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Full Length Research Paper

The role of curcuminoids in overcoming neurodegenerative disorders resulting from heavy metal overload

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Iron plays a vital role in brain where it is essential for production of several neurotransmitters, such as serotonin, dopamine, norepinephrine, and γ-aminobutyric acid. But, overload may have neurodegenerative effect due to oxidative metabolism. So, this study aimed mainly on how can overcome neurodegenerative disorders results from iron fortified diet using a natural product. We utilized curcumin (25 mg/kg/day) in normal and iron overload experimental rats against control. Iron overload was induced by packed biscuits (50 to 60 g/day) enriched with ferrous sulphate (0.2%, w/w) daily for 10 weeks. Rats were divided into four groups (n=10), Group I, rats received packed biscuits. Group II, rats received packed biscuits enriched with ferrous sulphate daily for 10 weeks. Group III, rats received curcumin orally. Group IV, rats were given packed biscuits enriched with ferrous sulphate concomitant with curcumin throughout the period of study referred to as treated group. All animals were killed after 10 weeks. The results showed that iron fortified elevated serum iron, ferritin, nitric oxide (NO) and peroxidation value (MDA) but reduced glutathione (GSH) and super oxide dismutase (SOD) were decreased. Also, Monoamine oxidase (MAO) was elevated leading to decrease of serotonin and dopamine. Serotonin, dopamine, GSH and SOD were alleviated significantly by curcumin. Both neuronal NOS (nNOS) expression and inducible NOS (iNOS) expression were elevated in rats fortified with iron overload and were decreased when treated with curcumin but endothelial NOS (eNOS) protein expression was absent in both controls, animals with iron overload. In conclusion, curcumin displayed effective neuroprotective potency. It has MAO inhibitory effects concerned with increasing of some neurotransmitters, such as serotonin, dopamine through iron chelators and antioxidant action.

Key words: Neurotransmitters, iron, serotonin, dopamine, ferritin, curcumin.

INTRODUCTION

The most abundant transition metal in the body is iron that present mainly in protein-bound forms such as heme and non-heme proteins, playing a major role in respiratory electron transfer and oxygen utilization (Boldt, 1999). Iron is required to sustain the brain's high respiratory activity, myelinogenesis and also essential for production of several neurotransmitters, such as serotonin, dopamine, norepinephrine, and γ-aminobutyric acid. So, it plays a vital role in brain (Moos and Morgan, 2004). This transition metal promotes free radical generation through Fenton and/or Haber–Weiss reactions, thus triggering secondary chain reactions in the oxidative modification of lipids, proteins, and DNA in different organs (Aust and Morehouse, 1985).

Iron-dependent oxidative stress, elevated levels of iron and of monoamine oxidase activity, and depletion of antioxidants in the brain may be major pathogenic factors in Parkinson's disease, Alzheimer's disease and related neurodegenerative diseases (Zheng et al., 2005). It has been reported that Fe^{2+} can increase oxidation of monoamines such as serotonin and dopamine Velez-(Pardo et al., 1997). In spite of its requirement in the body, high level of iron has neurodegenerative effect due to oxidative metabolism, which generates large amount of reactive oxygen species (ROS). Most of the proteins involved in maintenance of iron metabolism are expressed in brain, suggesting that brain cells follow similar homeostatic mechanism as do all other cells in the

body (Ponka, 2004). The brain is highly susceptible to oxidative damage because it consumes a large amount of oxygen and generates an abundance of free radicals as normal products of cellular metabolism (Sanz et al., 2005).

Reactive oxygen species (ROS) have been implicated in a wide range of biological functions, but they can be both essential and highly toxic to cellular homoeostasis (Halliwell et al., 1992). In normal aging, brain accumulates iron that suggests more in flow of iron into brain than out flow. This increased level of iron can disrupt the brain's iron homeostatic mechanism (Bishop et al., 2002; Kaur and Andersen, 2004). It was known that excess iron catalyzes the formation of ROS that cause oxidative damage and affect brain. However, iron accumulation in brain tissues has not been widely considered a primary cause of neurodegeneration (Jimenez et al., 1993). Under normal conditions, potentially toxic ROS are primarily generated by mitochondrial respiratory metabolism and efficiently neutralized by cellular antioxidant defense mechanisms. However, several conditions are known to disturb the balance between the ROS production and cellular defense, resulting in cellular destruction and dysfunction. Imbalance between pro- and anti-oxidant factors plays an important role in many processes, including excess of iron concentrations (McCullough and Bartfy, 2007).

