosidase protein as a most widely used biomarker for senescent cells [26]. The test was performed according to the manufacturer's protocol of commercial

senescence-associated β -galactosidase kit (cell signaling#9860). Briefly, cells were seeded at 80×10^4 cell/well in a six-well plate with or without 500 μ M crocin pretreatment for 24h followed by treatment with D-gal (200 mM) for 48 h. Cells were washed with PBS and incubated with SA- β -gal detection solution at 37°C for at least 12 h. After washing with PBS, cells were observed under light microscopy (Jenus, China) and positive staining cells were counted in 10 random fields of each well.

Cells were harvested and lysed in lysis buffer containing 50 mM Tris-HCl (pH: 7.4), 2 mM EDTA, 2 mM EGTA, 10 mM NaF, 1 mM sodium orthovanadate (Na_3VO_4), 10 mM β glycerophosphate, 0.2% W/V sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and complete protease inhibitor cocktail, then western blotting was performed as previously described [24, 27]. The total proteins were electrophoresed in 15% SDS-PAGE gels, transferred to polyvinylidene fluoride (PVDF) membranes. Then, the blots were blocked with 2% skim milk for 1 h at room temperature. After blocking, membranes were incubated with the primary antibodies at 1000-fold dilutions: anti-CML (ab27684) for overnight at 4°C and anti-GAPDH (#ab8245) for 2h at room temperature. After that the membranes were washed three times with 0.1% Tween 20 and TBST then incubated with Horseradish-peroxidase conjugated anti-rabbit antibody (#7074 Cell Signaling) at 1:3000 dilutions 90 min at room temperature. At the end of process protein bands were detected by enhanced chemiluminescence (ECL) reagent and Alliance 4.7 Gledoc (UK). Protein bands analyzing were performed using UVtec software (UK).

All data were expressed as means \pm SD and analyzed using analysis of variance (ANOVA) followed by the Tukey-Kramer post-hoc test for multiple comparisons. Differences were considered statistically significant when $P < 0.05$.

3. Results

Exposure of SH-SY5Y cells to different concentrations of D-gal (25-400 mM) for 24 and 48 h, concentration-dependently decreased cell viability. Significant reduction in cell viability was observed following 48 h exposure (fig 1A). As shown in fig 1B, pretreatment of cells with crocin (25-500 μ M), 24h before exposure to D-gal (200 mM) significantly attenuated the effects of D-gal at 100 μ M ($p < 0.05$), 200 μ M ($p < 0.05$) and 500 μ M ($p < 0.01$) as compared to D-gal-treated cells. Also, treatment of cells with different crocin concentrations (25- 500 μ M) alone for 72 h did not affect cell proliferation (fig 1C).

Treatment of cells with D-gal (200 mM), for 48 h elevated intracellular ROS production up to $132 \pm 22\%$ in SH-SY5Y cells ($p < 0.001$ vs control). This effect was reduced through incubation of cells with crocin 24h before adding D-gal. A significant reduction in ROS production was observed in 200 and 500 μ M of crocin concentrations ($p < 0.05$), $111.5 \pm 6\%$ and $108 \pm 5\%$, respectively ($p < 0.05$) (fig 2).

β -gal staining assay was performed according to above statement and the SH-SY5Y cells treated with D-gal 200 mM showed more positive blue staining cells when compared to control group. Pretreatment of cells with crocin (500 μ M) induced significant reduction in senescence

marker ($p < 0.05$) (fig 3A). The percentage of senescence cells showed in fig 3B was $11.9 \pm 1.18\%$ ($p < 0.05$) in D-gal incubated cells versus control group ($6.4\% \pm 0.43$). Crocin pretreated cells had $5.94 \pm 0.84\%$ cellular aging marker which was significantly less than D-gal group ($p < 0.05$).

