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ORIGINAL ARTICLE

Prophylactic neuroprotective propensity of Crocin, a carotenoid against rotenone induced neurotoxicity in mice: behavioural and biochemical evidence

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Abstract

Previously we have demonstrated the potential neuroprotective propensity of saffron and Crocin (CR) employing a *Drosophila* model of Parkinsonism. Rotenone (ROT) has been extensively used as a model neurotoxin to induce Parkinson's disease (PD) like symptoms in mice. In the present study, as a proof of concept we evaluated the efficacy of CR prophylaxis (25 mg/ kg bw/d, 7d) to attenuate ROT(0.5 mg/Kg bw/d,7d) -induced neurotoxic effects in male mice focussing on neurobehavioural assessments and biochemical determinants in the striatum. CR prophylaxis significantly alleviated ROT-induced behavioural alterations such as increased anxiety, diminished exploratory behaviour, decreased motor co-ordination, and grip strength. Concomitantly, we evidenced diminution of oxidative stress markers, enhanced levels of antioxidant enzyme and mitochondrial enzyme function in the striatal region. Further, varying degree of restoration of cholinergic function, dopamine and α-synuclein levels were discernible suggesting the possible mechanism/s of action of CR in this model. Based on our earlier data in flies and in worm model, we propose its use as an adjuvant therapeutic agent in oxidative stress-mediated neurodegenerative conditions such as PD.

Keywords Crocin · Neuroprotection · Rotenone · Parkinson's disease · Neurotoxicity

Introduction

Crocus sativus belonging to the family Iridaceae, is a perennial herb. The dried, red stigma of this plant is called saffron, which has an extensive record of use as a spice, medicine, and coloring agent (Srivastava et al. 2010). Saffron is known for a large number of medicinally important activities like anticonvulsant, antidepressant, antinociceptive, antihypertensive, antioxidant, anti-inflammatory, and relaxant activity (Hosseinzadeh and Jahanian 2010). Saffron is enriched with volatiles and aromatic compounds. The major characteristic bioactives of saffron are Crocin (CR), safranal, and picrocrocin. CR is responsible for the characteristic colour, safranal and picrocrocin are responsible for the distinctive flavour of saffron (Christodoulou et al. 2015). Saffron extracts and its bioactive constituents have been demonstrated to possess excellent antioxidant properties (Hosseinzadeh et al. 2009; Karimi et al. 2010; Mashmoul et al. 2013). Further, their learning and memory enhancing properties in animal models have been attributed to their antioxidant action (Amin et al. 2015; Papandreou et al. 2011). During the past decade, several attempts are being made to understand the neuroprotective effects of saffron and its bioactive component CR in animal models (Hosseinzadeh and Nassiri-Asl 2013; Hosseinzadeh and Sadeghnia 2007).

Numerous studies support that environmental toxin (pesticides, heavy metals, solvents and other pollutants) exposure is a key risk factor for the cause of Parkinson's disease (PD) (Betarbet et al. 2000; Cicchetti et al. 2009; Goldman 2014). Selective degeneration of dopaminergic (DA) neurons is the key feature of PD. These environmental contaminants alter mitochondrial respiratory complexes and antioxidant defense resulting in neuronal dysfunction (Caito and Aschner 2015). ROT, a characteristic mitochondrial complex I inhibitor, (Nisticò et al. 2011) is documented to induce oxidative stress, mitochondrial dysfunction, nigrostriatal DA degeneration, intracellular α-synuclein accumulation and motor impairment in

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experimental animals (Cannon and Greenamyre 2014; Murakami 2013; Nisticò et al. 2011). Accordingly, chronic systemic exposure to ROT is used to model PD in the rat, since it induces DA neurodegeneration, Parkinson's like behavior and occurrence of cytoplasmic inclusions similar to the lewy bodies (Betarbet et al. 2000). Evidence suggests that ROT in cell models induces apoptosis and aggregation of α-synuclein and ubiquitin, oxidative damage and endoplasmic reticulum stress (Sherer et al. 2002).

In recent times, the potential modulatory role of various phytonutrients to attenuate endogenous redox status is being considered as an effective approach to achieve neuroprotection (Dumont and Beal 2011; Prasad and Muralidhara 2012; Shinomol et al. 2012). One of the long –term objective of our research is to identify the neuroprotective properties of phytochemcials in invertebrate models such as Drosophila and *C. elegans* (Manjunath and Muralidhara 2015; Rao et al. 2016a, b; Rao et al. 2018) and further assess their efficacy in rodent models. In this regard previously, we have convincingly demonstrated the neuromodulatory potency of CR in a Drosophila model of Parkinsonism (Rao et al. 2016b). However, data on the prophylactic efficacy of CR in experimental models of PD is limited. Hence, our primary objective was to investigate the prophylactic efficacy of CR to attenuate ROT –mediated neurotoxic outcome in mice. In this communication we focused on its ability to alleviate ROT -induced neurobehaviour parameters and render the critical component of motor and reward system i.e., striatum of the brain less susceptible in terms of oxidative impairments and mitochondrial dysfunction. We also determined its potency to protect striatal region as evidenced by replenishment of cholinergic function, modulation of dopamine and α synuclein levels.

Materials and methods

Chemicals and reagents

Crocin (CR), rotenone (ROT), dopamine (DA), and other fine chemicals were procured from M/s Sigma Chemical Co., St. Louis, USA. All other chemicals used were of analytical grade.

Preparation of Crocin and rotenone

A stock of CR (10 mg/ml) was prepared in double distilled water. ROT stock was prepared (250 μg/ ml) in 1:1 solution of DMSO: Olive oil.

Animals and care

Male CFT-Swiss mice (8-week old) obtained from CSIR-CFTRI animal facilities were used for the studies. All animals were housed in standard polypropylene cages (27 X 21 X 14-cm; three/cage) with dust free paddy husk as a bedding material on a photoperiod 12-h light/dark cycle. The Animals were provided with commercial pellet and purified potable water all through the experiment.

The entire experimental design comprehensive of the animal treatment and all procedures were approved by the "Institutional Animal Ethics Committee" (IAEC). The guiding principles of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests, Climate change, Government of India were strictly followed. All efforts were made to minimize animals suffering and to reduce the number of animals used in the experiments.