Therefore, the formed oxygen free radical products can undergo covalent binding with free sulphydryl group. The latter is the component of proteins such as actin and "serotonin binding proteins" which are present in soluble brain extract (Velez-Pardo et al., 1997). Iron can increase the cytotoxicity of dopamine by increasing in its oxidation rate without intervention of monoamine oxidase B enzyme. These observations are relevant to the mechanism by which dopaminergic neurones are destroyed in neurodegenerative disorders such as Parkinson's disease (Velez-Pardo et al., 1997). Furthermore the role of iron in cerebral ischaemia is also very important where it seems to be associated with higher oxidative stress, excitotoxicity and inflammatory responses (Castellanos et al., 2002). Curcuminoids from curcuma longa are naturally occurring phytochemical possesses diverse pharmacologic effects including antioxidant, anti-inflammatory, anticancer and iron chelating activities (Srichairatanakool et al., 2007). Monoamine oxidase (MAO) enzyme play essential role in tyrosine and tryptophane metabolism that responsible for serotonin and dopamine synthesis (Nowakowska and Chodera, 1997).

The present work was undertaken to illustrate how can overcome neurodegenerative disorders resulted from iron fortified diet, mainly dopamine, serotonin and nitric oxide. So, the effect of both iron and curcumin on serum ferritin, malondialdehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD) were measured. Also, to investigate the effects of iron overload and curcumin on the expression of the three nitric oxide synthase (NOS) isoforms (endothelial, inducible and neuronal) as well as investigating the effects of iron overload exposure on NOS activity.

MATERIALS AND METHODS

Iron

Ferrous sulfate was obtained from Sd FiNE-CHEM LiMiTED, (INDIA) and was given in the maximum dose 2 gm/ kg/day (Shahidi and Naczk, 2003).

Curcumin

Curcumin (yellow coloured phenolic pigment (Cooper et al., 1994), obtained from powdered rhizome of *(C. longa)* Linn, (Family-Zingiberaceae) was obtained from Shanghai Seni Pharma- Tech Company Limited (Shanghai-China-Mainland) and was given in the dose 25 mg/kg/day (Saravanan and Par, 2005).

Forty male Wistar rats (160 to 180 g) were obtained from the National Research Centre Cairo, Egypt and kept under constant experimental conditions with free access to food and water. Animals were divided into four groups (n=10):

Group I, rats received packed biscuits (free of iron; 50–60 g/day) for 10 weeks (normal group).
Group II, rats received packed biscuits (50 to 60 g/day) enriched with ferrous sulphate (0.2%, w/w) daily for 10 weeks (Shahidi and Naczk, 2003). (Control group).
Group III, rats received curcumin (25mg/kg/day) orally.
Group IV, rats were given packed biscuits enriched with ferrous sulphate (0.2%, w/w) concomitant with curcumin throughout the period of study referred to as treated group. Iron fortified biscuits were fed to produce in vivo excess iron condition in rats.

Biochemical analysis

After completing the diet regimen, the rats were fasted over night and the blood were obtained via retro–orbital bleeding (Schemer, 1967) and centrifuged at 1000 *xg* for 15 min at 4°C. The sera were collected and stored at –70°C for estimation of serum iron, MAO, ferritin, MDA, GSH and SOD. Serum NO were determined spectrophotometrically according to Green et al. (1982). Peroxidation (MDA) was determined as described by Jain (1989) and Janero (1990). Monoamine oxidase enzyme was determined by Youdim and Tenne (1987) using Rat MAO ELISA Kit from BioAssay Systems/USA, following the instructions of the manufacturer.

