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Original article

Caffeic acid and resveratrol ameliorate cellular damage in cell and Drosophila models of spinocerebellar ataxia type 3 through upregulation of Nrf2 pathway

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ABSTRACT

Polyglutamine (polyQ)-expanded mutant ataxin-3 protein, which is prone to misfolding and aggregation, leads to cerebellar neurotoxicity in spinocerebellar ataxia type 3 (SCA3), an inherited PolyQ neurodegenerative disease. Although the exact mechanism is unknown, the pathogenic effects of mutant ataxin-3 are associated with dysregulation of transcription, protein degradation, mitochondrial function, apoptosis, and antioxidant potency. In the present study we explored the protective role and possible mechanism of caffeic acid (CA) and resveratrol (Res) in cells and *Drosophila* expressing mutant ataxin-3. Treatment with CA and Res increased the levels of antioxidant and autophagy protein expression with consequently corrected levels of reactive oxygen species, mitochondrial membrane potential, mutant ataxin-3, and the aggregation of mutant ataxin-3 in SK-N-SH-MJD78 cells. Moreover, in SK-N-SH-MJD78 cells, CA and Res enhanced the transcriptional activity of nuclear factor erythroid-derived-2-like 2 (Nrf2), a master transcription factor that upregulates the expression of antioxidant defense genes and the autophagy gene p62. CA and Res improved survival and motor performance in SCA3 *Drosophila*. Additionally, the above-mentioned protective effects of CA were also observed in CA-supplemented SCA3 *Drosophila*. Notably, blockade of the Nrf2 pathway by use of small interfering RNA annulled the health effects of CA and Res on SCA3, which affirmed the importance of the increase in Nrf2 activation by CA and Res. Additional studies are need to dissect the protective role of CA and Res in modulating neurodegenerative progression in SCA3 and other polyQ diseases.

1. Introduction

Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease (MJD), is a relatively rare but the most common subtype of inherited ataxia worldwide [1]. PolyQ diseases are late-onset, fatally inherited neurodegenerative disorders that result from an abnormal

Abbreviations: ARE, antioxidant response element; Bcl-2, B-cell lymphoma 2; CA, caffeic acid; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; HO1, heme oxygenase; Hsp27, heat shock protein 27; LC3, microtubule-associated protein 1 light chain 3; 3MA, 3-Methyladenine; MMP, mitochondrial membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NQO1, NAD(P)H: quinone oxidoreductase 1; Nrf2, nuclear factor erythroid-derived-2-like 2; PolyQ, polyglutamine; Res, resveratrol; ROS, reactive oxygen species; SCA3, spinocerebellar ataxia type 3; SIRT1, sirtuin1; SOD, superoxide dismutase; tBH, *tert*-butyl hydroperoxide

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polyQ expansion within the disease-specific protein prone to aggregation [2]. For example, SCA3 is caused by a polyQ expansion ranging in length from 60 to 87 repeats in the ataxin-3 protein, which contains 12–44 glutamine repeats in healthy individuals. Although ataxin-3 is widely expressed throughout the body and in neurons, the neurotoxicity and ultimately neurodegeneration induced by the mutant ataxin-3 protein is limited to specific brain regions, which leads to a progressive gait and limb ataxia, dysarthria, dysphagia, oculomotor dysfunction, and premature death [1]. Although effective treatment is presently limited, preclinical data have shown that induction of the autophagy pathway and reduction of oxidative stress can inhibit mutant polyQ protein expression, polyQ aggregation, and neuron cell death and thus may be a potential therapeutic strategy for polyQ diseases [3–6].

Oxidative stress, which results in disruption of redox signaling and control, is implicated in the pathogenesis of several late-onset neurodegenerative disorders including polyQ diseases [3,7]. Normal ataxin-3 protein has been shown to act as a transcriptional coactivator of the manganese superoxide dismutase 2 (SOD2) expression. However, this cytoprotective function against oxidative stress is impaired in lymphoblastoid cell lines of SCA3 patients [8]. Compared with those of healthy individuals, the leukocytes of SCA3 patients have higher amounts of the nuclear and mitochondrial DNA damage that is brought about by increased oxidative stress [9,10]. Moreover, data have shown that reactive oxygen species (ROS) production is increased, whereas glutathione (GSH) content, antioxidant enzyme expression and activity, and mitochondrial DNA copy numbers are reduced in cells expressing full-length mutant ataxin-3 protein [4,5,9]. Notably, the protein expression of heat shock protein 27 (Hsp27) and B-cell lymphoma 2 (Bcl-2), which protect against cell death, is decreased in neuronal SK-N-SH-MJD78 cells stably expressing mutant ataxin-3 with 78 glutamine repeats [11,12]. Our previous data showed that mutant ataxin-3 protein lead to more vulnerable to neuronal apoptosis as well as a reduction of life-span and climbing activity in cells and *Drosophila* treated with the oxidative stress inducer *tert*-butyl hydroperoxide (tBH), respectively [13].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that plays a protective role against neurodegenerative diseases [14]. Activated Nrf2 accumulates in the nucleus and transcriptionally promotes the expression of antioxidant response element (ARE)-mediated cytoprotective genes that encode antioxidant and phase 2 detoxifying proteins such as heme oxygenase 1 (HO1) and NAD(P)H: quinone oxidoreductase 1 (NQO1), glutathione reductase (GR), glutathione peroxidase (GPx), and glutamate-cysteine ligase. Expression of the ARE-mediated genes reduces oxidative stress and cytotoxicity and consequently promotes cell survival [15,16]. Additionally, the Nrf2 pathway is involved in the induction of autophagy which plays an important role in eliminating aggregation-prone proteins and is impaired in neurodegenerative disorders including SCA3 [17–19]. Studies of SCA3 and Huntington's disease have shown that polyQ mutant ataxin-3 and huntingtin proteins contribute to decreased expression and transcriptional activity of Nrf2, respectively, which are involved in the impaired mitochondrial dynamics and increased oxidative stress in cells expressing mutant polyQ proteins [4,5,20]. Moreover, over-expression and knockdown of Nrf2, respectively, reduce and augment the aggregation of mutant ataxin-3 in cells expressing mutant ataxin-3 with 75 glutamine repeats [4]. These findings strongly suggest that activation of the Nrf2 pathway may be an attractive treatment approach for SCA3 and other polyQ diseases.