As shown in fig 4, neuronal cells exposure to D-gal (200 mM) for 48 h, significantly increased CML formation to $139.5 \pm 14\%$ ($p < 0.01$ vs control group), whereas pretreatment of cells with crocin (500 μ M) for 24 h reduced CML generation to $107.6 \pm 14\%$ ($p < 0.05$ vs D-gal group).

4. Discussion

In this study, the anti-aging effects of crocin were evaluated in a reliable aging model induced by D-gal. Critical aging changes have been formed with D-gal in several *in vitro* and *in vivo* models [5, 28]. According to our results, D-gal not only increased cell death and intracellular ROS production but also induced cellular senescence and CML formation in SH-SY5Y cells. Pretreatment with crocin markedly suppressed cellular aging in SH-SY5Y cells through free radical scavenging activity. In support of these findings, previous results have shown that crocin has potent antioxidant and neuroprotective effects [29, 30].

SA- β -gal activity at pH=6 has been clearly elevated in different aged cells and tissues of old animals [31]. According to other research projects, in our study, SH-SY5Y cells which treated by D-gal 200 mM for 48h showed more β -gal senescence staining cells when compared to control group [5]. Betagalactosidase aging marker in the pretreated cells with crocin, significantly decreased in comparison to D-gal group.

In the different aging models, increased amounts of other cellular senescence markers such as ROS level and oxidative DNA damage have been observed [32]. Oxidative stress plays a crucial role in aging and ROS levels increase in the senescence cells with numerous mechanisms [33]. Crocin was shown obvious antioxidant effects in the different oxidative conditions. Ischemia-reperfusion-induced oxidative stress in kidney and skeletal muscle were reduced following pretreatment with crocin [34, 35].

Saffron and its carotenoid crocin reduced diabetic neuropathy in PC12 cells induced by high level of glucose through inhibition of ROS production [36].

Crocine administration, diminished ROS production, increased GSH content and SOD activity in rat retina after ischemia-reperfusion injury [37]. In our study, incubation of SHSY-5Y cells with D-gal 200 mM for 48h increased ROS production while pretreatment of cells with crocin ameliorated ROS formation in the dose-dependent manner.

It has been shown that D-gal increases lipid peroxidation and SOD activity while decreases catalase and glutathione peroxidase directly or via activation AGE/RAGE pathway [38]. Also, in the neurodegeneration disorders, the elevation of advanced glycation end products formation has been reported. AGEs molecules via AGE/RAGE pathway activate extracellular proteins such as β -amyloid peptides and lead to oxidative stress and inflammatory responses [39]. Recently crocin has been shown antiglycating effect in diabetic and atherosclerotic rats [22]. Similarly, in our

research, pretreatment with crocin (500 μ M) significantly decreased the most important advance glycation end product, CML, which increased in D-gal treated cells.

It seems that AGEs and ROS can stimulate the production of each other and both of them are the major mechanisms of brain aging. AGEs are produced from glucose and fructose under oxidative and antioxidative conditions. Also, AGE-modified proteins and free radicals lead to elevation of pro-inflammatory markers, including tumor necrosis factor α (TNF α), IL-1 β and NF- κ B [40]. The expression of pro-inflammatory cytokines in the cerebral cortex and hippocampus regions elevates in neurodegenerative disorders [41].

5. Conclusion

This study shows that crocin protects SHSY5Y cells against D-gal induced aging probably through the reduction of intracellular ROS and AGEs formation. Therefore, we should investigate molecular pathways affected by AGEs and ROS that contribute to brain aging mechanisms.

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Conflict of interest

All authors state that there are no actual or potential conflicts of interest.