Experimental design

Male mice ($n = 6$, 8 weeks old) were randomly assigned into four groups and were treated as follows:

Group 1: Control (CTR) group, mice received DMSO: Olive oil only.
Group 2: Crocin (CR) group, mice administered with CR (25 mg/kg bw/d; i.p) for 7 consecutive days.
Group 3: Rotenone (ROT) group, mice administered with rotenone (0.5 mg/ kg bw/d; i.p) for 7 consecutive days.
Group 4: CR + ROT, mice received CR prophylaxis (25 mg/ kg bw/d; i.p) for 7d followed by ROT challenge (0.5 mg/ kg bw/ d, i.p.) from day 8 onwards for the next 7 consecutive days.

Daily food intake of experimental animals was recorded and body weights of mice of all groups were measured on alternate days. Manifestations of behavioral responses were assessed on alternative days employing selected neurobehavioral tests. Terminally, 24 h after the last injection of ROT, mice were sacrificed by anesthetization. Brains region striatum was separated and processed to obtain both cytosolic and mitochondrial fractions. The biochemical investigations were conducted and 5–6 striatum (from each group) processed for histopathological examination.

Behavior studies

Open field test

The open field test (OFT) is a behavior test, which aims to assess the exploratory behavior and general activity of rodents. The open field apparatus was built of plywood and measured 38 X 38 cm with 18 cm walls. The lines divided the floor into sixteen 9.5 X 9.5 cm squares. A central square (9.5 cm × 9.5 cm) was drawn in the middle of the open field.

Mice were placed individually in the corner of the apparatus and their various behavioral activities were recorded. The number of line crosses, the frequency of rearing, grooming, duration of stay in the center square and the number of the entry to the center square are habitually used as measures of locomotor activity, nevertheless are also measures of exploration and anxiety. A high incidence/duration of these behaviors signifies increased exploratory behavior and low anxiety levels. Behaviors were scored for first 5 min for lines crossed, time spent moving, rearing, grooming, freezing, even defecation and urination are measures that were tabulated and reported (Gokul and Muralidhara 2014).

Elevated plus maze (EPM)

Elevated plus maze assay is a behavior test assessing anxiety-like behavior in rodents. This assay resolves a selection between relatively safe and comfortable surroundings (the closed arms) and an unsafe location (elevated open spaces) (Pellow and File 1986). EPM apparatus was made of wood and painted black, containing 4 arms, raised 40 cm above the ground level. Out of 4 arms, two were open without walls, and the other two were closed with 16 cm side walls. Both open and closed arms were 30 cm long and 5 cm wide. The arms were joined with a central place of size 5 X 5 cm. Each animal from every treatment group was put at the intersection of the open and closed arms, allowing for a free exploration for 5 min. The animal behavior was observed and the time spent in each arm was recorded. All the arms were cleaned with 10% ethanol after each trial.

Catalepsy score

The term implies the inability of an animal to correct an externally imposed posture. This behavior assay consists of placing an animal in an unusual position and recording the time taken to correct this posture (Sanberg et al. 1988). An apparatus made of wood having 2 parallel bars (20 cm long) connected with a thread (15 cm) was used to leave the animals in unusual positions. The animal is taken up by lifting its tail and is placed on its forepaws on a horizontal wooden bar. Each mouse was made to hold the thread using its forelimbs and the time taken by the animal to revert to its normal position was recorded.

Rotarod test

This behavior test is primarily employed to assess the motor coordination and capability of rodents. It requires rodents to balance on a revolving cylinder, the speed of which can be altered (Rozas et al. 1997). Each mouse was placed on the rotating cylinder (speed: 30 rpm) and observed for 3 min. The number of falls/s was recorded to score the performance of mice for their motor coordination. The apparatus was cleaned with 10% ethanol between each trial.

Sample preparation

The specific brain region- striatum was dissected on ice from both hemispheres of mice. Mitochondria and cytosol were separated by differential centrifugation practice (Moreadith and Fiskum 1984) using Tris–Sucrose buffer, 0.25 M, pH 7.4 and ice-cold Mannitol-Sucrose-HEPES buffer (pH 7.4).

Biochemical analysis

Measurement of oxidative stress markers

The markers of oxidative impairments were studied in the cytosolic portion. Reactive oxygen species (ROS) formed was quantified from dichlorofluorescen (DCF) standard curve following (Driver et al. 2000) method. Hydroperoxides (HP) generated was measured spectroscopically according to (Wolff 1994). The degree of lipid peroxidation was quantified using thiobarbituric acid following (Ohkawa et al. 1979). The levels of protein carbonyls were measured according to the previously described method (Levine et al. 1990).

Determination of nitric oxide (NO) content

The levels of NO were quantified in cytosol employing Griess reagent (1.5% sulphanilamide and 0.15% N-1-naphthyl-ethylene diamine in 1 N HCl) procured from M/s Sigma Chemicals. Nitric oxide levels were quantified from sodium nitrate standard curve measured at 560 nm. The Assay quantified nitric oxide content depending upon the enzymatic conversion of nitrate to nitrite by nitrate reductase (Choi et al. 2002).

Quantification of reduced glutathione (GSH) and total thiols (TSH)

The levels of Reduced GSH were determined by fluorescence detection method from cytosol following the previously mentioned method (Mokrasch and Teschke 1984). Determination of total thiol content from the cytosolic moiety was done (Ellman 1959).

Activities of antioxidant enzymes

Catalase (CAT) activity was estimated by observing the decomposition of hydrogen peroxide following the earlier detailed procedure (Aebi 1984). Superoxide dismutase (SOD) activity was measured by monitoring the inhibition of quercetin auto-oxidation (Kostyuk and Potapovich 1989). The

activity levels of glutathione-S-transferase (GST) and thioredoxin reductase (TR) were determined following previously mentioned protocol (Guthenberg et al. 1985; Luthman and Holmgren 1982).

Acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was estimated using acetylthiocholine iodide and DTNB following a standard procedure (Ellman et al. 1961).