It is well established that food phenolic compounds through regulation of a variety of molecular mechanisms exert valuable therapeutic benefits including in neurodegenerative diseases [21]. Caffeic acid (CA) is found in many agricultural products such as fruit, vegetables, wine, olive oil, and coffee and accounts for almost 90% of total dietary intake of phenolic acids, the primary representative phenolic compounds present in plants [22,23]. Resveratrol (Res), a polyphenolic stilbene, is present in variety of dietary sources such as red grapes, red wine, berries and peanuts [24]. Res, a sirtuin1 (SIRT1) agonist, can protect against neuron cell death and improve motor performance in cell and mouse models of SCA3 [25,26]. Moreover, Res treatment blocks ROS synthesis and enhances mitochondrial function and autophagy induction in Huntington's disease models. [27,28]. Recently, we showed that CA and Res through modulation of the p53 and Nuclear factor-κB pathways can decrease tBH-elicited neurotoxicity in cell and *Drosophila* models of SCA3 [13]. In connection with the therapeutic values of CA and Res, the aim of the present work was to investigate the protective effect and possible mechanisms of CA and Res against mutant ataxin-3-induced neuronal dysfunction in cell and *Drosophila* models of SCA3.

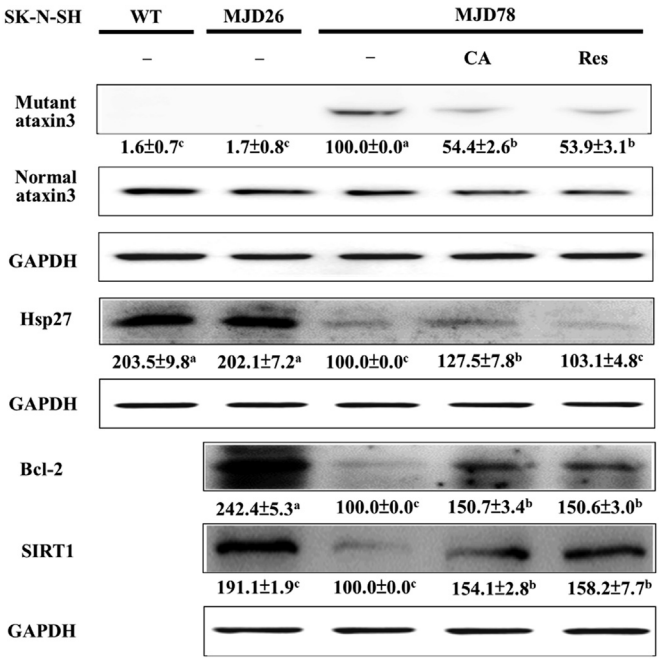


Fig. 1. Effects of CA and Res on protein expression of mutant ataxin-3, Hsp27, Bcl-2 and SIRT1 in SK-N-SH-MJD78 cells. SK-N-SH-WT, SK-N-SH-MJD26, and SK-N-SH-MJD78 cells were treated with or without DMSO vehicle control or 3 μM CA or Res for 24 h. Data are means ± SD of at least three separate experiments and are expressed as the percentage of SK-N-SH-MJD78 cells treated with the vehicle control. Values not sharing the same letter are significantly different ($P < 0.05$).

2. Materials and methods

2.1. Materials

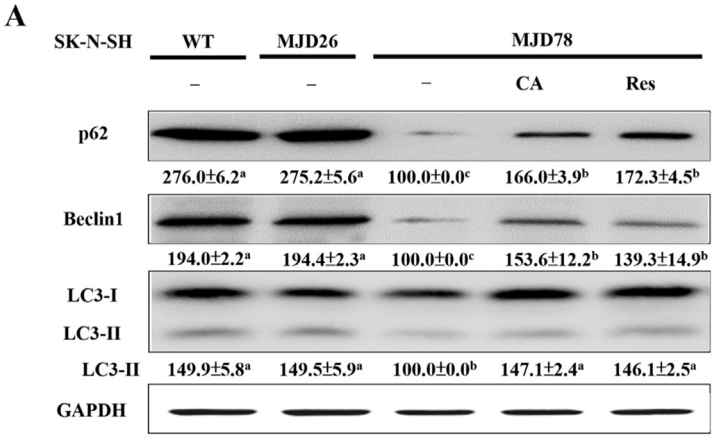
Human SK-N-SH neuroblastoma cells stably transfected with the full-length ataxin-3 gene with 26 or 78 CAG repeats (SK-N-SH-MJD26 and SK-N-SH-MJD78, respectively) were provided by Prof. Mingli Hsieh (Department of Life Science, Tunghai University, Taiwan). The UAS-MJDtr-Q27, UAS-MJDtr-Q78 and *elav*-Gal4 fly strains were purchased from the Bloomington *Drosophila* stock center (Indiana University, IN, USA). CA, Res and 3-Methyladenine (3MA) were got from Sigma Chemical Co. (St Louis, MO, USA). Antibodies against hsp27, NQO-1, Nrf2, Bcl-2, Beclin 1, Histone H1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Specific antibodies for the glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit (GCLM), SOD1, SOD2, catalase, GPx1, GPx2 and GR were obtained from Genetex Inc. (GeneTex, CA, USA). Specific antibodies for SIRT1, microtubule-associated protein 1 light chain 3 (LC3), HO-1 and β-actin were from Cell Signaling Technology Inc. (Beverly, MA, USA), MBL international (Woburn, MA, USA), Calbiochem (San Diego, CA, USA) and Millipore (Billerica, MA, USA), respectively. The specific antibodies to p62 and ataxin-3 were from Abcam (Cambridge, MA). The

Table 1
Effects of CA and Res on cell viability (MTT assay), protein aggregation and MMP (TMRE staining) in SK-N-SH-MJD78 cells*.

SK-N-SH	WT	MJD26	MJD78		
	DMSO	DMSO	DMSO	CA	Res
MTT#	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	96.4 ± 0.2	92.6 ± 0.2
protein aggregation#	8.0 ± 0.5^{e}	14.0 ± 1.4^{d}	100.0 ± 0.0^{a}	65.6 ± 2.1^{c}	73.0 ± 1.7^{b}
TMRE#	NT	360.4 ± 4.2^{a}	100.0 ± 0.0^{c}	245.4 ± 2.6^{b}	241.3 ± 1.6^{b}

* SK-N-SH-WT, SK-N-SH-MJD26, and SK-N-SH-MJD78 cells were treated with or without DMSO vehicle control or 3 μM CA or Res for 24 h except for measurement of the MMP level (4 h treatment).

Values are expressed as the percentage of SK-N-SH-MJD78 cells treated with the vehicle control. Data are means ± SD of at least three separate experiments and not sharing the same letter are significantly different ($P < 0.05$). NT: not tested.



