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Curcumin pre-treatment reduces toxic reactive oxygen species mediated damage in rats with reperfusion injury induced cerebral ischemia

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ABSTRACT

Curcumin is a compound whose anti-ischemic properties are well established in previous works. This study investigates the effect of curcumin on four marker enzymes for ischemia whose activities increase in ischemic conditions.Animals were classified into three groups. Group 1 was kept as sham-operated control. Groups 1 and 2 were induced cerebral ischemia by Bilateral Common Carotid Artery Occlusion for 30 min followed by reperfusion for three hours. Group 3 was treated orally with aqueous curcumin suspension (100 mg kg^{-1} of body weight) 24h prior to ischemic insult.Mitochondrial and cytosolic fractions were separated from a portion of the total brain extract. Levels of the products of lipid peroxidation (LPO) and the activity levels of each of the three ROS inhibiting enzymes were measured for each fraction of the extract.Baseline levels of LPO and the inhibiting enzymes' activities were significantly elevated in group 2 vs. group1 ($P<0.05$) after ischemic insult. In group 3 animals, neither of the four enzyme activities reported an increase as great as observed in group 2.Only in case of NADH Oxidase and Succinate Dehydrogenase enzymes did their activity levels fall below the baseline, after curcumin treatment.Curcumin helps to significantly check the ROS production in both cytosolic and mitochondrial fractions. The result may indicate a significant prospective role of curcumin to play in designing future management regimen towards cerebral ischemia in human beings; only after devising a perfect dose of the said drug.

Keywords: Curcumin; Cerebral ischemia; ROS; Lipid Peroxidase; ROS inactivating enzymes.

INTRODUCTION

Stroke is the world's second leading cause of mortality, resulting around 6,000,000 deaths annually [1]. Cerebral ischemia is a kind of stroke; an ischemic condition where the brain or parts of the brain do not receive enough blood flow to maintain normal neurological function [2]. Cerebral ischemia causes a reduction in oxygen supply to the brain, which leads to drop in the cellular ATP biosynthesis [3]. Due to the decreased ATP concentrations, cellular levels of calcium is increased, since the loss of cellular high-energy compounds virtually eliminates three of the four mechanisms of cellular calcium homeostasis [4]. The elevation of Ca^{++} dependent enzymes viz., phospholipase, proteases, nucleases, Na^{+}/K^{+} ATPases and Adenylate Cyclase leads to disorganization of neurons and finally exerts irreversible damage to neuronal cells [5]. The disturbed Na^{+}/K^{+} gradient leads to reverse glutamate transport (efflux) in affected neurons and astrocytes, and depolarization increases downstream synaptic release of glutamate [6].The elevation of these afore said enzymes leads to the formation of free radicals. These free radicals have been implicated in neuronal death in acute CNS injury and chronic neurodegenerative disorders [7]. After ischemia and reperfusion, elevation of conjugated diene levels, lipid peroxidation and cell damage were reported in the rat cerebral cortex region [8].

Cerebral ischemia-reperfusion injury has long been considered as an ideal laboratory model for cerebral stroke which occurs by occlusion of the carotid artery followed by embolus formation (embolic-ischemic stroke) and

subsequent thrombus (thrombotic-ischemic stroke) or cerebro-vascular haemorrhage (haemorrhagic stroke) [9]. Toxic reactive oxygen species (ROS) are generated due to oxidative stress in ischemia reperfusion injury [10] and exert a potential threat to neuronal survival [11]. Antioxidants are now being looked upon as a credible therapeutic, against solemn neuronal loss, as they have capability to combat by neutralizing free radicals [12]. Many such antioxidants occur in plants and products derived from them [13]. Such phyto-antioxidants being present in abundance are always a better alternative for use as drugs compared to their synthetic alternatives. Recently various plant products have been tried to sort out cerebral ischemia in effective and sustainable ways [14]. Curcumin, the active principle of *Curcuma longa* has been tested for its role to prevent the oxidative attack during cerebral ischemia and reperfusion in Sprague-Dawley (SD) rat model [14–19]**.**