Measurement of mitochondrial enzyme activity

NADH-cytochrome C reductase (complex I– III) and Succinate cytochrome C reductase (complex II–III) activities were determined by measuring the decrease in the absorbance when mitochondrial protein was added to the substrate and 0.1 mM cytochrome C for 3 min at 550 nm (e-19.6/mM/cm). The enzyme activities were expressed in nmol cytochrome C reduced/min/mg protein (Navarro et al. 2004; Pennington 1961). *MTT Reduction Assay* (Berridge and Tan 1993): An aliquot of protein of was combined with Mannitol-sucrose-HEPES buffer (pH 7.4) containing sodium succinate (20 mM) and MTT (3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide) (75 mg/ml) and incubated at 37 °C for 60 min. The formazan crystals produced were dissolved in aqueous 10% SDS-45% DMF buffer (*V*/V; pH 7.4). The optical density was measured at 570 nm and the value expressed as absorbance/mg protein.

Determination of ADP/ATP ratio: ADP/ATP ratio in striatum sample was measured by ApoSENSOR™ ADP/ATP Ratio Bioluminescent Assay Kit (from Biovision Catalog #K255–200) following manufacturer's instructions. This kit employed bioluminescent detection of the ADP and ATP intensities.

Dopamine (DA) quantification

DA levels in the striatum region were determined by HPLC method (Dalpiaz et al. 2007). Homogenised and filtered samples were allowed to pass through microbore LC-18 analytical column from Sigma-Aldrich (150 mm 9 4.6 mm, 5 lm particle size) connected to a UV detector (280 nm). The mobile phase was a mixture of methanol and deionized water (70:30, *v*/v; pH 3.5) with 0.2% trifluoroacetic acid. The flow rate was 1 mL/min. DA levels in the sample were calculated from external standard and expressed as μg DA/mg protein.

Quantification of α-synuclein

α-synuclein was quantified in the striatum region of mice brain utilizing Enzyme-linked Immunosorbent Assay (ELISA) Kit from Invitrogen (Cat No. KHB0061) following manufacturer's instructions.

Protein estimation

The protein content was determined following a well-known procedure (Classics Lowry et al. 1951) using Lowry's reagent (2% Na_3CO_3 in 0.1 N sodium hydroxide containing 1% copper sulphate and 2% sodium potassium tartrate). The absorbance was measured at 750 nm, and the concentration of protein determined using BSA as the standard.

Statistical analysis

The data obtained from all the studies were expressed as the mean ± standard error (SE). Statistical analyses were carried out by one-way analysis of variance (ANOVA) using Tukey post-tests for comparison. $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using GraphPad Prism version-5.0.

Results

Effects of CR and ROT supplementation on body weight and food intake

Administration of CR per se did not elicit any behavioral alterations during the experimental period. No significant changes in food intake were also evident. Further, mice administered with ROT showed no decrease in body weight during the treatment period.

Protective efficacy of Crocin treatment against ROT induced behavioral phenotype

CR improved the performance of mice in open field test

The protective effect of prophylactic treatment of CR on ROT induced locomotory phenotype was evidenced by assessing the motor activity in open field test (Fig. 1a-f). Mice administered ROT only showed a significant decrease in the number of lines crossed, number of centre square entries and duration of stay in the centre square. However, mice provided with CR prophylaxis and administered ROT exhibited a higher quality performance by increasing the number of lines crossed, the number of centre square entries and duration. Further ROT only treatment severely affected the behavior of animals by reducing their grooming and rearing activities, while CR prophyalxis improved the behavior pattern (50–55%). An evident augmentation in freezing behavior was noticed among ROT only treated mice, while CR prophylaxis significantly reduced the freezing behavior of ROT treated mice.