Figure 1A

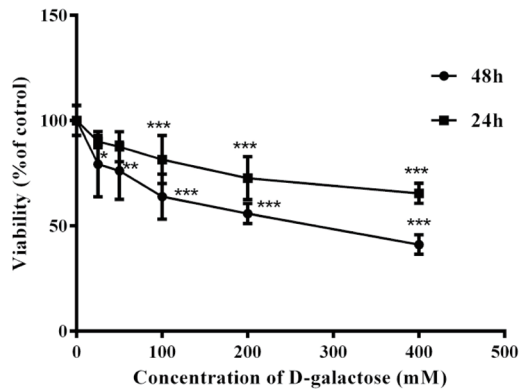


Figure 1B

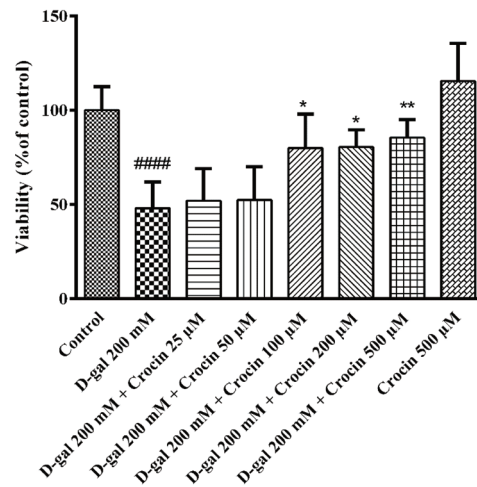


Figure 1C

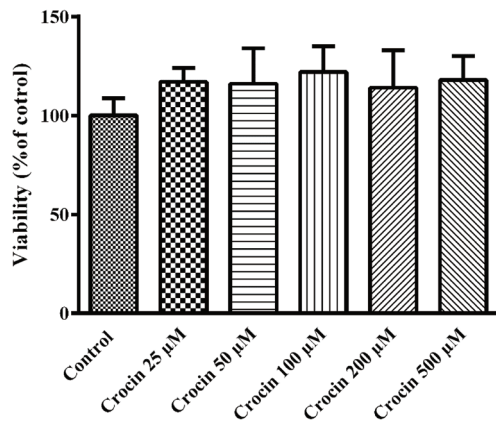


Figure 1 (A) Viability of SH-SY5Y cells incubated with 25-400 mM D-galactose for 24 and 48h. (B) Viability of SH-SY5Y cells incubated with 200 mM D-galactose (DG) with or without 24h pretreated crocin (25-500 μM). (C) Viability of SH-SY5Y cells incubated with crocin (25-500 μM) alone for 72h. Values are expressed as means ± SD (n=6). #P<0.05, ##p < 0.01 and ###p < 0.001 vs. control, *p < 0.05 and **p < 0.01 vs, D-gal at the same incubation time.

Figure 2

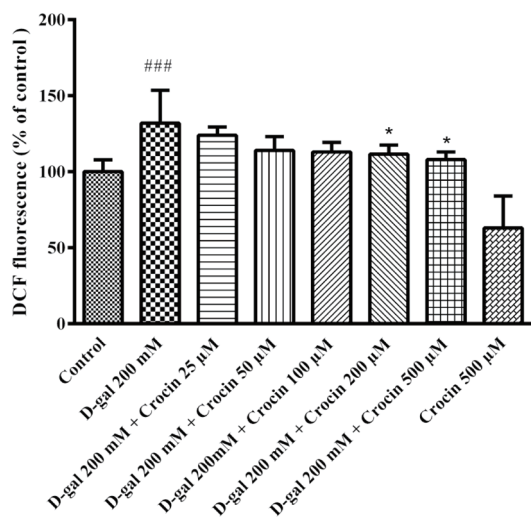


Figure 2 Effect of crocin on D-gal induced intracellular ROS formation in SH-SY5Y cells after 30 min incubation with 20μM, 70-dichlorofluorescein (DCF). Data are expressed as means ± SD (n=6). ###P < 0.05 vs. control, *P < 0.05 vs. D-gal treated cells at the same incubation time.