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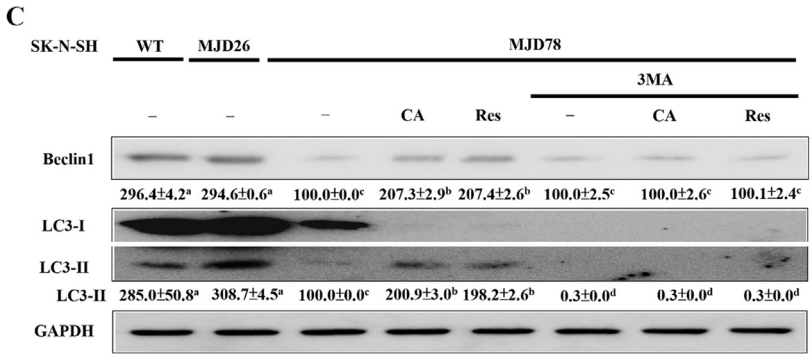
SK-N-SH	WT	MJD26	MJD78					
						3MA		
	DMSO	DMSO	DMSO	CA	Res	DMSO	CA	Res
autophagy	503.8±14.4^{a}	505.6±21.8^{a}	100.0±0.0^{c}	355.5±15.5^{b}	340.8±19.3^{b}	97.7±1.1^{c}	98.0±1.5^{c}	98.9±1.0^{c}
protein aggregation	7.6±0.4^{e}	14.2±0.2^{d}	100.0±0.0^{a}	64.7±0.7^{c}	70.7±0.8^{b}	102.6±1.3^{a}	103.4±1.8^{a}	102.8±1.6^{a}



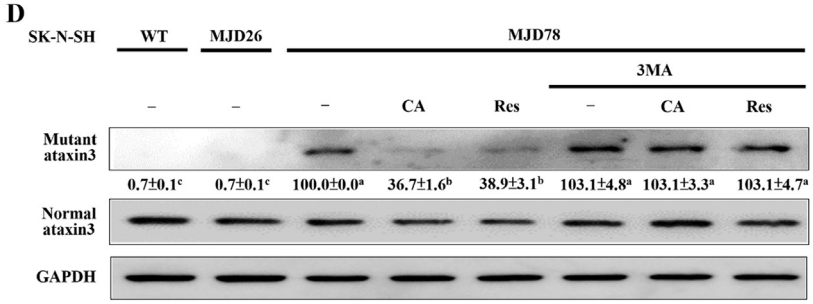


Fig. 2. Effects of CA and Res on autophagy in SK-N-SH-MJD78 cells. Cells were treated with or without DMSO vehicle control or 3 μM CA or Res for 24 h in the absence or presence of pretreatment with 1 mM 3MA for 1 h. (A) and (C) Protein expression of p62, Beclin-1, and LC3-II. (B) Autophagic cells and protein aggregation were measured by acridine orange staining and the PROTEOSTAT* protein aggregation assay, respectively, and were quantified by flow cytometry. (D) Protein expression of mutant and normal ataxin-3. Values are expressed as the percentage of SK-N-SH-MJD78 cells treated with the vehicle control. Data are means ± SD of at least three separate experiments and not sharing the same letter are significantly different ($P < 0.05$).

Table 2
Effects of CA and Res on ROS and GSH levels in SK-N-SH-MJD78 cells*.

SK-N-SH	WT	MJD26	MJD78		
	DMSO	DMSO	DMSO	CA	Res
H_2DCFDA#	8.1 ± 0.2[c]	11.5 ± 0.8[c]	100.0 ± 0.0[a]	30.2 ± 1.3[b]	31.7 ± 1.1[b]
MitoSOX#	12.6 ± 0.5[c]	12.6 ± 1.0[c]	100.0 ± 0.0[a]	31.2 ± 3.3[b]	30.3 ± 3.3[b]
GSH#	NT	333.8 ± 3.2[a]	100.0 ± 0.0[c]	235.1 ± 3.1[b]	232.4 ± 3.0[b]

* Cells were treated with or without DMSO vehicle control or 3 μM CA or Res for 24 h except for measurement of H_2DCFDA (18 h treatment).
# Values are expressed as the percentage of SK-N-SH-MJD78 cells treated with the vehicle control. Data are means ± SD of at least three separate experiments and not sharing the same letter are significantly different ($P < 0.05$). NT: not tested.

PROTEOSTAT® Protein aggregation assay was from Enzo Life Sciences (Farmingdale, NY, USA).

2.2. Cell culture and treatment

The neuroblastoma cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with l% penicillin/streptomycin, 2 mM glutamine, 1% non-essential amino acids, and 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT) at 37 °C under a humidified atmosphere of 5% CO_2. Medium containing 100 μg/mL G418 (InvivoGen, San Diego, CA, USA) was used to maintain exogenous protein expression in SK-N-SH-MJD26 and SK-N-SH-MJD78 cells. When the cells reached 90% confluence, they were treated with dimethyl sulfoxide (DMSO) vehicle control or 3 μM CA or Res.

2.3. Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl) − 2,5-diphenyltetrazolium bromide (MTT) assay was used as an indicator of cell viability. After treatment with CA or Res for 24 h, cells were incubated in DMEM containing 0.5 mg/mL MTT for 3 h. After removal of the media, isopropanol was added and the cells were centrifuged at 5000 × *g* for 5 min. The absorbance of the supernatant from each sample was read at 570 nm in a VersaMax™ Tunable Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA).

2.4. Protein extraction and Western blot

RIPA lysis buffer as well as hypotonic and then hypertonic extraction buffer were used to prepare total protein and nuclear protein extracts, respectively [29]. Equal amounts of proteins were denatured and separated on SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (New Life Science Product, Inc., Boston, MA, USA). Proteins were visualized with specific antibodies and horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Bio-Rad, Hercules, CA, USA), which were developed using the enhanced chemiluminescence kit (Perkin–Elmer Life Science, Boston, MA, USA). Immunoblots were scanned by luminescent image analyzer (LAS-1000 plus, Fuji Photo Film Company, Japan) and quantified by AlphaImager 2200 (Alpha Innotech Corp., San Leandro, CA, USA).

2.5. Measurements of ROS, mitochondrial membrane potential (MMP), autophagic cells and protein aggregation

Levels of ROS in cell and fly models of SCA3 were determined with CM-H_2DCFDA and MitoSOX Red. Acridine orange, TMRE staining and PROTEOSTAT® Protein aggregation assay were used to quantify autophagic cells, MMP and protein aggregation, respectively. Fluorescence intensity was measured by use of the FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and FlexStation 3 Multi-Mode Microplate Reader (Applied Biosystems, Foster City, CA, USA).