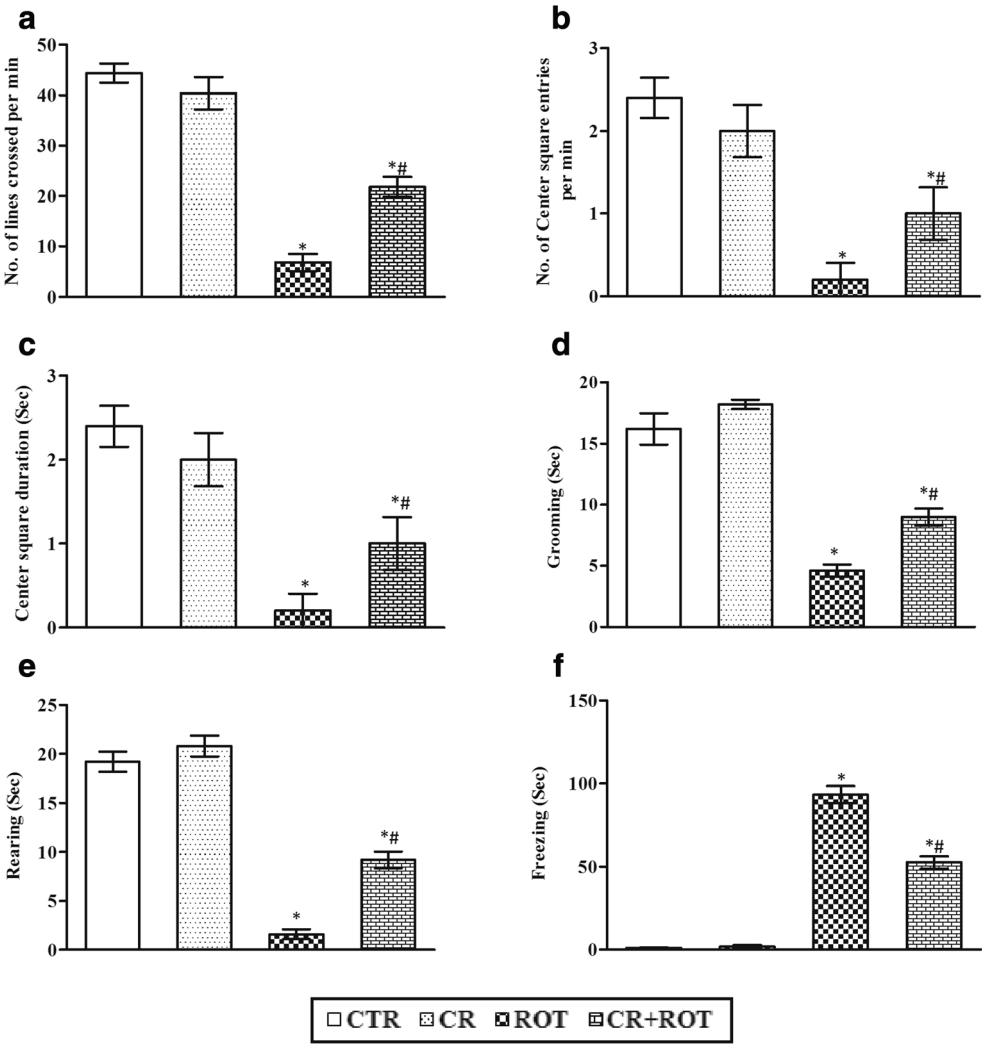

Fig. 1 Prophylactic efficacy of CR on ROT induced behaviour abnormalities of mice in Open field test (OFT) on day 7. Values are mean ± SE ($n = 6$); Data analyzed by one-way ANOVA followed by post hoc Tukey test ($p < 0.002$). * = significantly different from CTR; # = significantly different from ROT. CTR = Control; CR = Crocin (25 mg/kg b.w) (i.p) prophylaxis for 7consecutive days; ROT = Rotenone (0.5 mg/kg b.w) (i.p) for 7 consecutive days; CR + ROT = CR prophylaxis for 7d followed by ROT challenge for 7d

Elevated plus maze test

In this test, CR prophylaxis markedly alleviated the anxiety-Like phenotypic conduct induced by ROT. ROT administered mice demonstrated lesser activity as manifested by a significant increase in the duration of stay in close arm number than in the open arm. On the other hand, mice provided with CR prophylaxis exhibited nearly 40% increase in the basal activity levels (Fig. 2a, b).

Rotarod test

CR prophylaxis decreased ROT induced motor deficits as assessed by the rotarod test. Motor co-ordination which was affected by ROT administration was improved significantly in mice given CR prophylaxis. An evident improvement (45%) in motor behavior was noticed among mice given CR (Fig. 3a).

Catalepsy score

A typical catalepsy test consists of time taken by an animal to recover from an abnormal position to the normal position. We monitored animals for 120 s. While ROT administered mice needed more time to correct their posture, those given CR prophylaxis performed better than ROT mice by taking lesser time than ROT group (Fig. 3b). The ROT provoked increased catalepsy score was normalized (36%) by CR prophylaxis.

Protective effects of CR in striatum: Biochemical evidences

Attenuation of oxidative stress and activities of antioxidant enzyme

Rotenone administration resulted in a significant increase in the levels of ROS (35%), HP (42%), MDA (49%) and PC (32%) in the striatum (Table 1). In contrast, mice which have received CR prophylaxis exhibited a marked decrease in the

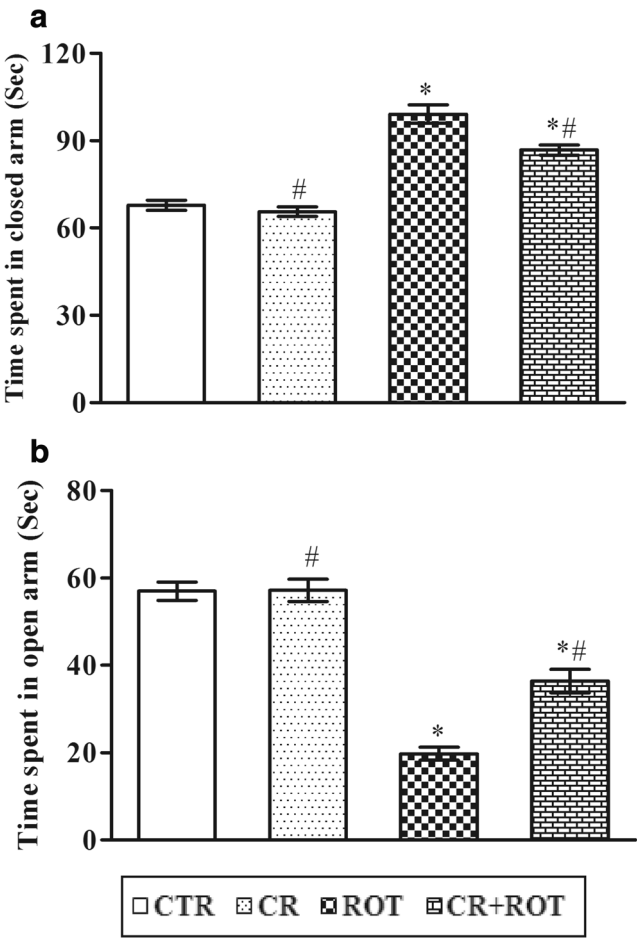

Fig. 2 Crocin treated mice exhibited reduced anxiety-like behaviour induced by ROT in elevated plus maze (EPM) assay (A, B). Values are mean ± SE (n = 6); Data analyzed by one-way ANOVA followed by post hoc Tukey test ($p < 0.002$). * = significantly different from CTR; # = significantly different from ROT. CTR = Control; CR = Crocin (25 mg/kg b.w) (i.p) prophylaxis for 7consecutive days; ROT = Rotenone (0.5 mg/kg b.w) (i.p) for 7 consecutive days; CR + ROT = CR prophylaxis for 7d followed by ROT challenge for 7d

