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RESEARCH ARTICLE

Protective effect of curcuminoids on age-related mitochondrial impairment in female Wistar rat brain

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Abstract The present study demonstrated the neuroprotective effect of curcuminoids, the active polyphenols of *Curcuma longa* (L.) rhizomes on mitochondrial dysfunctioning in middle aged and aged female Wistar rat brain. Rats were orally treated with curcuminoids (100 mg/kg) for 3 months and their brain was collected for evaluation of mitochondrial enzymes and complexes activity, ultra structural changes in mitochondria, neuronal nitric oxide synthase (*nNOS*) protein expression, adenosine triphosphate (ATP) and lipofuscin content. Significant alterations were observed in all the tested parameters in highly aged rat brain when compared with young control. Long term curcuminoids administration prevented this age associated loss of mitochondrial enzymes and complexes activity in middle aged rat brain except for malate dehydrogenase, Complex II and IV activity when compared with young control. Among aged rats, curcuminoids treatment specifically elevated isocitrate and NADH dehydrogenase, cytochrome c oxidase, Complex I and total ATP content. A significant down-regulation of *nNOS* protein expression along with reduced lipofuscin content was also observed in curucminoids treated middle aged and aged rats. Thus, it was suggested that curcuminoids may act as a putative drug candidate for the prevention of deleterious effects of ageing and age associated neurodegenerative disorders through amelioration of aberrant mitochondrial functioning.

Keywords Ageing · Mitochondria · Curcuminoids · NO

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Introduction

Ageing is defined as the progressive decline in cellular, metabolic and defence functions with increased likelihood to develop age associated neurodegenerative disorders. In ageing brain declined mitochondrial respiratory chain complexes and enzymes activity, increased rate of somatic mitochondrial DNA mutations and accumulation of higher percentage of defective mitochondria were reported (Ojaimi et al. 1999; Yan et al. 1997; Michikawa et al. 1999). The

progressive loss of mitochondrial functioning with ageing further rendered neurons vulnerable to develop age associated neurodegenerative pathologies. Keeping the above facts in view, it was hypothesized that therapeutic interventions which improve mitochondrial function may promote the healthy brain ageing and prevent age related neurodegenerative disorders. This was further supported by the studies where administration of mitochondrial nutrients such as acetyl-L-carnitine (ALCAR) and lipoic acid (LA); polyphenolic compounds from herbs and spices like curcumin significantly improved the behavioral decrements and reduced the oxidative damage in the brain of aged rodents (Crouch et al. 2007; Haripriya et al. 2004; Belviranl et al. 2013; Sharma et al. 2009). Curcumin is active biological compound of *Curcuma longa* (L.) rhizomes which in turn is reputed medicinal plant of Indian System of Medicine. Despite of immense pharmacological potential of curcumin, its efficacy has been masked due to poor bioavailability. In recent study (Ahmed and Gilani 2009, 2013) it was reported that curcuminoids mixture exerted better neuroprotective effect than curcumin and concluded that the other two components viz. bis-demethoxycurcumin and demethoxycurcumin significantly contributed to the pharmacological profile of curcuminoids mixture. Moreover, few metabolites of curcuminoids have also been reported to be active, which may further explain the in vivo efficacy of curcuminoids (Wang and Qiu 2013). In our previous studies we have reported that curcuminoids treatment significantly ameliorated mitochondrial complexes activity in diabetic rat brain (Rastogi et al. 2008). The present study aims to investigate the neuroprotective effect of curcuminoids on age related mitochondrial dysfunctioning. To our knowledge this is the first report providing evidence for the activity of curcuminoids over mitochondrial dysfunction in aged brain and its influence in promotion of healthy brain ageing.

Material and methods

All chemicals used were of analytical grade and purchased from Sigma-Aldrich (USA) and Merck (Germany). Antibody for Immunoblotting of neuronal nitric oxide synthase (*nNOS*) was purchased from Santa Cruz Biotechnology Inc.

Extraction and quantification of curcuminoids

The standard extract of *Curcuma longa* (L.) rhizome was dipped overnight in the mixture of hexane and 95 % ethanol (2:8) at room temperature. The marc was discarded and the remaining fraction was concentrated and then precipitated by adding petroleum ether. The % yield of total polyphenols was found to be approximately 94–95 % and was quantified by HPLC with PDA detector (mobile phase, acetonitrile and water in the ratio of 85:15 (v/v); flow rate, 1 mL/min; detection wave length, 425 nm) for major components. The extracted polyphenols majorly comprised of 78.1 % curcumin, 16.5 % demethoxycurcumin and 5.4 % bis-demethoxycurcumin.

Experimental design

All the experiments were carried out using female Albino Wistar rats according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (Approval No.19/SASTRA/IEAC/RPP). Rats were caged under controlled temperature, 19 ± 3 °C; relative humidity, 30–70 %; 12 h light/dark cycle and were fed with standard laboratory diet and RO water ad libitum. 100 mg/kg of curcuminoids dissolved in 5 % Tween 80 was orally administered daily for 3 months in the following experimental design ($n = 6$ each group).