2.6. Determination of GSH content

GSH content was assayed by using the dithio nitrobenzene method with some modifications [30]. Cells were scraped into PBS and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was deproteinized by the addition of 5% perchloric acid solution containing 2.5 mM phenanthroline. After centrifugation at 5000 × *g* for 5 min, supernatant was neutralized with 20 mM Tris/EDTA and then 10 mM 5,5′-dithiobis-2-nitrobenzoic acid was added. GSH was measured by the production of 5-thio-2-nitrobenzoic acid, which was photometrically determined at 412 nm. The protein concentration in each sample was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc.).

2.7. Plasmids and transient transfection

The pGL3 promoter-luciferase plasmid expressing a 2xARE fragment containing tandem repeats of double-stranded oligonucleotides spanning the Nrf2 binding site, 5′-TGACTCAGCA-3′, was used to assay Nrf2 transcriptional activity [31]. Cells were transiently transfected with plasmid or control vector by use of Lipofectamine 2000 transfection reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions.

2.8. Reporter gene assay

Nrf2 transcriptional activity was measured by using the Luciferase Assay System corrected on the basis of β-galactosidase activity by using the β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer from Promega Co.

2.9. Nrf2 small interfering RNA (siRNA) transfection

To knockdown the Nrf2 expression, SK-N-SH-MJD78 cells were transfected with predesigned siRNA against Nrf2 and non-targeting control (NTC) siRNA (MDbio, Inc., Taipei, Taiwan) by using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. After 16 h of transfection, the cells were treated with CA or Res as described in the figure legends.

2.10. Drosophila stocks and crosses

All fly stocks were raised on a standard cornmeal medium at 25 °C on a 12-h light-dark cycle at 60% relative humidity. Virgin female flies carrying the driver *elav-Gal4* on the X chromosome were crossed to male flies carrying UASQ27 or UASQ78, and F1 offspring selectively expressed ataxin-3tr-Q27 or ataxin-3tr-Q78 in the nervous system.

2.11. Survival and climbing activity

After eclosion, female flies were grown on the standard media added with or without DMSO vehicle control, 0.5 mM or 1 mM CA, or 0.5 mM Res. The medium was changed and the survival rate and climbing activity were assessed every 3 days. The survival data were

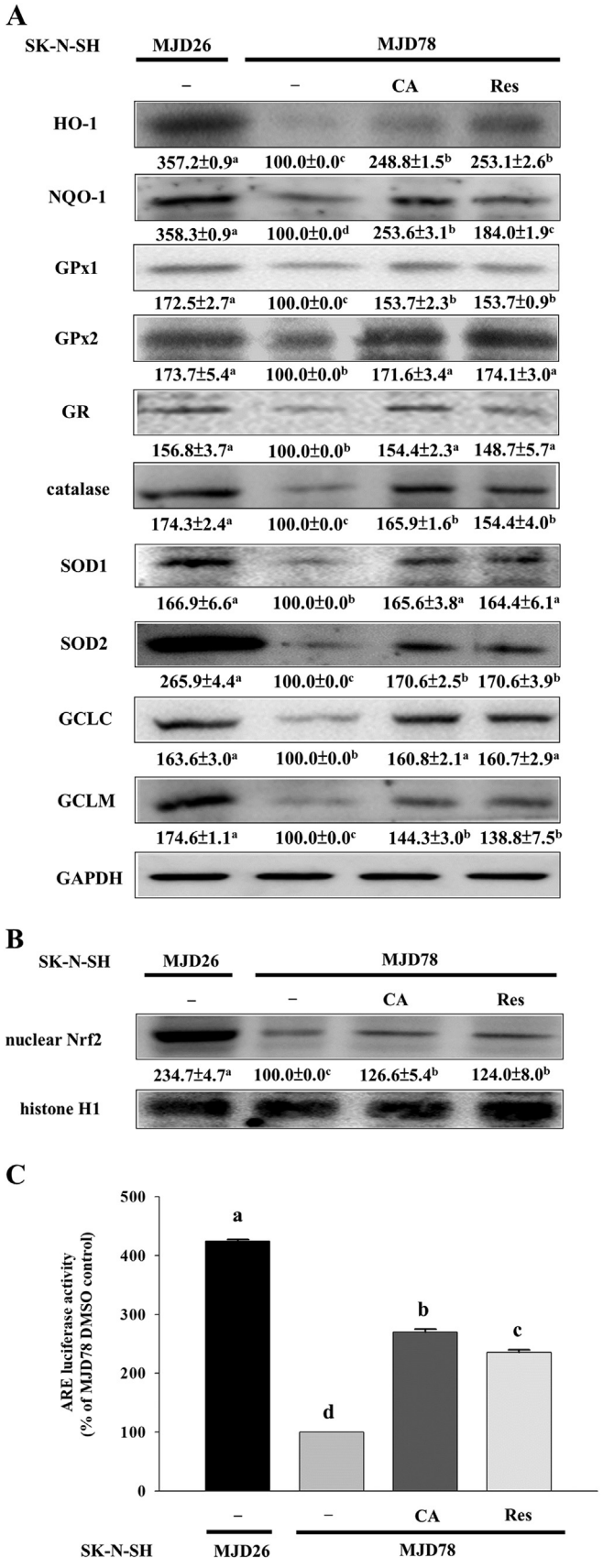


Fig. 3. Effects of CA and Res on ROS, antioxidant enzyme expression, and Nrf2 activation in SK-N-SH-MJD78 cells. Cells were treated with or without DMSO vehicle control or 3 μM CA or Res for 24 h. (A) and (B) Protein expression of HO-1, NQO1, GPx, GR, catalase, SOD, GCLC, GCLM, and nuclear Nrf2. (C) Before treatment with CA and Res, cells were transfected with ARE-luciferase reporter construct for 16 h. Nrf2 reporter gene activity was measured by luciferase activity level, which was normalized by β-galactosidase activity level. Data are means ± SD of at least three separate experiments and are expressed as the percentage of SK-N-SH-MJD78 cells treated with the vehicle control. Values not sharing the same letter are significantly different ($P < 0.05$).

graphed and compared by using Kaplan–Meier log rank survival statistics in SigmaStat V3.5 software (Systat Software, Inc., San Jose, CA, USA). For measurement of climbing activity, flies were tapped down and the number of flies reaching 5 cm in 18 s was recorded. The climbing activity (%) was determined as $N_{top}/N_{total} \times 100$, where N_{total} and N_{top} represented the numbers of total flies and the number of flies at the top (over the 5-cm line), respectively.