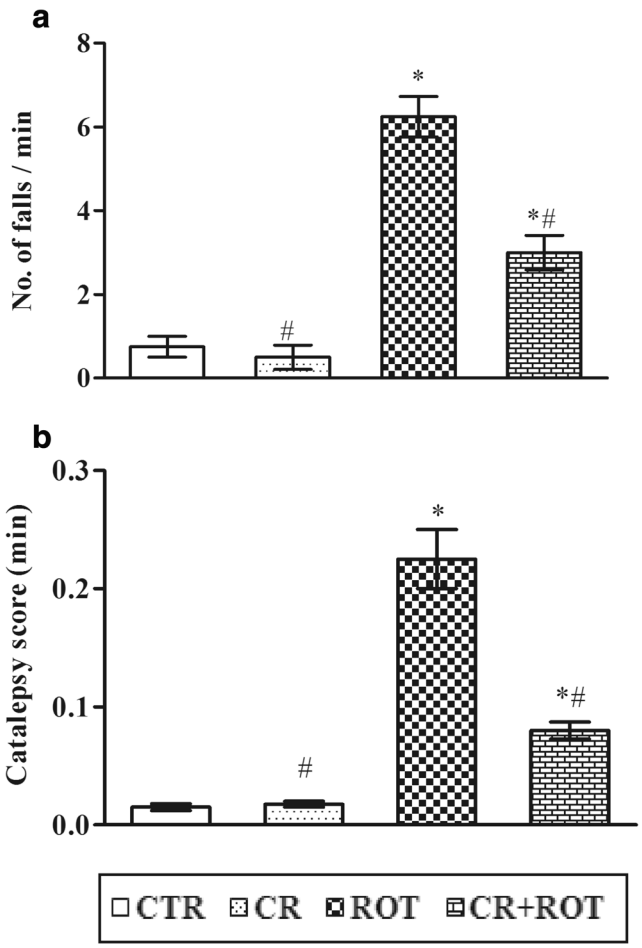

Fig. 3 Crocin improved the performance of mice in Rotarod test (A) and eased ROT induced enhanced catalepsy score (B). Values are mean ± SE (n = 6); Data analyzed by one-way ANOVA followed by post hoc Tukey test ($p < 0.002$). * = significantly different from CTR; # = significantly different from ROT. CTR = Control; CR = Crocin (25 mg/kg b.w) (i.p) prophylaxis for 7consecutive days; ROT = Rotenone (0.5 mg/kg b.w) (i.p) for 7 consecutive days; CR + ROT = CR prophylaxis for 7d followed by ROT challenge for 7d

levels of all these above biochemical endpoints. The reduced glutathione and total thiol levels were markedly reduced (39–49%) by ROT challenge were restored by CR prophylaxis (Table 1). While ROT caused a significant diminution in the activity levels of antioxidant enzymes such as CAT (48%), SOD (31%), TR (22%) and GST (22%), in the cytosol, CR prophylaxis resulted in an increase in the levels of antioxidant enzyme levels in comparison to the toxin group animals (Table 1).

Modulation of mitochondrial function

ROT administration caused significant perturbation in the activity levels of mitochondrial electron transport chain (ETC) enzymes. A significant diminution in the activity levels of Complex I-III (66%) and Complex II-III (38%) and a marked decrease in MTT reduction (34%) ensued with ROT administration. CR prophyalxis normalized the levels of mitochondrial enzyme function and ameliorated MTT reduction. While ROT administration enhanced (76%) the ADP/ATP ratio, CR prophylaxis reduced (54%) the same (Fig. 4 a-d).

CR prophylaxis modulates the levels of dopamine, α-synuclein and AChE activity

ROT administration significantly elevated the activity levels of AChE (33%), while CR prophylaxis restored the levels. Further, DA levels which were depleted (42%) as result of ROT administration, were significantly (23%) restored with CR prophylaxis. Furthermore, α-synuclein levels which were enhanced (30%) with ROT were significantly reduced (17%) by CR prophylaxis (Fig. 5).

Table 1 Efficacy of crocin prophylaxis on ROT- induced perturbations on oxidative stress markers and antioxidant defence in striatum

Parameter	CTR	CR	ROT	CR + ROT
ROS[a]	9.77 ± 0.52	6.21 ± 0.56$^{\#}$	14.95 ± 0.62*	11.23 ± 1.17$^{\#}$
HP[b]	10.23 ± 2.26	7.28 ± 0.93*$^{\#}$	17.29 ± 1.40*	12.66 ± 1.14$^{\#}$
MDA[c]	4.36 ± 0.11	3.71 ± 0.21$^{\#}$	8.46 ± 0.82*	6.26 ± 0.45*$^{\#}$
PC[d]	38.32 ± 2.39	33.19 ± 2.10$^{\#}$	56.75 ± 3.67*	44.67 ± 2.44*$^{\#}$
NO[e]	7.88 ± 0.34	6.51 ± 0.61$^{\#}$	8.94 ± 0.23*	7.68 ± 0.33$^{\#}$
GSH[f]	21.54 ± 1.64	24.27 ± 2.20$^{\#}$	11.09 ± 1.53*	12.59 ± 0.93$^{\#}$
TSH[g]	29.17 ± 1.77	33.15 ± 2.62*$^{\#}$	19.68 ± 1.65*	24.17 ± 2.39$^{\#}$
CAT[h]	4.46 ± 0.46	5.27 ± 0.61*$^{\#}$	2.32 ± 1.14*	3.67 ± 0.41*$^{\#}$
SOD[i]	134.32 ± 3.25	163.7 ± 4.26*$^{\#}$	92.72 ± 4.12*	116.3 ± 4.86*$^{\#}$
GST[j]	33.10 ± 1.34	36.05 ± 1.56	21.28 ± 2.66*	25.69 ± 2.39*$^{\#}$
TR[k]	14.77 ± 1.49	18.75 ± 1.23*$^{\#}$	11.49 ± 0.70*	13.92 ± 0.66$^{\#}$

Values are mean ± SE (n = 6); Data analyzed by one-way ANOVA followed by post hoc Tukey test ($p < 0.005$). * = significantly different from CTR; # = significantly different from ROT

CTR = Control; CR = Crocin (25 mg/kg b.w) (i.p) prophylaxis for 7consecutive days; ROT = Rotenone (0.5 mg/kg b.w) (i.p) for 7 consecutive days; CR + ROT = CR prophylaxis for 7d followed by ROT challenge for 7d

ROS- Reactive oxygen species; MDA- Melandialdehyde; HP- Hydroperoxides; PC-Protein carbonyls; NO- Nitric oxide; GSH- Reduced glutathione; TSH- Total thiols; CAT- Catalase; SOD- Superoxide dismutase; GST- Glutathione-S-transferase; TR- Thioredoxin reductase