Group I: young (2–3 months) (6–8 % life span)
Group II: aged (24–25 months) (80–84 % life span)
Group III: middle aged + curcuminoids (17–18 months) (50–55 % life span)
Group IV: aged + curcuminoids (83–87 % life span)

After completion of the treatment schedule, the rats were euthanized by ketamine. Their brain was quickly collected and cerebellum and brain stem was removed. The remaining cortex was maintained at −80 °C and used for further investigations.

Estimation of lipofuscin content

Lipofuscin content was detected spectrofluorimetrically by the method as described by Wilhelm and Herget (1999) at the excitation/emission

wavelength of 350/445 nm. Standard curve was obtained by running quinine sulphate in the range of 2–100 μg/mL.

Mitochondrial enzymes

Brain mitochondria were isolated by the method of Takasawa et al. (1993). Brain cortex was homogenized (10 % w/v) in ice cold buffer A (0.3 M sucrose, 5 mM Tris and 2 mM EGTA with pH 7.4) and the homogenate were centrifuged at 2,000×*g* for 5 min. The supernatant obtained were again centrifuged twice at 12,000×*g* for 10 min each to obtain the pellet which was suspended in 0.1 mL of buffer A and quantified for its protein content by Lowry method. Finally, mitochondria was suspended in buffer B (0.1 mM KCl, 3 mM HEPES, 1 mM EGTA, 5 mM KH_2PO4, 1 % BSA with pH 7.2) to make a final conc. of 1 mg/mL and was used for quantification of mitochondrial enzymes activity viz., Isocitrate dehydrogenase activity, α-ketoglutarate dehydrogenase, malate dehydrogenase, succinate dehydrogenase, NADH dehydrogenase and cytochrome c oxidase according to the methods described in Sudheesh et al. (2009). All mitochondrial enzyme activities were expressed as IU/mg protein.

Mitochondrial complexes

Isolation of mitochondria

Brain mitochondria were isolated by the method as described in our previous report (Rastogi et al. 2008). Briefly, brain cortex was homogenized as 10 % (w/v) in buffer A (100 mM KCl, 0.5 M Tris HCl, 5 mM $MgCl_2$, 1 mM adenosine triphosphate (ATP)-Mg, 1 mM EGTA and 0.08 v/v protease inhibitor cocktail) and centrifuged at 800 g for 10 min. The supernatant obtained were centrifuged twice at 800 g for 10 min and 10,000 g for 15 min respectively to obtain the crude mitochondrial pellet. The pellet was washed thrice with 0.25 M sucrose by centrifugation at 8,000, 5,000 and 3,500×*g* respectively and the final pellet was suspended in 1 mL of buffer B (0.25 M sucrose, 0.1 mM EGTA). The total protein content of the isolated mitochondria was determined by Lowry method. Further, the isolated mitochondrial pellet was carefully topped on Percoll solution (30 % in buffer B) and was centrifuged at 100,000×*g* for 15 min. Intact mitochondria will separate out in the density layer 1.09–1.13 g/mL while lysosomes and broken mitochondria in the density layer of 1.05 g/mL just above the mitochondrial layer. The lower band was separated carefully and was re suspended in sucrose followed by centrifugation at 10,000×*g* for 10 min. The pellet obtained was kept in aliquots under ice for the determination of protein content and mitochondrial complexes activity.

Complex I: NADH:ubiquinone:oxidoreductase

The complex I activity was measured by the method as described by Shults et al. (1995). The reaction mixture containing 0.1 M phosphate buffer (pH 7.4), 15 mM NADH, 2.5 mM coenzyme Q1, 0.5 M KCN, 1 M sodium azide was kept for incubation for 10 min at room temperature. The reaction was initiated by the addition of mitochondrial protein and the decrease in absorbance was recorded at every 30 s for 5 min to measure the total activity. The above experiment was repeated in the presence of 0.5 mM rotenone in-order to calculate rotenone insensitive activity. The rotenone-sensitive complex I activity was calculated by subtracting the activity measured in the presence of rotenone from the total activity. The values were expressed as IU/mg protein calculated by using molar extinction coefficient (ε) of NADH as 6.81 mM^{-1} cm^{-1} L^{-1}.

Complex II: Succinate:ubiquinone oxidoreductase

Complex II activity was determined by the method of Birch-Machin et al. (1994). To the assay buffer (25 mM phosphate buffer, pH 7.2 and 5 mM $MgCl_2$), 1 M sodium succinate and mitochondrial protein (10–50 μg) were added and kept for incubation for 10 min at room temperature. To this antimycin A (1 mg/mL), rotenone (1 mg/mL), KCN (1 M) and DCIP (5 mM) were added and absorbance was recorded at an interval of 30 s for 3 min at 600 nm. Further, 2.5 mM of ubiquinone was added to the reaction mixture and absorbance was recorded as earlier. The values were expressed as IU/mg protein calculated by using molar extinction coefficient (ε) of DCIP as 19.1 mM^{-1} cm^{-1} L^{-1}.