Figure 3B

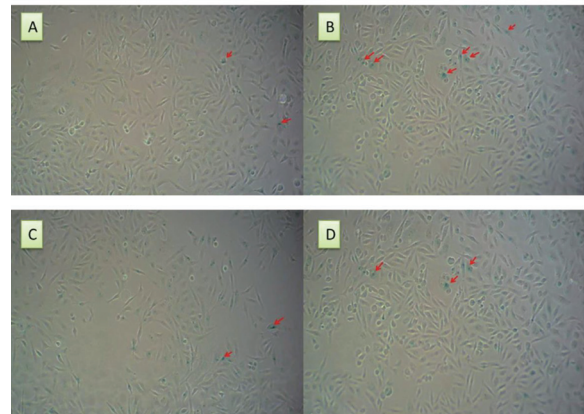
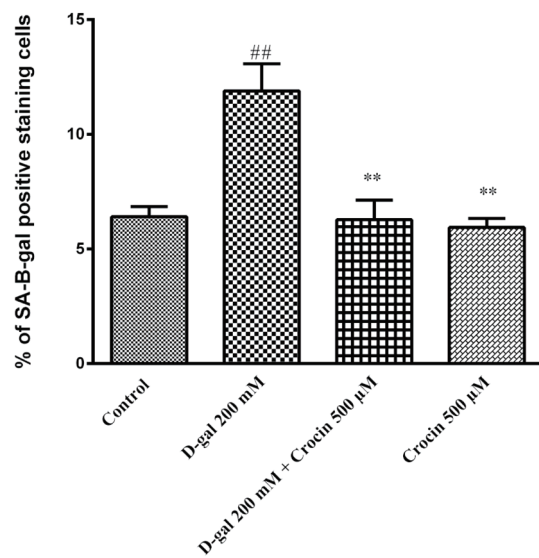


Figure 3 (A) Effect of crocin on D-gal induced cellular senescence in SH-SY5Y cells. Cells were treated with SA-b-gal for at least 12h. Arrows show staining senescent cells. (B) The percentage of SA-B-gal positive cells. Data are expressed as means \pm SD. ## $p < 0.01$ vs. control, ** $p < 0.01$ vs. D-gal treated cells at the same incubation time. A, control group B, D-gal 200mM group C, D-gal 200 mM + crocin 500μM group D, crocin 500μM group.

Figure 4A

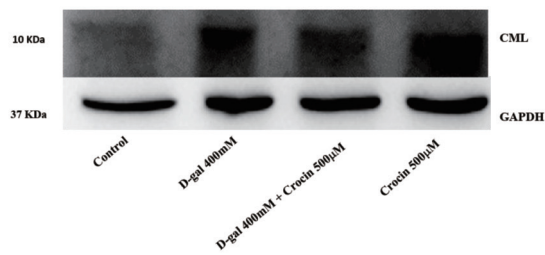


Figure 4B

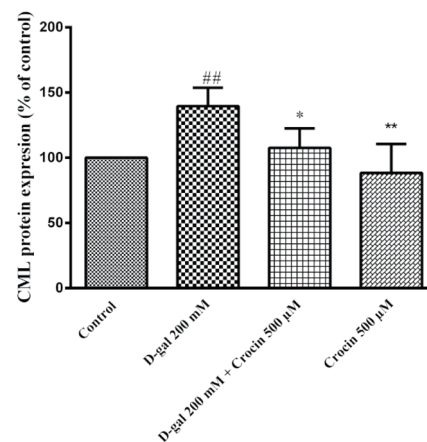


Figure 4 Expressions of N-ε-(carboxymethyl)lysine (CML) protein in SH-SY5Y cells treated of 200 mM D-gal with or without crocin pretreatment (500 μM) for 24 h. (A) Western blots of CML protein (B) The quantitative protein levels of CML. Data are expressed as the mean \pm SD of four separate experiments. ## $P < 0.01$ vs. control, * $P < 0.05$, ** $p < 0.01$ vs. D-gal treated cells.

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