[a] pmol DCF/min/mg protein

[b] nmol HP/min/mg protein

[c] nmol MDA/mg protein

[d] nmol protein carbonyls/mg protein

[e] nmol nitrites/mg protein

[f] μg GSH/mg protein;

[g] nmol DTNB oxidized/min/mg protein; h- nmol H_2O_2 hydrolyzed/min/mg protein;

[i] Units/mg protein; j- nmol adduct formed/min/mg protein;

[k] nmol NADPH oxidized/min/mg protein

Discussion

CR, a pharmacologically-active component of *Crocus sativus* L. (saffron), possess a broad spectrum of pharmacological properties, including anti-hyperglycemic, anti-oxidant, anti-tumor and anti-inflammatory activity in several rodent disease models. In the past decade, the neurobeneficial effects of CR have been intensively explored (Alavizadeh and Hosseinzadeh 2014; Hosseinzadeh and Sadeghnia 2007). Numerous studies (in-vivo, in-vitro and few clinical trials) have also documented the neuro-beneficial effects of CR which is mainly due to its interactions with cholinergic, dopaminergic and glutamatergic systems which results in anti-Alzheimer and anti-Parkinsonism (Khazdair et al. 2015). Recently, we demonstrated its efficacy to attenuate experimentally induced neurotoxicity in invertebrate models such as Drosophila (Rao et al. 2016b) and *C. elegans* (Rao et al. 2018). Owing to its potential to attenuate behavioural phenotype and neurotoxic outcome in these models, as a proof of concept the present study aimed to assess the prophylactic protective effect employing a well-known model of ROT neurotoxicity in mice.

Efficient mitochondrial function is necessary for the high-energy demand of the brain. Mitochondrial dysfunction is a primary cause of neurodegenerative diseases. Increased ROS production is a key culprit in neurodegeneration that disturbs the endogenous antioxidant system resulting in devastation of cellular components. The relevance of the mitochondrial enzyme complex I-III and II-III in ROS production (Wen et al. 2011; Vrbacký et al. 2007) prompted us to conduct study along this line. ROT a classic mitochondrial toxin is well known to replicate characteristics of PD when administered to model organisms in both acute and chronic treatment regimen (Betarbet et al. 2002; Cannon and Greenamyre 2014; Lapointe et al. 2004; Karlsson et al. 2016). Even though, rotenone is a well-known complex-I inhibitor, it does inhibit other enzyme complexes of the TCA cycle. Motor impairments have been a characteristic feature among experimental animals administered low multiple doses of ROT (Borland et al. 2008; Hattori 2004; Höglinger et al. 2005; Lapointe et al. 2004). The dose of ROT used in this study is based on our previous findings (Shinomol et al. 2012).

In the present study, ROT administered mice exhibited time dependent increase in motor deficits as assessed by battery of

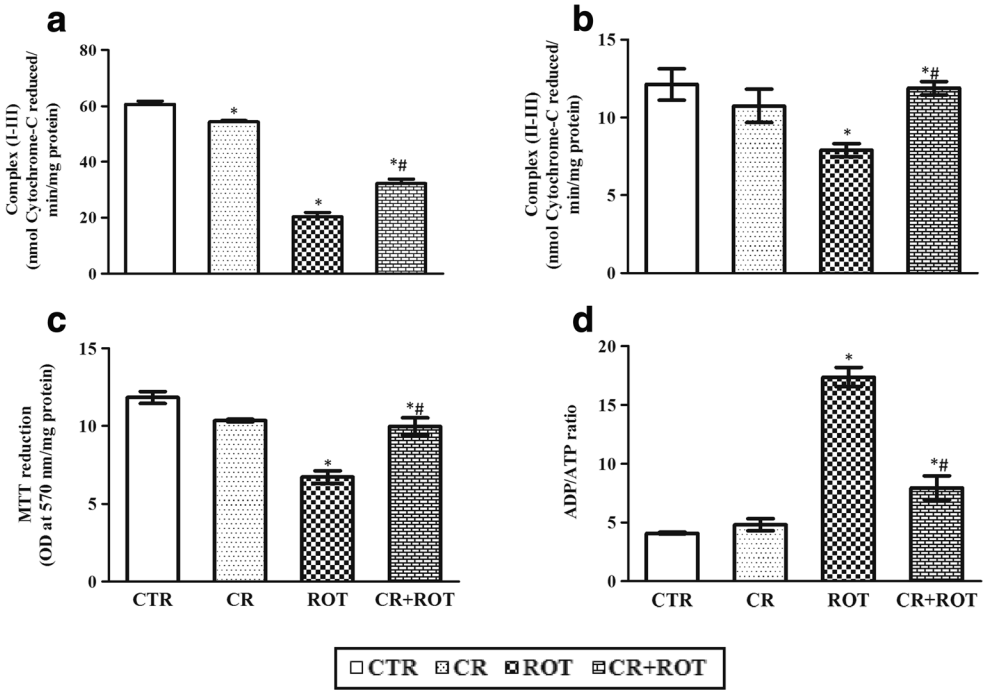

Fig. 4 Ameliorative effect of crocin treatment against rotenone caused mitochondrial enzyme alterations and disruption of ADP/ ATP ratio in striatum region of mice. Values are mean ± SE (n = 6). Data analyzed by one-way ANOVA followed by post hoc Tukey test ($p < 0.002$). * significantly different from CTR; # significantly different from ROT. CTR = Control; CR = Crocin (25 mg/kg b.w) (i.p) prophylaxis for 7consecutive days; ROT = Rotenone (0.5 mg/kg b.w) (i.p) for 7 consecutive days; CR + ROT = CR prophylaxis for 7d followed by ROT challenge for 7d. A- Complex I-III- NADH-cytochrome-C reductase; B- Complex II-III- Succinate-cytochrome-C reductase; C- MTT reduction-OD/mg protein; D- ADP/ATP- ADP/ATP ratio