Complex II–III: Succinate cytochrome c reductase

The method was adopted as indicated by Chuang et al. (2002). To the assay medium (25 mM phosphate buffer pH 7.2, 2 mM KCN, 2 μg/mL antimycin A), rotenone (1 mg/mL) and mitochondrial protein (10–50 μg) were added and kept for incubation for 10 min at 30 °C. To this, 20 μL of cytochrome c was added and increase in absorbance was recorded at 550 nm at an interval of 30 s for 3–4 min. The values were expressed as IU/mg protein calculated by using molar extinction coefficient (ε) of cytochrome c as 19.5 $mM^{-1}cm^{-1}L^{-1}$.

Complex IV: Cytochrome c oxidase

The assay of cytochrome c oxidase was performed as described by Gibson and Hilf (1983). The reaction mixture contains 20 mM phosphate buffer (pH 7), 3 mM cytochrome c, 30 mM dodecyl maltoside, 3 M potassium hexacyanoferrate and 10–15 μg of mitochondrial protein. The absorbance was taken at 550 nm at an interval of 30 s for a time period of 3 min. The values were expressed as IU/mg protein calculated by using molar extinction coefficient (ε) of cytochrome c as 19.5 $mM^{-1}cm^{-1}L^{-1}$.

Immunoblotting of neuronal nitric oxide synthase (*nNOS*)

The mitochondrial pellets as obtained by density gradient centrifugation correspond to an enriched non-synaptic mitochondrial fraction with reportedly higher *nNOS* protein expression (Czerniczyniec et al. 2006). 100 μg of total mitochondrial protein was separated on SDS PAGE with 5 % loading gel (pH 6.8) and 10 % running gel (pH 8.4) in the presence of 0.1 % (v/v) SDS. Molecular weight marker (R & D Systems, Minneapolis, USA) was employed for the confirming of protein transfer and the molecular weight orientation. SDS-PAGE was blotted into a nitrocellulose (Amersham Hybond ECL) membrane and probed primarily with *nNOS* rabbit polyclonal antibodies (dilution 1:500) from Santa Cruz Biotechnology Inc., CA. The nitrocellulose membranes were then incubated with a secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (dilution 1:5,000), followed by development of chemiluminescence with the ECL reagent for 2–4 min. Analysis and quantification of blots was done by Quantity One image analysis software (Bio-Rad).

ATP quantification by HPLC

The quantification of adenosine triphosphate (ATP) content was performed by the method of Tekkanat and Fox (1988) with little modifications as described elsewhere.

Detection of ultra structural changes in mitochondria by transmission electron microscopy (TEM)

Briefly, the isolated mitochondrial fractions were fixed in 3 % glutaraldehyde fixative in phosphate buffer (pH 7.2) for 24 h and subjected to routine transmission electron microscopy (TEM). The pellets were post fixed in 1 % osmium tetroxide, followed by dehydration in grades of ethyl alcohol and cleared in propylene oxide. Further, the samples were embedded in Araldite CY212 resin and polymerized at 60 °C for 48 h. The blocks were cut on Leica EM UC6 ultramicrotome (M/S Leica Mikrosysteme, Austria). 1 μm thick sections were stained with 1 % toluidine blue and after initial screening several 600–700 Å ultra thin sections were collected on copper grids and stained by double staining method as described by Frasca and Parks (1960) using uranyl acetate (4 %) and lead citrate. Later the sections were scanned under Tecnai G2 Spirit Bio-twin (FEI Netherlands) at 80 KVA and related images were captured using Megaview-III digital CCD camera.

Statistical analysis

All results were presented as mean ± SD. The inter-group variation was measured by one way analysis of variance (ANOVA) followed by LSD *Posthoc* analysis through SPSS software. The level of significance was considered at $P < 0.05$. The determination of correlation coefficient and linear regression plots were performed by Graph Prism Pad software.

Results

Anti ageing effect of curcuminoids in middle aged and aged rat brain cortex

A significant accumulation of ageing biomarker lipofuscin was observed in aged rat brain cortex

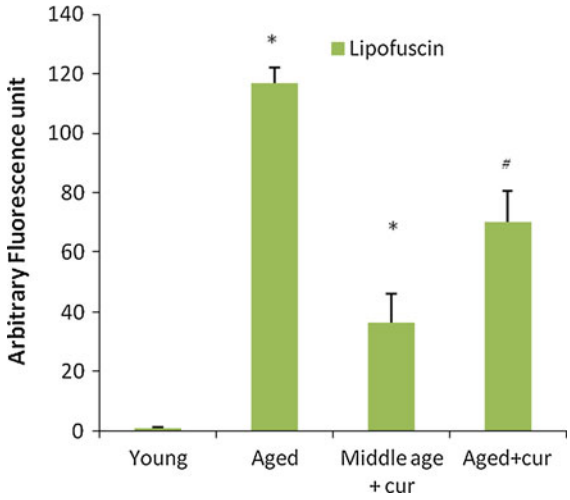

Fig. 1 Concentration of lipofuscin content in the brain of young, middle aged and aged treated or untreated rats. Values were expressed as mean ± SD of six animals in each group. Level of significance as calculated by one way ANOVA followed by *Posthoc* LSD shows $^{*}P < 0.05$ for young versus aged or middle aged treated rats and $^{\#}P < 0.05$ for aged versus aged treated group