2.12. Statistical analysis

Data are expressed as the means ± SD from at least three independent experiments. Differences among treatments were examined statistically by using the Statistical Analysis System (Cary, NC, USA) by one-way ANOVA and Tukey's multiple-range test. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of CA and Res on protein aggregation, mutant ataxin-3, Hsp27, Bcl-2, SIRT1 and MMP in SK-N-SH-MJD78 cells

SK-N-SH-MJD78 cells showed increases in mutant ataxin-3 protein expression and protein aggregation and decreases in Hsp 27, Bcl-2 and SIRT1 expression and the MMP compared with parental SK-N-SH cells (SK-N-SH-WT cells) and/or SK-N-SH-MJD26 cells. Incubation of SK-N-SH-MJD78 cells with CA or Res reduced the amount of protein aggregation and mutant ataxin-3 protein expression and restored levels of Hsp27, Bcl-2, SIRT1 and MMP without influencing cell viability ($P < 0.05$, Fig. 1 and Table 1).

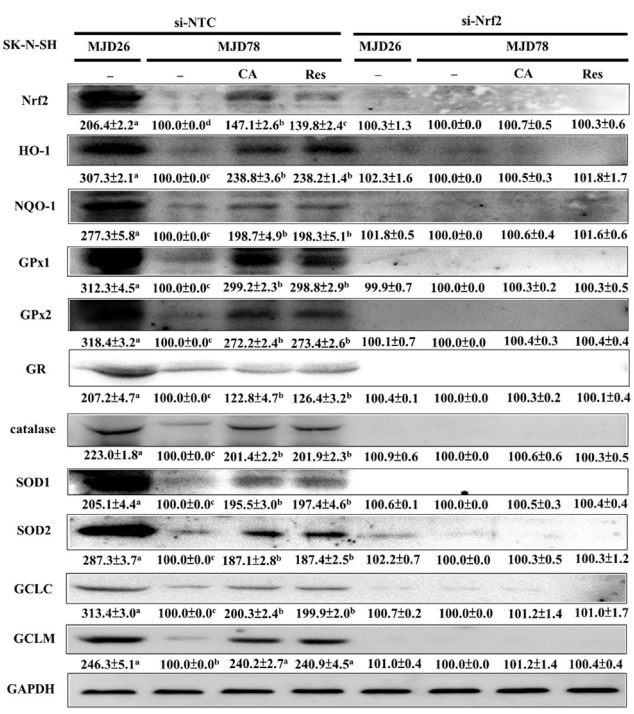
3.2. Effects of CA and Res on autophagy in SK-N-SH-MJD 78 cells

Upregulation of autophagy enhances the clearance of mutant ataxin-3 and its aggregation, which helps to alleviate the neurotoxicity in SCA3 [19,32]. Compared with SK-N-SH-WT and SK-N-SH-MJD26 cells, autophagy levels in SK-N-SH-MJD78 cells as measured by the protein expression of p62, beclin 1, and LC3-II and staining with the lysosomotropic agent acridine orange were reduced. Treatment with CA and Res augmented the autophagy levels in SK-N-SH-MJD78 cells ($P < 0.05$, Fig. 2A and B). Notably, the ability of CA and Res to inhibit mutant ataxin-3 expression and protein aggregation in SK-N-SH-MJD78 cells was cancelled by pretreatment with 3MA, a specific inhibitor of autophagy ($P < 0.05$, Fig. 2B, C and D).

3.3. Effects of CA and Res on oxidative stress and antioxidant protein expression in SK-N-SH-MJD 78 cells

Oxidative stress plays an important role in polyQ protein aggregation and neurodegeneration in SCA3 and other polyQ diseases [8,9,33,34]. Compared with that in SK-N-SH-WT and SK-N-SH-MJD26 cells, total and mitochondrial ROS levels were increased in SK-N-SH-MJD78 cells as determined by H_2-DCFDA and MitoSOX fluorescence assays, respectively ($P < 0.05$,Table 2). Moreover, the antioxidant system was damaged in SK-N-SH-MJD78 cells as evidenced by the decreased protein expression of HO-1, NQO1, GPx, GR, catalase, and SOD ($P < 0.05$, Fig. 3A). Furthermore, GSH levels as well as the expression

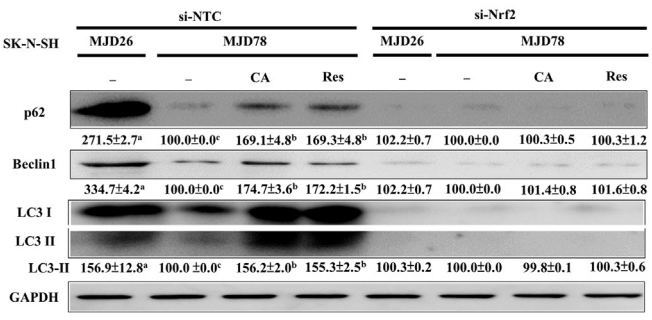
A



B

	si-NTC				si-Nrf2			
SK-N-SH	MJD26	MJD78			MJD26	MJD78		
	DMSO	DMSO	CA	Res	DMSO	DMSO	CA	Res
ARE luciferase activity	426.2±3.5^a	100.0±0.0^d	265.6±2.5^b	238.4±2.3^c	105.9±1.3^a	100.0±0.0^b	103.4±1.4^a	104.0±1.8^a
H_2DCFDA	11.3±0.2^c	100.0±0.0^a	32.1±0.5^b	32.5±0.5^b	97.4±0.4^b	100.0±0.0^a	97.2±0.8^b	99.3±0.7^a
MitoSOX	13.7±0.5^d	100.0±0.0^a	37.1±1.7^b	30.2±1.7^c	91.9±0.6^b	100.0±0.0^a	100.0±1.3^a	101.0±0.7^a
GSH	343.5±1.8^a	100.0±0.0^d	224.2±2.6^b	221.3±1.5^c	100.3±0.1^a	100.0±0.0^a	100.4±0.1^a	100.5±0.1^a
protein aggregation	12.6±0.2^d	100.0±0.0^a	65.0±0.6^c	69.7±1.1^b	70.2±3.8^b	100.0±0.0^a	105.6±3.2^a	108.2±3.5^a
TMRE	381.8±4.7^a	100.0±0.0^c	267.4±3.5^b	262.6±1.3^b	108.0±8.3^a	100.0±0.0^b	100.0±1.2^b	102.6±12.0^b