behavior assessments. Depression and anxiety are usually associated with non-motor symptoms of PD (Poewe 2008). Although the major symptoms of PD are motor-related, DA loss is possibly involved in the depression and anxiety-like behavior in PD patients this is because of the adverse effects caused by neurotransmitters, adrenergic and serotonergic neurons due to PD pathogenesis (Prediger et al. 2012; Taylor et al. 2010; Santiago et al. 2010). In the present study, ROT administered mice exhibited signs of depression as evident from the open field test. ROT exposed mice often froze, crossed less number of lines in the block, spent less time on grooming and rearing activities than control animals, evidently exhibiting induction of depression. Further ROT induced anxiety-like behavior and concomitantly decreased exploratory nature of animals which was evident from the lesser duration of time spent by mice in the open arm of elevated plus maze. Furthermore, ROT significantly impaired neuromuscular coordination performance as evidenced by increased number of falls/min on the rotating rod than control mice consistent with earlier reports (Swarnkar et al. 2010). Previously, ROT induced a dose-dependent increase in catalepsy score and motor disruption (Alam and Schmidt 2002) in rats. Consistent with this, ROT administration to mice increased the time taken by the animal to revert to normal position from an abnormal posture, indicating the drop in the energy level of mice. However, mice given CR prophylaxis and then challenged with ROT, exhibited improved performance in open field test by decreased freezing, increased exploratory behavioural activities. Similarly, these mice performed better in elevated plus maze, rotarod, and catalepsy test, clearly suggesting the potential of CR to abrogate rotenone-induced behavioral deficits. Our results are in agreement with previous evidence on the protective efficacy (anxiolytic, hypnotic, improving motor function, counteracting hypermotility, stereotypes, and ataxia) of CR against behavior deficits in rodents (Amin et al. 2015; Georgiadou et al. 2014; Hosseinzadeh et al. 2009; Hosseinzadeh and Noraei 2009).

Increased oxidative stress leads to neurotoxicity and neuronal death. Given the importance of redox mechanisms in neurodegeneration, several studies have utilised the antioxidant capacity of several natural compounds to enhance the enzymatic (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidise, thioredoxine reductase) and non-enzymatic (reduced glutathione and thiols) defence system to protect the neuronal cells (Aruoma et al. 2003; Wąsik and Antkiewicz-Michaluk 2017). Several studies (Ferrante et al. 1997) have documented the damage produced by ROT in various brain regions and observed the selective vulnerability of striatum in rats. Oxidative impairments and microglia activation were more prominent in striatum and nigra than in other parts of the ROT- exposed rat brain (Sherer et al. 2003). Hence, we examined the potential of CR to attenuate

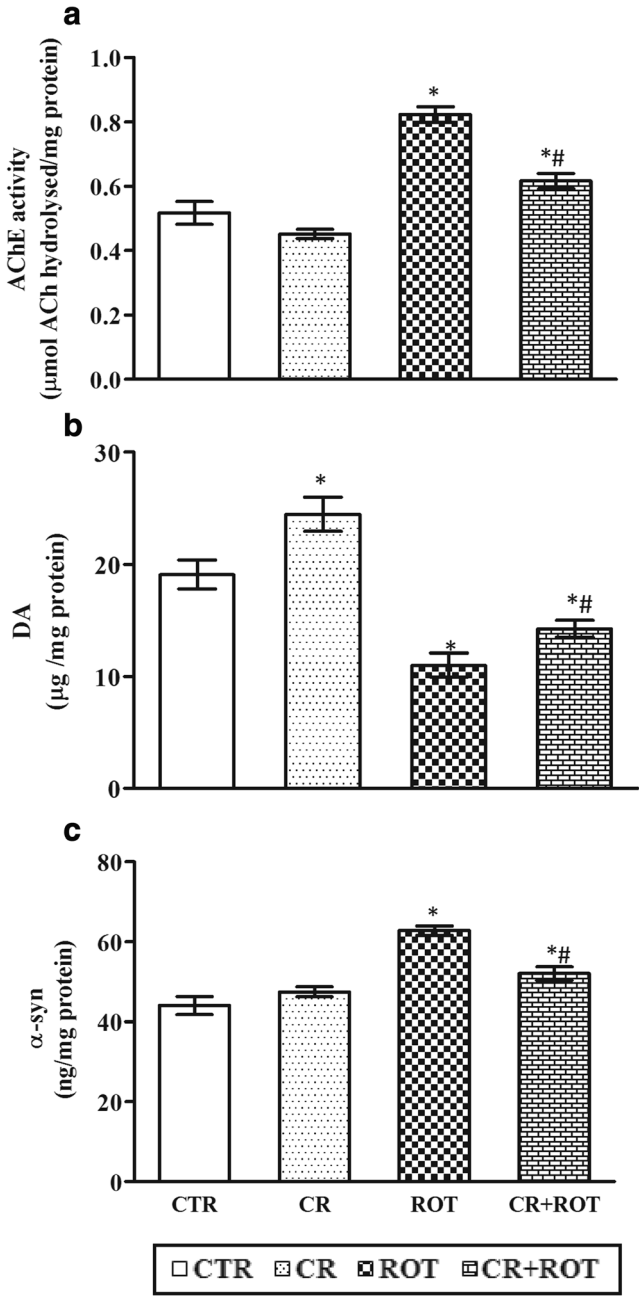

Fig. 5 Modulatory efficacy of Crocin supplementation on ROT induced alterations in the levels of acetylcholinesterases, Dopamine and α-synuclein levels in striatum of mice. Values are mean ± SE (n = 6); Data analyzed by one-way ANOVA followed by post hoc Tukey test ($p < 0.005$). * = significantly different from CTR; # = significantly different from ROT. CTR = Control; CR = Crocin (25 mg/kg b.w) (i.p) prophylaxis for 7consecutive days; ROT = Rotenone (0.5 mg/kg b.w) (i.p) for 7 consecutive days; CR + ROT = CR prophylaxis for 7d followed by ROT challenge for 7d

ROT-induced oxidative impairments in the striatum. Oxidative stress, mitochondrial dysfunction, and alterations in the antioxidant mechanism are the important issues in rotenone-mediated neurotoxicity (Nisticò et al. 2011). Biochemical analysis in the striatum of mice indicated that ROT caused significant oxidative damage as evidenced by enhanced levels of ROS, HP, and MDA. CR prophylaxis significantly decreased the elevated levels of these markers with a concomitant increase in glutathione and total thiols. Previous reports of CR have evidenced the antioxidant property in different experimental organisms (Chen et al. 2015; Chen et al. 2008; Hosseinzadeh et al. 2009; Rao et al. 2016b). CR treatment also proved effective in reducing the ROT-induced enhanced levels of nitric oxide and protein carbonyls. The present result corroborate with the previous findings (Jnaneshwari et al. 2013; Kim et al. 2014) who have demonstrated the inhibitory effect of CR on iNOS expression, NO production and the protective effect of CR to ameliorate toxin-induced oxidative stress and alterations in antioxidant enzyme system.