(Fig. 1). Long term treatment with curcuminoids (100 mg/kg) significantly prevented the aggregation of lipofuscin in the middle aged and aged rat brain cortex.

Effect of curcuminoids on mitochondrial enzymes

Age dependant depletion in the activity of mitochondrial enzymes was observed in the rat brain cortex (Table 1). The activity of dehydrogenases enzymes viz., isocitrate, α-ketoglutarate, succinate, malate and NADH dehydrogenase were found to be reduced to 59.38, 68.24, 10.53, 48.8 and 6.43 % respectively in aged rat brain cortex in comparison to young group (100 %). In addition, the enzyme cytochrome c oxidase was also decreased to 11.54 % in aged rat brain cortex. Treatment with curcuminoids significantly prevented the age associated depletion of enzyme activity, except for malate dehydrogenase activity in the middle aged rat brain, when compared with the young control. In the curcuminoids treated aged rats, the activity of enzymes isocitrate dehydrogenase, NADH dehydrogenase and cytochrome c oxidase were found to be elevated to 77.21, 34.29 and 53.85 % respectively, however the other enzymes activity showed no significant changes.

Amelioration of mitochondrial complexes activity on curcuminoids treatment in middle aged and aged rat brain

Impairment in the mitochondrial complexes activity was observed in brain of ageing rats (Figs. 2, 3, 4, 5). The activity of Complex I, II, II–III and IV were down regulated to 9.85, 43.48, 21.74 and 35.29 % respectively in aged rats in comparison to young control (100 %). Oral administration of curcuminoids prevented the age associated decline in mitochondrial complexes, except for Complex IV activity in middle aged rats, when compared with young control. Curcuminoids treatment specifically elevated the Complex I activity to 57.41 % in aged rat brain with no significant effect over remaining three Complexes activity.

Analysis of ATP content in aged brain mitochondria: Effect of curcuminoids

Age-related alterations in the ATP level was observed in the rat brain cortex as represented in Fig. 6. The total ATP content was found to be reduced to 53.77 % in aged group of rats as compared with the young group (100 %). Treatment with curcuminoids significantly prevented this age-associated alteration in the middle aged rats and restored the ATP content to 86.97 % in aged brain cortex.

Effect of curcuminoids over *n*NOS protein expression

In-order to understand the age-associated NO production in ageing rat brain cortex, the study has been extended to analyze the expression of *nNOS* activity in mitochondrial fractions. The expression of *nNOS* was significantly up-regulated in the aged brain cortex which was significantly down regulated on curcuminoids treatment in middle aged as well as aged rat brain mitochondria (Fig. 7a, b).

Analysis of ultrastructural changes in mitochondria by transmission electron microscopy

In the present study, the age-associated alterations and the effect of curcuminoids treatment on mitochondrial morphology were examined qualitatively by

Table 1 Effect of curcuminoids treatment on mitochondrial enzymes activity in rat brain

Groups	Isocitrate dehydrogenase (IU/mL)	α-Ketoglutaarate dehydrogenase (IU/mL)	Succinate dehydrogenase (IU/mL)	Malate dehydrogenase (IU/mL)	NADH dehydrogenase (IU/mL)	Cytochrome c oxidase (IU/mL)
Young	12.68 ± 0.64	3.4 ± 0.28	0.19 ± 0.06	2.91 ± 0.71	1.4 ± 0.18	0.26 ± 0.09
Aged	7.53 ± 0.84*	2.32 ± 0.19*	0.02 ± 0.007*	1.42 ± 0.34*	0.09 ± 0.04*	0.03 ± 0.01*
Middle aged + Curcuminoids	12.92 ± 1.26	3.19 ± 1.02	0.23 ± 0.07	1.77 ± 0.55**	1.17 ± 0.23	0.25 ± 0.07
Aged + Curcuminoids	9.79 ± 1.49$^{\#}$	2.23 ± 0.18	0.08 ± 0.01	1.52 ± 0.25	0.48 ± 0.13$^{\#}$	0.14 ± 0.03$^{\#}$

Curcuminoids at the doses of 100 mg/kg were orally administered for 3 months. Values were expressed as mean ± SD of six animals

* $P < 0.05$ for young versus aged or middle aged treated rats; ** $P < 0.05$ for middle aged treated versus young rats; $^{\#}$ $P < 0.05$ for aged versus aged treated rats