Curcumin [*1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione*] is a polyphenol derived from the rhizome of turmeric (*Curcuma longa*; Zingiberaceae). It possesses diverse anti-inflammatory and anti-cancer properties following oral or topical administration [20–22]. Apart from its potent antioxidant capacity [23], its mechanisms of action in preventing cerebral ischemia reperfusion injury include inhibition of several cell signalling pathways at multiple levels, effects on cellular enzymes, immuno-modulation and effects on angiogenesis, increased mitochondrial biogenesis and cell–cell adhesion [24]. The ability of curcumin to affect gene transcription [25] and to induce apoptosis [26–28] in preclinical models is likely to be of particular relevance to chemoprevention and chemotherapy in patients. It is also known to us that curcumin can affect the expression and activity of a variety of enzymes, such as cyclooxygenase [29], lipoxygenase [30], glutathione-S-transferase [31] and cytochrome P450 [32]. An attempt to verify the extent of changes occurring due to the activity of curcumin on lipid peroxidation and activities of ROS inactivating enzymes viz. Catalase, NADH Oxidase and Succinate Dehydrogenase has been taken in this experiment. The process of lipid peroxidation depends entirely on the activity of the Lipid Peroxidase enzyme. This process has a significant positive correlation with the levels of ROS present in the cell [33]. Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyses the decomposition of hydrogen peroxide to water and oxygen [34]. It is a very important enzyme in protecting the cell from oxidative damage by ROS via dose-dependent suppression of the same [33]. NADH oxidase proteins are cell surface-associated and growth-related hydroquinone (NADH) oxidases with protein disulphide–thiol interchange activity [35]. They are also responsible for the carcinogenic loss of cellular growth control [36] and are a form of antioxidant machinery of the plasma membrane associated with ROS degradation [37]. The mitochondrial electron-transport chain is the main source of ROS during normal metabolism [38] and its rate is increased in a variety of pathologic conditions including hypoxia [39], ischemia [40] and reperfusion [40]. Succinate Dehydrogenase is an enzyme complex, bound to the inner mitochondrial membrane of mammalian mitochondria and many bacterial cells. The activity of this enzyme was assayed as it is the only enzyme that prevents the production of ROS in mitochondria [41].

Previously, a rise in activity levels of these four enzymes has been proved to be specific markers for cerebral ischemia [42–44]. It has also been discovered that curcumin can effectively prevent the occurrence of ischemia [16,45]; but the specific mechanisms of such an action is still not understood. Here, a unique approach has been taken in understanding this mechanism by verifying the effect curcumin has on these four enzymes;so that curcumin can be used as a prophylactic measure in potential ischemia patients. The activity levels of these four enzymes viz., Lipid peroxidase, Catalase, NADH oxidase and Lipid peroxidase were measured in cerebral tissues of sham-operated control, ischemic and curcumin-treated SD rat models. This experiment may help to clarify any correlation between curcumin treatment, rate of lipid peroxidation and activity of these ROS inactivating enzymes.

MATERIALS AND METHODS

2.1. Chemicals used

The chemicals used for the experiment were obtained from the Sigma-Aldrich Corporation, USA.

2.2. Animal experiment

A total of 45 female Sprague-Dawley rats weighing about 300 gm. were used. Rats were sub-divided into three randomly-assigned groups, each group of 5 animals and the whole experiment was replicated three times. First group was kept as sham-operated control. Second group were used as untreated control for cerebral ischemia and reperfusion (30 min. ischemia and 3 hours reperfusion). The last group was treated orally with aqueous curcumin suspension 24h prior to ischemic insult. The dose of curcumin was 100 mg per kg of body weight [18]. Investigators responsible for surgical procedures and drug treatments were appropriately blinded. End point assessments were also performed by investigators blinded to the groups for which each animal was assigned. All experimental procedures were approved by the institutional Animal Ethics Committee which is in accordance to the guidelines as stipulated by theCommittee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India.

2.3. Induction of global cerebral ischemia

Animals were anaesthetized by *i.p*. injection of urethane (35 mg kg^{-1}) and made ischemic by Bilateral Common Carotid Artery Occlusion for 30 min. Blood flow was restored and after 3 hours rats were killed by decapitation [46]. Rat brain was isolated immediately and surface rinsed with ice cold saline (0.9 percentNaCl solution). The subarachnoid membrane was removed gently and the brain was dissected into several coronal sections. A fraction of it was used to isolate mitochondria. The rest part of the brain was homogenized in 0.05 mol/L phosphate buffered saline with a Teflon coated homogenizer.