C



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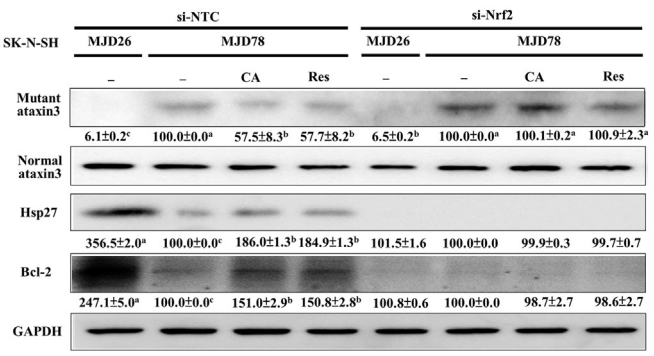

Fig. 4. Effects of CA and Res in SK-N-SH-MJD78 cells transfected with si-Nrf2. Cells were transiently transfected with si-NTC or si-Nrf2 as well as with or without ARE-luciferase reporter construct for 16 h and then treated with or without DMSO vehicle control or 3 μM CA or Res for 24 h except for measurement of H_2DCFDA (18 h treatment). (A) Protein expression of Nrf2, HO-1, NQO1, GPx, GR, catalase, SOD, GCLC, and GCLM. (B) Levels of ARE-luciferase reporter gene activities, H_2DCFDA, MitoSOX, GSH, protein aggregations, and TMRE. (C) and (D) Protein expression of p62, Beclin-1, LC3-II, mutant and normal ataxin-3, Hsp27, and Bcl-2. Data are means ± SD of at least three separate experiments. Within treatments with the same plasmid transfection, values are expressed as the percentage of SK-N-SH-MJD78 cells treated with the vehicle control, and values not having the same letter are significantly different ($P < 0.05$).

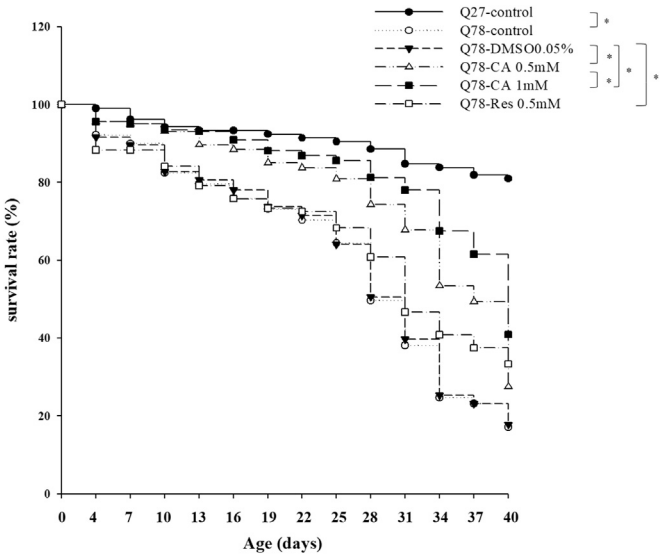

Fig. 5. Effects of CA and Res on survival in ELAV-SCA3tr-Q78 transgenic *Drosophila*. Survival rates were compared across groups by Kaplan-Meier log rank analysis. The mean life-span and SD are shown, * $P < 0.01$ (n = 320).

of GCLC and GCLM (subunits of glutamate-cysteine ligase, the rate-limiting enzyme of glutathione synthesis) were decreased in SK-N-SH-MJD78 cells compared with SK-N-SH-MJD26 cells ($P < 0.05$, Fig. 3A and Table 2). Treatment with CA and Res decreased oxidative stress by lessening ROS production and reconditioning antioxidant protein expression in SK-N-SH-MJD78 cells ($P < 0.05$, Fig. 3A and Table 2).

3.4. Effects of CA and Res on Nrf2 activation in SK-N-SH-MJD 78 cells

We then examined whether CA and Res could induce Nrf2 and the importance of Nrf2 activation in the health benefits of CA and Res in SK-N-SH-MJD78 cells. As expected, nuclear Nrf2 protein expression and Nrf2 transcriptional activity were lower in SK-N-SH-MJD78 cells than in SK-N-SH-MJD26 cells. Addition of CA and Res increased Nrf2 activation in SK-N-SH-MJD78 cells ($P < 0.05$, Fig. 3B and C). We used Nrf2 siRNA to knock down Nrf2 expression and transcriptional activity induced by CA and Res. The ability of CA and Res to recover the aforementioned protein expression involved in antioxidation and autophagy was diminished in SK-N-SH-MJD78 cells transfected with Nrf2 siRNA ($P < 0.05$, Fig. 4A, B and C). Of note was that the therapeutic effects of CA and Res on mutant ataxin-3, Hsp 27, and Bcl-2 expression; on protein aggregation, and on MMP in SK-N-SH-MJD78 cells were cancelled by silencing Nrf2 ($P < 0.05$, Fig. 4B and D).

3.5. Effects of CA and Res on life-span and climbing activity in ELAV-SCA3tr-Q78 transgenic Drosophila

To further confirm the protective effects of CA and Res on SCA3 phenotypes, we analyzed the survival rates and climbing activity of ELAV-SCA3tr-Q78 transgenic flies. When compared with ELAV-SCA3tr-Q27 flies, the ELAV-SCA3tr-Q78 flies had reduced life-span and climbing activity and these decreases were reversed by administration of CA and Res ($P < 0.05$, Fig. 5 and Table 3).

3.6. Effects of CA on protein aggregations and expression of mutant ataxin-3, Hsp27, and autophagy molecules in ELAV-SCA3tr-Q78 transgenic Drosophila

In agreement with the in vitro data, CA affected exogenous mutant ataxin-3 expression and protein aggregation, depleted Hsp 27 expression, and the molecular expression of proteins involved in autophagy in ELAV-SCA3tr-Q78 transgenic flies ($P < 0.05$, Fig. 6A and Table 4).

3.7. Effects of CA on oxidative stress, Nrf2 and antioxidant enzyme expression in ELAV-SCA3tr-Q78 transgenic Drosophila

Compared with that in ELAV-SCA3tr-Q27 flies, the ROS level was increased as well as Nrf2 and antioxidant enzyme expression was reduced in ELAV-SCA3tr-Q78 transgenic flies. Addition of CA ameliorated oxidative stress in ELAV-SCA3tr-Q78 transgenic flies as evidenced by the decline in total and mitochondrial ROS levels as well as the increase in Nrf2 and antioxidant enzyme expression ($P < 0.05$, Fig. 6B and Table 4).