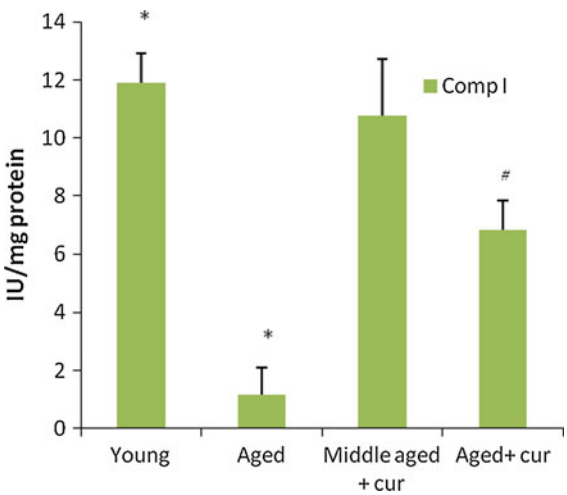

Fig. 2 Values were expressed as mean ± SD for six rats in each group. Specific activity of mitochondrial Complex I (NADH-coenzyme Q oxidoreductase) was determined spectrophotometrically as elaborated in materials & methods. *$P < 0.05$ for young versus aged and $^{\#}P < 0.05$ for aged versus aged treated group

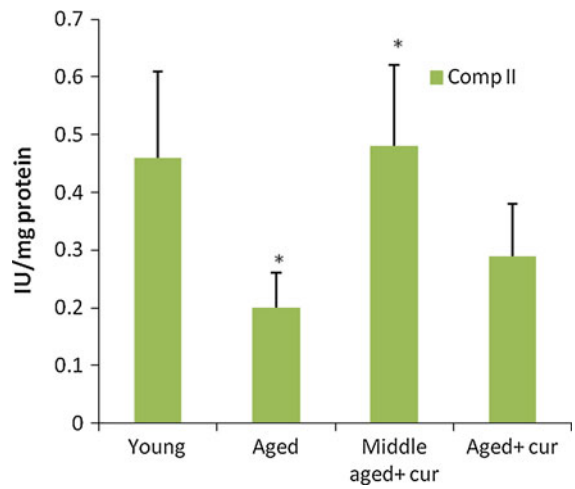

Fig. 3 Values were presented as mean ± SD for $n = 6$. Succinate-cytochrome c reductase activity was expressed as IU/mg protein. *$P < 0.05$ for young group versus aged or middle aged treated group

transmission electron microscopy (Fig. 8). Diffused structures were obtained due to the use of mitochondrial pellets isolated from rat brain cortex, nevertheless, an age-associated and the drug induced alterations can be identified in the same. A range of mitochondrial abnormalities- mitochondria with partially and/or completely damaged cristae, the presence of membrane disruptions and the mitochondria derived lysosomal structures due to loss of identifiable cristae were observed in aged brain cortex. A significant decrease in the prevalence of damaged mitochondria were observed in curcuminoids treated middle aged rat mitochondria. Most of the mitochondria showed presence of reduced cristae and broken outer membrane indicating amelioration of age associated disruption of mitochondrial integrity on curcuminoids treatment.

Correlation studies between ageing biomarker and mitochondrial parameters in curcuminoids treated middle aged and aged treated group

In curcuminoids treated aged rats, the ageing marker lipofuscin was significantly correlated with cytochrome c oxidase ($r = -0.899$, $P = 0.015$) suggesting the prevention and/or delay of the ageing progression in part due its potential activity on mitochondrial functioning (Fig. 9).

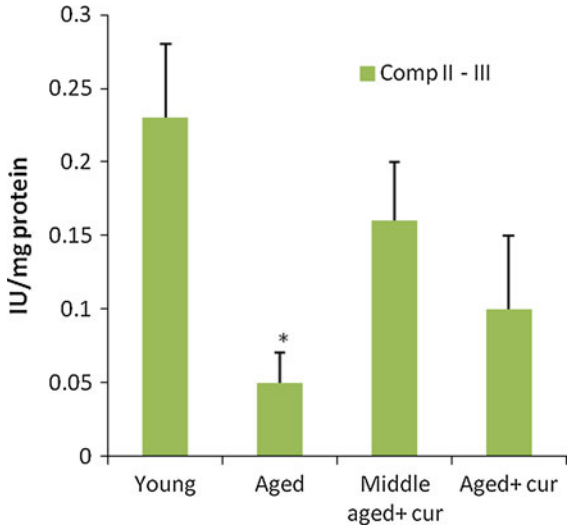

Fig. 4 Values were mean ± SD and the significance of differences was analyzed by one way ANOVA with *PostHoc* test. $*P < 0.05$ for young versus aged group. No significant changes were observed in any of the treated groups