2.4. Isolation of mitochondria from rat brain and preparation of cytosolic fraction

Dissected rat brain from experimental animals were homogenized (10 percent w/v) in extraction buffer containing 0.02 mol/L HEPES-KOH, 0.01 mol/L KCl, 0.0015 mol/L $MgCl_2$, 0.001 mol/L EDTA, 0.001 mol/L EGTA, 0.001 mol/L DTT, 0.0001 mol/L PMSF and centrifuged at 750g for 5 min in Sorvall High Speed Cold Centrifuge at 4°C; resulting supernatant was further centrifuged at 2000g for 20 min. A part of this supernatant was kept at –20°C for enzymatic and biochemical studies. The remaining portion was centrifuged at 18000g in Sorvall SM24 rotor at 4°C. The supernatant (cytosolic fraction) was collected and kept at –20°C. The pellet was resuspended in 500 µl of extraction buffer and again centrifuged at 18000g for 20 min. Finally the mitochondria were resuspended in 200 µl extraction buffer and kept at –20°C until used.

2.5. Protein estimation method

Estimation of protein concentrations was done with the help of QuantiPro™Bicinchoninic Acid Assay Kit, from the Sigma-Aldrich Corporation, USA; with Systronics® UV-VIS Double Beam Spectrophotometer (Type–2203) [manufactured by Systronics India Ltd].

2.5.1. Lipid peroxidation level

The content of malondialdehyde (MDA), a final product of lipid peroxidation, was determined using the method described by Dhindsa*et al.* [47].A 0.5 ml aliquot of extract was added to 1ml of 20 percent (v/v) trichloroacetic acid and 0.5 percent (v/v) thiobarbituric acid. The mixture was heated in a water bath at 95°C for 30 min. After cooling down to room temperature and centrifuged at 10,000g for 10 min, the supernatant was read for absorbance at 532 and 600 nm. The absorbance for nonspecific absorption at 600nm was subtracted from the value at 532nm. The amount of MDA was calculated using the adjusted absorbance and the extinction coefficient 155 $(mol/L)^{-1}$ cm^{-1} [48]. Specific activity is expressed as units per milligram of protein.

2.5.2. Catalase activity

Catalase activity was measured using the method of Chance and Maehly [49]. The reaction solution (3ml) contained 0.05 mol/L phosphate buffer (pH 7.0), 0.015 mol/L H_2O_2 and 0.1 ml of enzyme extract. Reaction was initiated by adding the enzyme extract. Due to the linear decline of absorbance at 240 nm within the first 3 min, changes of the absorbance were read every minute. Specific activity is expressed as units per milligram of protein.

2.5.3. NADH Oxidase activity

NADH oxidase activity was determined, using the method of Morré*et al.*[50]. The disappearance of NADH measured at 340 nm in a reaction mixture containing 0.025 mol/L Tris-Mes buffer (pH 7.2), 0.001 mol/L KCN to inhibit low levels of mitochondrial oxidase activity, and 150 µM NADH at 37°C with stirring. Continuous recording over two intervals of 5 min each were taken. A millimolar extinction coefficient of 6.22 was used to determine specific activity. Specific activity is expressed as units per milligram of protein.

2.5.4. Succinate dehydrogenase activity

Succinate dehydrogenase activity was measured using the method of Slater and Bonner [51]. 1.0 ml of phosphate buffer, 0.1 ml of EDTA, 1.0 ml of KCN were added and made up to 2.9 ml with water. The extinction at 455nm was noted and then the reaction was started by addition of enzyme and followed the change in extinction during the first two minutes. Initial rates were taken as a measure of activity. A blank rate (all reagents except succinate) must be determined separately. Concentration of potassium ferricyaniderates can be measured by following the reaction at 420nm (ε =1.03×10 cm). The enzyme activity is expressed as units per milligram of protein.