ROT exposure is well known to decrease the endogenous antioxidant enzyme function in both cell and animal models (Molina-Jiménez et al. 2005; Testa et al. 2005). In the present study, ROT-induced decreased activity levels of antioxidant enzymes were significantly improved due to CR prophylaxis indicating the potential of CR in enhancing the defensive mechanism.

Several Studies (Höglinger et al. 2005; Höglinger et al. 2003; Li et al. 2003) have demonstrated that ROT administration leads to inhibition of complex-I activity, in particular, increase in mitochondrial ROS production, and decrease in ATP synthesis. Consistent with this, ROT caused significant diminution in the activity levels of mitochondrial enzymes and MTT reduction potential. Interestingly, CR prophylaxis restored the mitochondrial enzyme function and MTT reduction property. The increased ratio of ADP/ATP is indicative of decreased ATP levels. Further, ROT induced increase in the ADP/ATP ratio was also normalized indicating the potential of CR to ameliorate ROT-induced mitochondrial dysfunction. While the precise mechanism/s by which CR prophylaxis renders protection against ROT induced mitochondrial dysfunction aren't speculated in this study, further studies are necessary to delineate the specific pathways, which are involved.

A body of evidence has suggested a significant role of cholinergic neurons in the pathophysiology of neurodegenerative diseases (Zhang et al. 2013). Consistent with this finding, we evidenced that CR prophylaxis CR prophylaxis could normalize ROT-induced elevated activity levels of AChE, suggesting its efficacy to attenuate cholinergic function. This finding also corroborates the previous investigations on the potential AChE inhibitory action of saffron extract and its constituents in molecular docking studies (Geromichalos et al. 2012) as well as their protective effect in animal models of AD and PD (Akhondzadeh et al. 2010; Rao et al. 2016b).

ROT is well recognized to interact with the mitochondrial electron transport chain, and it is known to affect the survivability of dopaminergic neurons and deplete the DA content in experimental models. This may be responsible for the

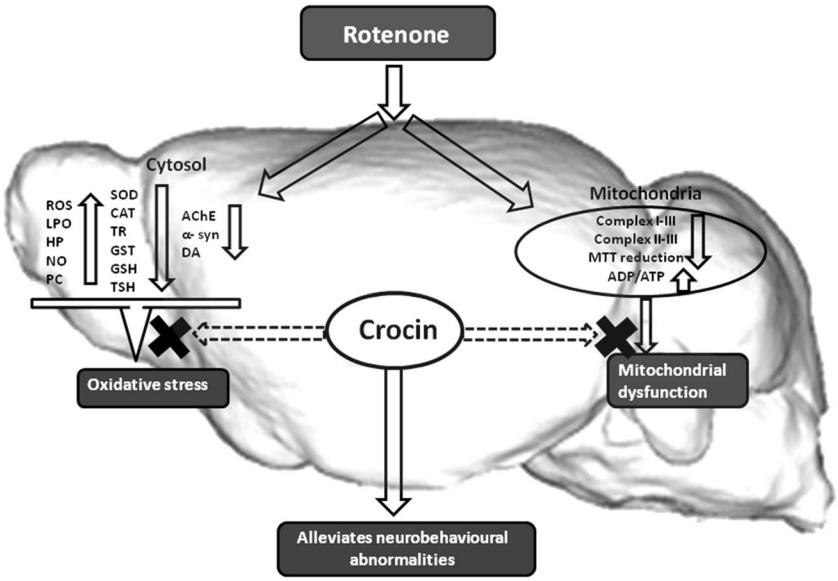

Fig. 6 A Schematic representation of possible mechanisms underlying the prophylactic effects of Crocin to alleviate rotenone-induced behavioural and biochemical alterations in striatum of brain. ROS- Reactive oxygen species; LPO = Lipid peroxidase; HP-Hydroperoxides; NO- Nitric oxide; PC-Protein carbonyls; SOD-Superoxide dismutase; CAT- Catalase; TR- Thioredoxin reductase; GST- Glutathione-S-transferase; GSH- Reduced glutathione; TSH-Total thiols; AChE- Acetyl cholinesterase; α-syn- α-synuclein; DA- dopamine; complex I-III- NADH-cytochrome C reductase; complex II-III-succinate-cytochrome C reductase; ADP/ATP- adenine diphosphate to adenine triphosphate ratio

locomotory phenotypes observed in this study. Interestingly, CR prophylaxis normalized the DA content of ROT administered mice. This is in agreement with our previous report of neuroprotective efficacy of CR in ROT model of toxicity (Rao et al. 2016b). The formation and accumulation of a misfolded protein i.e., α-synuclein is the characteristic feature of PD (Recasens et al. 2014). CR pretreatment evidently, decreased the ROT-induced elevation in the levels of α-synuclein in striatum suggesting the therapeutic potential of CR against oxidative stress-mediated neuronal dysfunction. Collectively these findings suggest that CR prophylaxis renders significant multiple levels of protection against the ROT–induced neurotoxic effects.

Conclusion

Prophylactic treatment of mice with Crocin significantly improved the performance of animals in behavior tests as it attenuated rotenone-induced anxiety-like behavior and depression, improved the performance of animals in the rotarod test. Crocin prophylaxis markedly offset rotenone-induced oxidative stress, antioxidant status and mitochondrial enzyme dysfunctions in the striatum of the brain (Fig. 6). Further, Crocin restored cholinergic fucntion, levels of straital dopamine and α-synuclein. Based on these findings we propose its use as a promising therapeutic agent and can be utilized as supplement, adjuvant or as a prophylactic agent since it is safe with zero side effects and combats oxidative stress- mediated neurodegenerative conditions such as PD among humans.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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