4. Discussion

Increasing evidence underscores that a reduced capability to counteract oxidative stress and a decreased induction of autophagy are part of the neuropathological changes induced by mutant ataxin-3 in SCA3 [3–6]. Previous data have shown that mutant ataxin-3 have reduced expression of hsp27, Bcl2 and SIRT1 protein as well as of antioxidant proteins such as NQO1, SOD 1 and 2, GCLC, and GPx1 and that these changes result in increases in ROS, protein aggregation and susceptibility to apoptotic inducers in the cell and animal models of SCA3 [4,5,8,9,11,12,25]. Moreover, the autophagy pathway is impaired in advanced SCA3 [19,32]. This pathway is crucial for clearing mutant polyQ tracts and misfolded protein aggregations [18,19,32]. Induction of the autophagy pathway by use of mTOR inhibitor, temsirolimus, or over-expression of the autophagy protein beclin-1 can decrease the levels of mutant ataxin-3 and its aggregations, which has a neuroprotective role in cell and transgenic mouse models of SCA3 [19,32]. Along these lines, our data showed that treatment with CA and Res increased the expression of GSH, the expression of Hsp27, Bcl-2, SIRT1 and the expression of antioxidant proteins, e.g., HO-1, NQO-1, catalase, SOD, GPx, and GR. As a result, total and mitochondrial ROS were eliminated and the MMP was corrected in SK-N-SH-MJD78 cells. Moreover, CA and Res not only decreased the expression of mutant ataxin-3 and its aggregation but also increased autophagy as evidenced by the expression of autophagy proteins and staining of cells with a lysosomotropic agent. Notably, pretreatment with the autophagy inhibitor 3MA blocked the inhibitory effects of CA and Res on the levels of mutant ataxin-3 and its aggregation. This finding suggests the importance of autophagy in the beneficial properties of CA and Res in SK-N-SH-MJD78 cells.

In our previous study we showed that treatment with CA and Res

Table 3
Effects of CA and Res on climbing activity in ELAV-SCA3tr-Q78 transgenic *Drosophila*.

Climbing activity (%)[#]	ELAV-SCA3tr-Q27	ELAV-SCA3tr-Q78				
	Control	Control	DMSO	CA 0.5 mM	CA 1 mM	RSV 0.5 mM
7 day-old	96.2 ± 1.6^{a}	78.0 ± 4.5^{c}	78.3 ± 6.1^{c}	83.5 ± 3.9^{b}	84.6 ± 3.1^{b}	78.3 ± 0.0^{c}
19 day-old	92.4 ± 1.6^{a}	63.1 ± 4.4^{c}	62.8 ± 9.2^{c}	76.8 ± 4.7^{b}	79.1 ± 4.9^{b}	65.8 ± 7.2^{c}
28 day-old	88.3 ± 0.5^{a}	39.2 ± 3.9^{e}	39.4 ± 4.0^{e}	67.8 ± 4.0^{c}	74.7 ± 3.2^{b}	53.3 ± 7.2^{d}
40 day-old	80.0 ± 3.4^{a}	3.3 ± 1.8^{d}	2.4 ± 1.1^{d}	7.5 ± 3.0^{c}	17.6 ± 2.6^{b}	12.8 ± 1.3^{b}

[#] Within the same age, values not sharing the same letter are significantly different ($P < 0.05$).

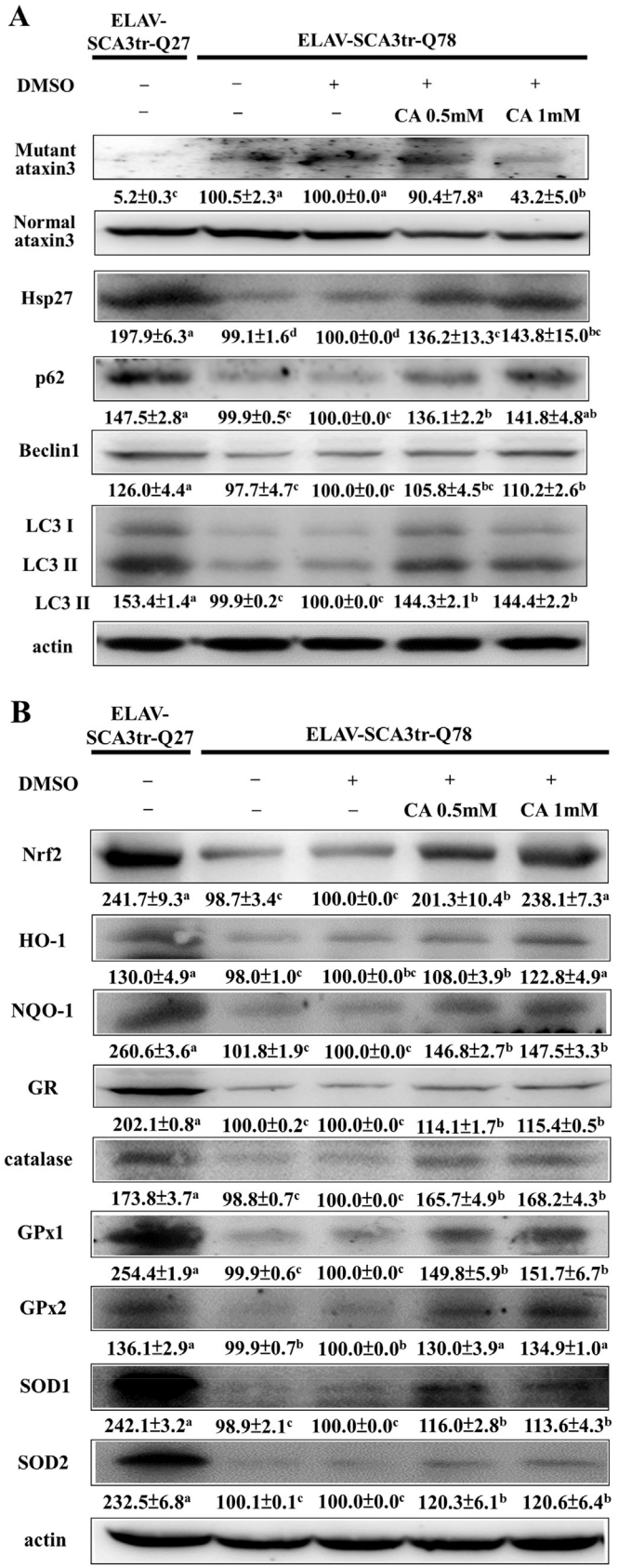

Fig. 6. Effects of CA on ELAV-SCA3tr-Q78 transgenic *Drosophila*. In 19-day-old female ELAV-SCA3tr-Q78 flies, (A) and (B) protein expression of mutant and normal ataxin-3, Hsp27, p62, Beclin-1, LC3-II, Nrf2, HO-1, NQO1, GPx, GR, catalase, and SOD. Values are means ± SD, n = 30 female flies in three separate experiments. The values are expressed as the percentage of ELAV-SCA3tr-Q78 flies treated with the vehicle control. In ELAV-SCA3tr-Q78 fly groups, values not sharing the same letter are significantly different ($P < 0.05$).