2.6. Statistical Analysis

The presence of any significant differences between the activity values of the four enzymes for the three sets of rat brain tissues tested was checked by performing one-way analysis of variance (ANOVA) followed by Tukey tests; with the help of R (version 3.2.3) [52]. A P value<0.05 was considered to be statistically significant.

RESULTS

The mean activity values for the four enzymes viz., Lipid peroxidase, Catalase, NADH oxidase and Succinate dehydrogenase are given with their standard deviations, in Table 1. There was a statistically significant difference ($P<0.05$) in enzyme activity between each group of sham-operated control (C), ischemic (Is) and curcumin-treated (D) rat brain tissues for every enzyme. So, the result indicates that curcumin treatment affects the activities of all the enzymes studied.

Table 1: Enzymatic profile.The total (t), mitochondrial (mt) and cytosolic (c) fractions of the four enzyme's activities (in Units/mg/minute) in sham-operated control(C), ischemic (Is) and curcumin-treated (D) rat brain tissues are provided. (all values are expressed in mean ± S.D.)

		LIPID PEROXIDASE	CATALASE	NADH OXIDASE	SUCCINATE DEHYDROGENASE
C	t	5.16*± 0.19	80.51*± 0.61	32.03*± 2.09	–
	mt	2.30*± 0.25	35.52*± 0.65	17.94*± 0.78	4.48* ± 0.18
	c	6.50*± 0.23	26.61*± 0.47	22.58*± 1.39	–
Is	t	6.13*± 0.47	193.24* ± 2.08	42.80*±1.44	–
	mt	2.48*± 0.27	46.98*± 0.99	32.11*± 1.12	5.17*± 0.12
	c	8.11*± 0.30	40.35*± 0.93	38.78*± 0.52	–
D	t	5.36*± 0.09	61.66*± 0.55	37.35*± 0.70	–
	mt	2.33*± 0.26	43.56*± 1.39	23.93*± 0.74	2.94*± 0.18
	c	7.10*± 0.16	22.46*± 1.66	24.98*± 0.47	–

The values represented by (*) differ significantly between C, Is and D brain tissue samples ($P<0.05$)

The total activity levels of Lipid Peroxidase (Figure 1A) between ischemic and curcumin-treated rats have experienced a drop of about 13 percent. The induction of cerebral ischemia does not have much effect on the activity of the mitochondrial fraction of this enzyme; causing a mere eight percent rise in activity.

The total activity levels of Catalase (Figure 1B) between ischemic and curcumin-treated rats have experienced a massive drop of about 68 percent. The mitochondrial fraction of this enzyme has experienced a moderate decrease of about seven percent in its activity. Here also, the cytosolic fraction plays the main role as it alone experiences an increase of about 52 percent upon induction of ischemia, as well as a decrease of 44 percent upon curcumin treatment. Also another interesting fact about its activity is that, after treatment with curcumin its total activity decreases about 23 percent below its total control level of 80.51 (± 0.61) U/mg/min.

As Succinate Dehydrogenase is present only in mitochondria; hence there is no further division of its activity (Figure 1C). Its activity levels between ischemic and curcumin-treated rats have experienced a huge drop of about 43 percent. After treatment with curcumin its activity decreases about 34 percent below its control level of 4.48 (± 0.18) U/mg/min.

The total activity levels of NADH Oxidase (Figure 1D) between ischemic and curcumin-treated rats have experienced a drop of about 13 percent. The cytoplasmic fraction of this enzyme underwent a drastic inhibition in curcumin-treated rats showing a drop of about 36 percent and an escalation of about 72 percent upon ischemic insult. Due to cerebral ischemia, the activity of the cytoplasmic fraction of this enzyme undergoes a rapid increase.