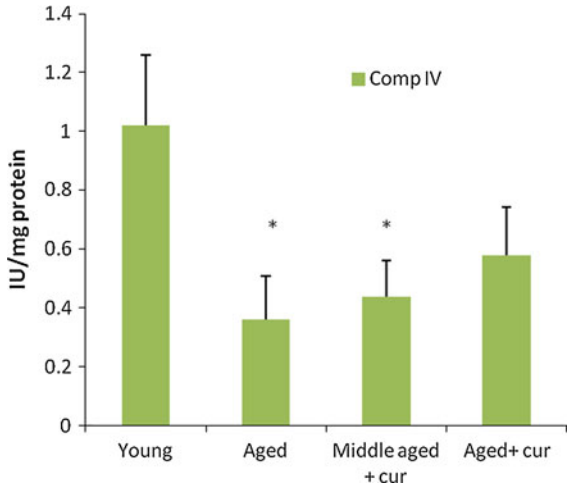

Fig. 5 Values were mean ± SD and the significance of differences was analyzed by one way ANOVA with *PostHoc* test. $*P < 0.05$ for young versus aged or middle aged group

Discussion

The present study demonstrated the overall effect of curcuminoids against age associated mitochondrial dysfunction at the enzymatic and structural levels. In the present investigation, all the mitochondrial enzymes showed age related decline in their activities which were in accordance with the previous reports

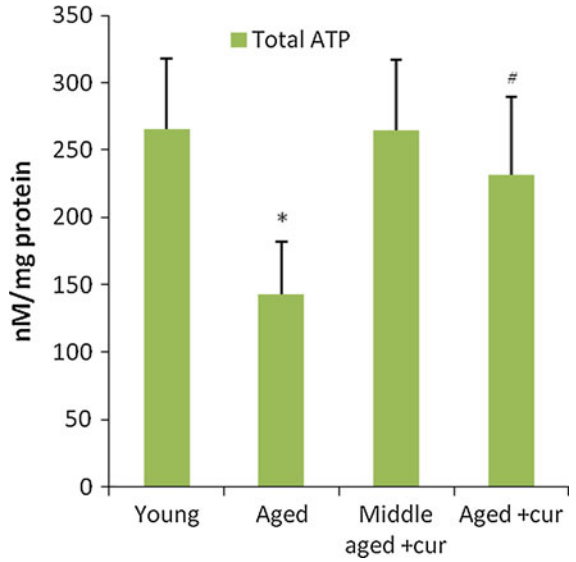

Fig. 6 Effect of curcuminoids on the ATP levels as analyzed in brain mitochondria of young, middle aged or older rats by HPLC–PDA detector. Each value represents the mean ± SD of 6 animals. $*P < 0.05$ young versus aged and $^{\#}P < 0.05$ for aged versus aged treated group

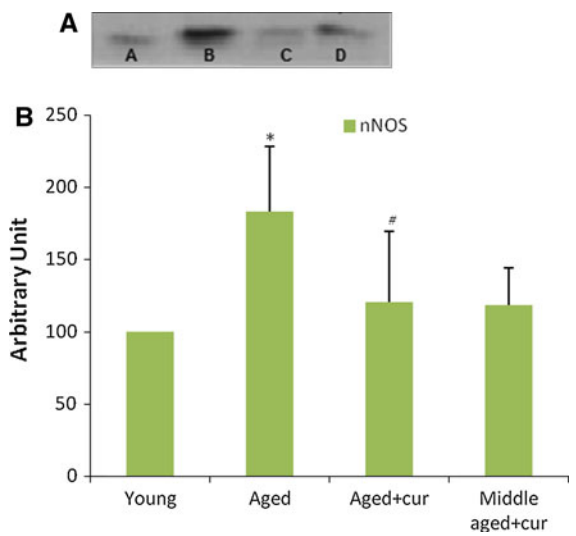

Fig. 7 **a** Represents the *nNOS* protein expression in young, middle aged and aged treated or untreated group. Representative blot was obtained after separating the brain cortex mitochondrial extracts by SDS-PAGE and transblotting onto nitrocellulose membranes, which were then probed with anti-*nNOS* antibody, as described in "Materials and Methods". (*A*) Young, (*B*) aged, (*C*) aged + curcuminoids, (*D*) middle aged + curcuminoids. **b** Shows mean ± SD and values were found to be statistically significant for $*P < 0.05$ young versus aged and $^{\#}P < 0.05$ for aged versus aged treated group