can alleviate the mitochondrial apoptosis caused by oxidative stress inducer in a neural cell model of SCA3 [13]. The Nrf2 pathway is an important cellular defense mechanism against ROS that acts by inducing the expression of various antioxidant and detoxification enzymes. Additionally, activation of the Nrf2 pathway through an increase in mitophagy and resistance to oxidative stress-induced mitochondrial permeability transition impacts mitochondrial integrity, which is damaged in many neurodegenerative diseases, including polyQ diseases [35–37]. Notably, data have demonstrated molecular cross-talk between the Nrf2 pathway and autophagy. The autophagy-related protein p62 can facilitate Nrf2 nuclear translocation and transcriptional activity through binding with Kelch-like ECH-associated protein 1. Because p62 contains a functional ARE element in the promoter region of its gene, the increased Nrf2 transcriptional activity in turn can induce p62 expression [17]. Thus, this positive feedback loop of Nrf2 activation and p62 expression may provide therapeutic benefits in neurodegenerative disorders including polyQ diseases. Herbal medicines augmenting the Nrf2 pathway may decrease not only ROS but also polyQ aggregations in cells with mutant ataxin 3 [4,5]. As aforementioned, consistent with antioxidant and autophagy-related protein expression, the results of the present study showed that Nrf2 activation was significantly lower in SK-N-SH-MJD78 cells than in SK-N-SH-MJD26 cells and that treatment with CA and Res augmented nuclear Nrf2 expression and its transcriptional activity. When we used Nrf2 siRNA to block Nrf2 activation, we found that CA and Res induced antioxidant and autophagy-related protein expression and subsequently decreased oxidative stress in SK-N-SH-MJD78 cells through an Nrf2-dependent manner. Notably, the effects of CA and Res on mutant ataxin-3 and its aggregation and on MMP in SK-N-SH-MJD78 cells were also cancelled when the cells were transfected with Nrf2 siRNA. These data suggest that enhancing Nrf2 activation is important in the neuroprotective effects of CA and Res on SCA3.

ELAV-SCA3tr-Q78 transgenic flies which show neuron-specific expression of the 78-residue polyQ tract of ataxin-3 have features similar to human SCA3, with late-onset, progressive neurodegeneration and abnormal protein aggregation. Compared with ELAV-SCA3tr-Q27 control flies, there is no remarkable degeneration of the adult eye in ELAV-SCA3tr-Q78 flies due to the neuron-specific promoter elav. However, the ELAV-SCA3tr-Q78 flies have a shorter life-span than the GMR-SCA3tr-Q78 flies where the ataxin-3 polyQ tract of 78 residues is selectively expressed in the developing eyes [38,39]. Previous data from our lab showed that supplementation with CA and Res prolongs life-span and restores locomotor activity in tBH-treated ELAV-SCA3tr-Q78 transgenic flies [13]. In the present study, we showed that supplementation with CA and Res significantly enhanced life-span and climbing activity in unstressed ELAV-SCA3tr-Q78 transgenic flies. Moreover, we found that CA administration diminished ROS production, protein aggregation, and the expression of mutant ataxin-3, Hsp27, Nrf2 as well as antioxidant and autophagy molecules in ELAV-SCA3tr-Q78 flies under unstimulated conditions.

Data from epidemiological and experimental studies have proven that increased consumption of polyphenols or polyphenol-rich foods is associated with decreased risk of neurodegenerative diseases [40]. The present study showed for the first time that CA and Res through induction of Nrf2 activation can correct disorders induced by mutant ataxin-3 in human neuroblastoma cell models of SCA3. Moreover, the finding that supplementation with CA and Res improved life-span and climbing activity in SCA3 *Drosophila* strengthens the therapeutic value of CA and Res in SCA3. Our data suggest that further in-depth studies to reinforce the health effects of CA and Res in SCA3 and other polyQ diseases are warranted.

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Table 4
Effects of CA on protein aggregation and ROS in ELAV-SCA3tr-Q78 transgenic *Drosophila*[*].

	ELAV-SCA3tr-Q27	ELAV-SCA3tr-Q78			
	Control	Control	DMSO	CA 0.5 mM	CA 1 mM
protein aggregation[#]	10.8 ± 1.9[c]	99.1 ± 1.2[a]	100.0 ± 0.0[a]	42.7 ± 1.8[b]	41.3 ± 3.7[b]
H_2DCFDA[#]	1.3 ± 0.2[d]	100.1 ± 1.3[a]	100.0 ± 0.0[a]	62.3 ± 2.7[b]	12.6 ± 0.9[c]
MitoSOX[#]	2.1 ± 0.3[c]	100.4 ± 1.3[a]	100.0 ± 0.0[a]	36.1 ± 1.4[b]	34.7 ± 1.9[b]

[*] Levels of protein aggregation, H_2DCFDA, and MitoSOX were measured in 19-day-old female flies.
[#] Values are means ± SD, n = 30 female flies in three separate experiments. Values are expressed as the percentage of ELAV-SCA3tr-Q78 flies treated with the vehicle control. Values not sharing the same letter are significantly different ($P < 0.05$).

Author contributions

Conceived and designed the experiments: Y.L.W., J.C.C., C.S.L. and K.L.L. Performed the experiments: Y.L.W. Analyzed the data: Y.L.W., J.C.C., C.C.L., W.Y.L. W.T.W. and K.L.L. Contributed reagents/materials/analysis tools: J.C.C., M.H., W.Y.L., C.C.L., T.S.W., W.T.W., C.S.L. and K.L.L. Writing of the manuscript: Y.L.W., H.W.C. and K.L.L. All authors have reviewed the manuscript.

Competing interests

The authors declare no competing financial interests.

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