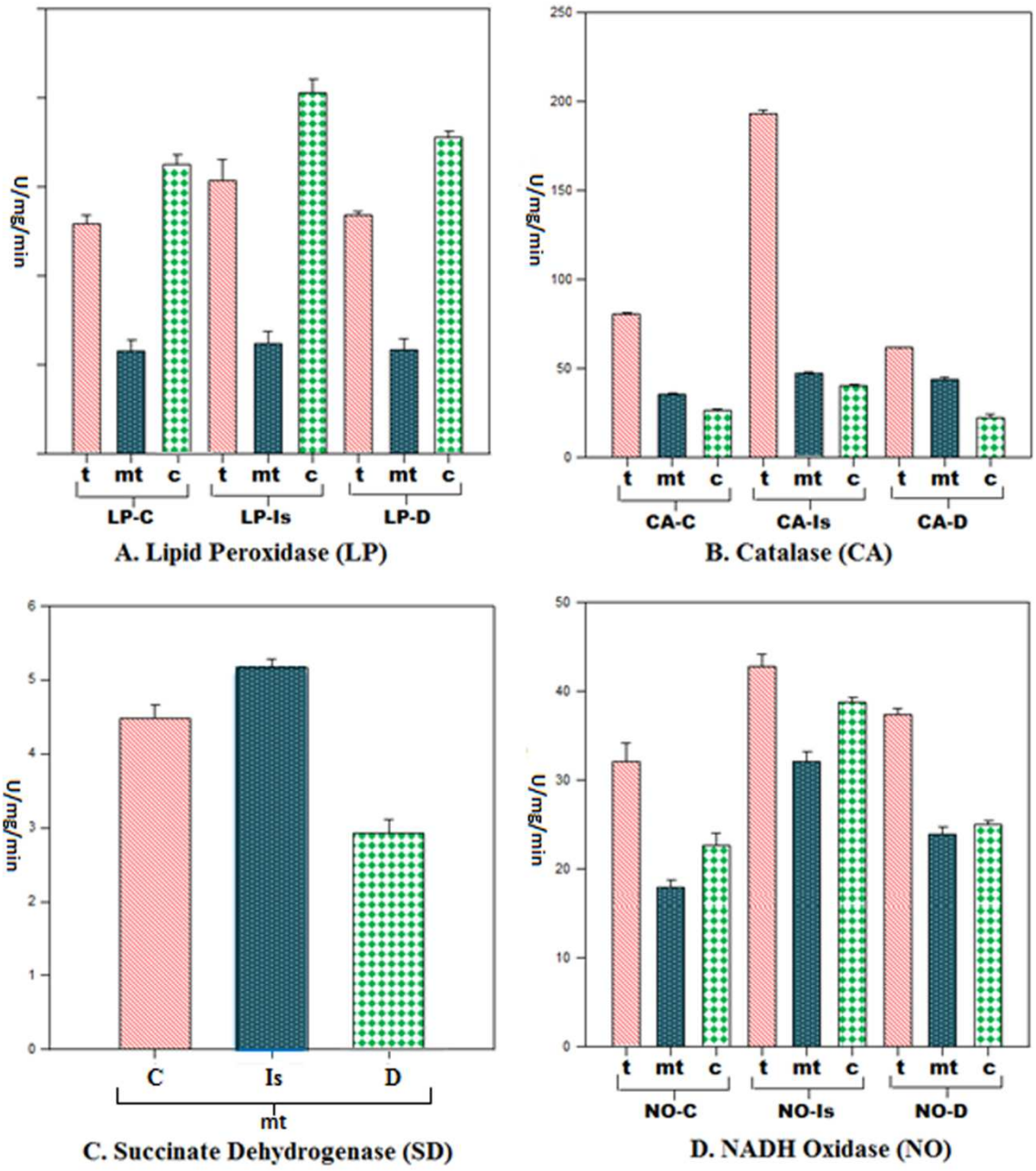

Figure 1: Comparative histogram showing total (t) [cytosol+ mitochondria], mitochondrial (mt) and cytosolic (c) fractions of the four ischemic stress related enzymes' activities in control (C), ischemic (Is) and curcumin-treated (D) rat brain tissues along with their respective standard deviations

DISCUSSION

Cerebral ischemia-reperfusion injury occurs mostly due to hypoxic conditions created in the brain tissues due to reduced blood flow [2]. This reduced blood flow, in turn, causes oxidative stress in the brain tissues due to reduced supply of oxygen and metabolites. Due to this oxidative stress, ROS are produced in the brain tissues [10], which cause heavy neuronal damage [11] leading to the symptoms of cerebral ischemia. Thus, treatment of this disease actually focuses itself on effectively reducing the ROS concentrations. For this purpose, use of natural antioxidants has gained a significant role. Our study focuses on such a phyto-antioxidant curcumin, derived from the rhizome of *Curcuma longa*.