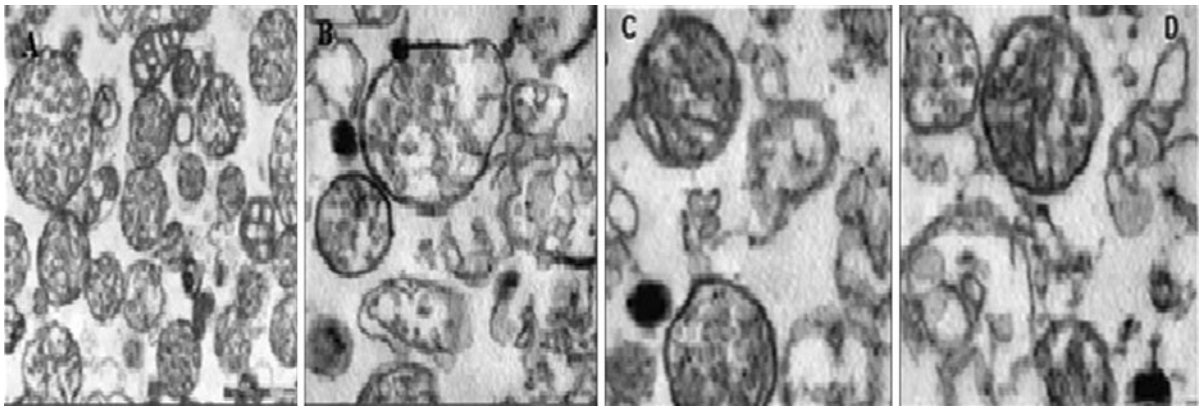

Fig. 8 Ultrastructural characteristics of mitochondria in **a** young, **b** aged, **c** middle aged + curcuminoids, **d** aged + curcuminoids. Young rats showed mitochondria with intact outer membrane and cristae, whereas mitochondria isolated from aged brain cortex indicate the presence of a range of mitochondria lesions (explained in "Results"). Mitochondria in middle aged and aged rats treated with curcuminoids demonstrated the much intact morphology. Curcuminoids treatment significantly reduced the number of damaged mitochondria when compared with aged untreated group. (Magnification ×49,000)

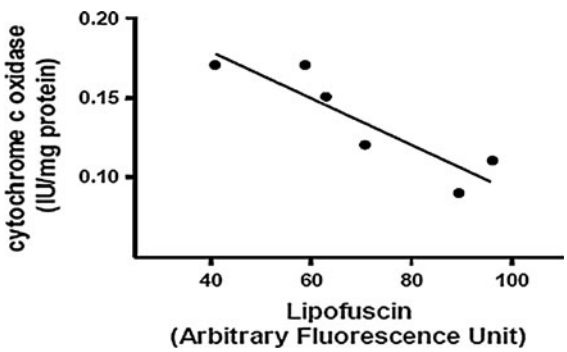

Fig. 9 Demonstrates that the elevation in the mitochondrial enzyme cytochrome c oxidase on curcuminoids treatment was involved behind the anti-ageing effect of the drug with $r = -0.899$, $p = 0.015$

(Haripriya et al. 2004). Deficient activity of three mitochondrial enzymes viz., α-keto glutarate dehydrogenase (KGDH), pyruvate dehydrogenase and cytochrome c oxidase had been specifically reported in ageing and associated neurodegenerative disorders (Gibson et al. 1998; Remus and Firman 1995). The inhibition of these mitochondrial enzymes lead to decreased rate of transfer of reducing equivalents to molecular oxygen (Garcia-Ruiz et al. 1995) resulting into excessive generation of ROS which further deteriorate oxidative phosphorylation chain and depleted energy content (Haycock et al. 1996). This in turn will impose a negative impact over neurobehavioral and neurochemical functioning with ageing (Hinerfeld et al. 2004; Shi et al. 2011).

In the present study curcuminoids treatment significantly prevented the loss of almost all the NAD^+ dependant dehydrogenases enzymes activity in the middle aged rat brain. However, in aged rat brain cortex curcuminoids specifically elevated the NADH dehydrogenase and cytochrome c oxidase activity. A recent study has reported the restoration of mitochondrial enzymes activity in various brain regions on curcumin treatment in aluminium induced neurotoxicity (Sood et al. 2011). The amelioration of NADH dehydrogenase and cytochrome c oxidase activity in aged brain on chronic administration of curcuminoids was perhaps due to the prevention of ROS mediated enzyme dysfunctioning, since it failed to elevate the level of enzyme KGDH, responsible for maintaining the appropriate levels of mitochondrial enzymes. The present study also demonstrated that enhanced activity of cytochrome c oxidase on curcuminoids treatment was associated with the reduced lipofuscin content in the aged rat brain, indicating the suppression of ageing progression due to efficient functioning of mitochondrial enzymes.

The present study also demonstrated the age associated reduction of mitochondrial complexes activity which was in corroboration with the previous reports (Navarro and Boveris 2007). Defects in the electron transport chain have been reported widely in the etiology of age associated neurodegenerative disorders like Alzheimer's disease (AD) (Bubber et al. 2005; Navarro & Boveris 2004). Curcuminoids administration

specifically prevented the functional loss of Complex I activity in middle aged and aged treated group which was attributed to its direct inhibition of ROS (O_2^-) and RNS ($ONOO^-$) species and the elevation of NADH dehydrogenase activity. Studies suggested that chronic exposure of $ONOO^-$ modifies the tyrosine content abundant in Complex I to 3-nitrotyrosine (3-NT) at post translational levels, thus leading to impaired Complex I activity (Murray et al. 2003). From the results, it was suggested that curcuminoids treatment could have prevented this post translational modifications by scavenging the O_2^- and $ONOO^-$ radicals due to its polyphenolic structures consequently leading to the synthesis of functional NADH dehydrogenase and thus Complex I activity (Mythri et al. 2007).

Curcuminoids administration was observed to prevent the age associated loss of Complex II, II–III and IV activity in the middle aged treated group, however in the aged treated group, the activity of three Complexes largely remains unaltered. Bolanos along with his coworkers (Bolanos et al. 1997) have reported the inhibition of Complex II and II–III activity as a consistent feature in neurons, astrocytes and isolated brain mitochondria exposed to $ONOO^-$. Similarly, decline in complex IV activity was the most consistently reported age-related alteration after Complex I and was attributed to NO-mediated neurotoxicity (Kwong and Sohal 2000; Navarro & Boveris 2004). The inactivity of chronic administration of curcuminoids in alleviating the Complex II, II-III and IV activity implies an irreversible damage in the aged brain due to the prolonged exposure of $ONOO^-$.

Since, NO and its reactive species appears to be the key intermediates behind the disruption of mitochondrial complexes, the present study was extended to investigate that whether curcuminoids exerted neuroprotective activity over neuronal nitric oxide synthase (*nNOS*) enzyme. In the present study, the expression of *nNOS* was found to be significantly up-regulated in the aged brain, which was in accordance with previous reports (Jesko et al. 2003). According to the NO hypothesis of ageing, a decrease in NADPH diaphorase-positive neurons (containing *nNOS*) ultimately leads to its expression in NADPH diaphorase-negative neurons, thus increasing the NO content (Calabrese et al. 2000; McCann et al. 1998). NO is also produced by mitochondria by *mtNOS* which is the isoform of *nNOS* (Elfering et al. 2002). Moreover, the inactivation of complex I was also reported to make *mtNOS* pro-oxidative in nature which in turn generate superoxide anions and augment mitochondrial peroxynitrite formation (Parihar et al. 2008). The present study demonstrated the significant down regulation of *nNOS* activity on chronic administration of curcuminoids in middle aged and aged rat brain. Recently, in one study curcumin has been reported to directly inhibit the *nNOS* activity better than the contemporary polyphenols in quinolinic acid induced excitotoxicity in human neuronal cell culture (Braidy et al. 2010). Based upon the present results it was suggested that the prevention of age dependant decline of mitochondrial electron transport chain on curcuminoids was in—part attributed to their activity over *nNOS* protein expression which was significantly down regulated on long term treatment consequently preventing the excessive NO generation.

The present study demonstrated the depletion of ATP content in the aged rat brain which further confirmed the impaired mitochondrial functioning with ageing. Previous reports also demonstrated the age dependant ATP depletion due to the decreased H^+-driven ATP synthesis (Navarro and Boveris 2005), reduction in the number of mitochondria or perhaps the bio-energetically efficient mitochondria (Sastre et al. 1998; Wakabayashi 2002) and the impaired mitochondrial electron transport chain (Davey et al. 1998). Curcuminoids administration significantly prevented the age associated loss of ATP content due to the maintenance of mitochondrial enzymes and complexes functioning and reduced oxidative burden in the middle aged and aged rat brain cortex.

Moreover, in order to assess the overall impact of curcuminoids over ageing mitochondria, the ultra structural changes in mitochondria were evaluated by TEM. The present study data indicated a significant decrease in the intact mitochondria and increase in the damaged mitochondria (mitochondria with broken cristae and vacuoles) in aged brain which was in accordance with the previous reports (Cash et al. 2003). A recent morphometric study found a significant reduction in intact mitochondria in different cellular compartments of AD and AD-like rodent brain (Aliyev et al. 2005; Obrenovich et al. 2006). Chronic administration of curcuminoids significantly ameliorated the age associated mitochondrial damage as evident by marked decrease in partially and completely damaged mitochondria in aged rat brain

cortex. The prevention of age dependant ultrastructural decay may further be responsible for restoration of ATP content on curcuminoids treatment among the aged brain.

To conclude, the present research findings suggested that curcuminoids may act as an effective therapeutic candidate against age associated mitochondrial impairment and thus may promisingly delay ageing and associated neurodegenerative disorders. This neuroprotective effect has been attributed but not limited to the direct inhibition of ROS and RNS species along with downregulation of *nNOS* protein expression. Recently, curcumin has been reported to exhibit antioxidant properties through hormetic effect via stress response pathways like Nrf2 and HO-1 (Demirovic and Rattan 2011). HO-1 hyperactivity reportedly induces mitochondrial bioenergetic failure through pathological iron deposition and macroautophagy (Schipper 2011). Therefore, it is hypothesized that the effect of long term curcuminoids treatment over mitochondrial dysfunctioning may also be through the activation of hormetic pathways. In-addition, based upon the present findings the early intervention is highly recommended, as many of the mitochondrial enzymes and complexes activity was found to be unaltered in the highly aged rat brain.

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