Previous studies regarding the activity of curcumin have pointed out its specific neuro-protective antioxidant functions [23]. Since the mechanism via which this is achieved is still unknown; its role on various enzymes are being checked. We have further added on to this search, by examining the role of curcumin on the activities of Lipid

Peroxidase, Catalase, NADH Oxidase and Succinate Dehydrogenase. Upon induction of cerebral ischemia, the activity level of these enzymes increases significantly over control (Figure 1).

The increase in Lipid Peroxidase activity caused due to cerebral ischemia [42], has been further proved with the help of this experiment. Due to increased rate of lipid peroxidation, the concentration of cytosolic ROS is also increased. This increased ROS level is in turn related to the higher activity of the Catalase and NADH oxidase enzyme, which has to degrade ROS molecules before they cause any serious damage.

Curcumin actually down-regulates the activity of all the four enzymes studied. The retarding action of curcumin on lipid peroxidation [15–16] has also been supported by our results. However, the actual mechanism of inhibiting their action is not yet known; which can either be by inhibiting their transcription [53–54], thereby decreasing their concentration within the cell or it may act as an inhibitor to the enzyme's function [55] and in turn reducing its effective concentration in the cell cytoplasm.

The Lipid Peroxidase, NADH Oxidase and Catalase enzymes are most affected in their cytoplasmic fraction by both ischemic induction and curcumin treatment. After curcumin treatment, lipid peroxidase activity has undergone a significant decrease; which has further translated into the reduced formation of ROS in brain tissues. Again, due to decreased ROS levels, the activities of the cytosolic fraction of Catalase and NADH oxidase have also decreased significantly. Thus, we can say that ROS concentration in the cytoplasm undergoes significant increase upon induction of ischemia and is substantially checked after curcumin treatment.

Cerebral ischemia also results in increased production of mitochondrial ROS [40], which can be estimated by the observed increase in Succinate Dehydrogenase activity. The main source of this ROS is not the membrane lipid peroxidation but the mitochondrial electron-transport chain [38]. Succinate Dehydrogenase is the main ROS inactivating enzyme in the mitochondria, since the other two do not show much of a change in their mitochondrial fractions upon ischemic insult or curcumin treatment. Here also, curcumin shows its effect by decreasing the mitochondrial ROS production which in turn decreases Succinate Dehydrogenase activity. The exact mechanism of preventing this is not well understood; hence further research regarding this subject is needed.

Another interesting find was that Catalase and Succinate Dehydrogenase are inhibited by curcumin to such a degree, that they become hypoactive *i.e.* their activity falls below that observed in control. Henceforth, a suitable dose of curcumin must be determined before assigning it off as a drug capable of mediating recovery from cerebral ischemia-reperfusion injury.

This work can be further translated into making novel research about perfecting the dose of curcumin required for prophylactic measures against ischemia. The dose should be such as to not significantly bring down its effects on Lipid Peroxidase and NADH Oxidase activity, but at the same time, keeping the drug from causing over-inhibition of Catalase and Succinate Dehydrogenase enzymes.

CONCLUSION

Our results extend past observations made on curcumin on quite a few fronts, regarding its role in alleviating cerebral ischemia by controlling the ROS levels. We have determined that curcumin affects mostly the cytoplasmic fraction of the lipid peroxidase enzyme, which in turn decreases the level of cytoplasmic ROS. This decreased ROS level translates into the lower activities of the cytosolic ROS inactivating enzymes like Catalase and NADH Oxidase. Curcumin also effectively controls the mitochondrial ROS generation, thereby bringing down the activity of Succinate Dehydrogenase after treatment. The results indicate that curcumin plays a vital role in alleviation of any neural damage due to cerebral ischemia reperfusion injury by preventing lipid peroxidation, which thereby regulates ROS levels in brain cells and activity of the cytoplasmic as well as mitochondrial ROS inactivating enzymes. Finally, the results also suggest a promising role of curcumin in future management regimen towards cerebral ischemia in humans.

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