Iron concentration determined by Andrews (1999)

ferritin according to Cox et al. (2002) reduced glutathione (GSH) was assayed colourimetrically at 412 nm according to the method of Hu et al. (2003) Serotonin (5-HT) and dopamine (DA) were determined fluorometrically (Schlumpf et al., 1973).

Preparation of brain homogenate

Whole brain tissues were removed quickly on ice and homogenized. Serotonin, dopamine, NO contents and NO synthtase gene expression were assayed in the brain homogenate.

Whole brains were washed in phosphate-buffered saline. Homogenizing solution (20 mmol/l HEPES, pH 7.5 with 0.1 mmol/l EDTA, 1 mmol/l DTT and mammalian protease inhibitor cocktail) 2.5 ml per 0.5 g tissue was added to the samples. Homogenized tissue was transferred to 50 ml centrifuge tubes and centrifuged at 1000 g, 4°C for 20 min. Supernatant was decanted into fresh tubes and pellets were discarded. Then supernatant was centrifuged at 10 000 g, 4°C for 20 min. Supernatant was analyzed for NOS protein expression using Western blot analysis.

Western blot analysis

Homogenates were analyzed for NOS protein expression using Western blot analysis (Sheehy et al., 1997). Protein was quantified using Coomassie protein assay reagent. Protein extracts (15 µg) were separated on polyacrylamide gels. Separated protein was electrophoretically transferred to nitrocellulose membranes. Membranes were first blocked in TBS/0.1% Tween containing 5% non-fat dry milk, then incubated with a primary antibody to neuronal- (nNOS), endothelial- (eNOS) or inducible- (iNOS) NOS. The anti-nNOS rabbit antibody used as described by Sheehy et al. (1997). The anti-eNOS and anti-iNOS mouse antibodies were obtained from Transduction Laboratories. Membranes were then probed with secondary antibodies raised against the appropriate species. After washing with TBS/0.1% Tween, membranes were developed using a horseradish peroxidase chemiluminescent technique (Super Signal West Femto Super Sensitive Substrate). Blots were imaged and results quantified using an Image Kodak Digital Science.

Nitric oxide synthase *(NOS) activity analysis*

Homogenates were analyzed for NOS activity utilizing the arginine-citrulline conversion assay originally described by Bush et al (1992). Tissue samples (50 µg) were added to reaction tubes kept on ice. Specificity for NOS activity was demonstrated by pre-exposing brain homogenates to Nω-nitro-L-arginine methyl ester HCL (L-NAME), a non-specific NOS inhibitor. Then a reaction mixture containing L-arginine, FAD, BH_4 and NADPH was added. $MgCl_2$ was then added.

Reactions were run both with and without $CaCl_2$ and calmodulin (CaM). [^{3}H]arginine was then added to each reaction tube and samples were incubated in a shaking water bath (37°C) for 1 h such that no more than 20% of the [^{3}H]arginine was metabolized, to ensure that the substrate was not limiting. Final concentrations within the final reaction mixture were L-arginine (8 µmol/l), [^{3}H]arginine (17 nmol/l), NADPH (1 mmol/l), FAD (5 µmol/l), BH_4 (14 µmol/l), $MgCl_2$ (1 mmol/l), $CaCl_2$ (3 mmol/l) and calmodulin (25 units). Then the reaction was stopped with ice cold stop buffer (20 mmol/l Na citrate, pH 5.0 containing 1 mmol/l citrulline, 2 mmol/l EDTA 2 mmol/l and 0.2 mmol/l). Reactions mixtures were immediately poured through Dowex-50 W columns, followed by 2 ml distilled H_2O. Eluted fluid was collected in 15 ml scintillation vials, scintillation cocktail (ScintiVerse, Scintanalyzed; Fisher Scientific) 10 ml was added to each vial, and vials were counted for ^{3}H using a multipurpose scintillation counter (Beckman). NOS activity was estimated by the differences between counts in the presence and absence of L-NAME.

Animal approval committee

An approval was taken from the University committee resident in College of Medicine/Minia University.

Statistical analysis

All obtained data were represented as mean ±SE. Differences between the mean values were statistically analyzed by using one-way analysis of variance (ANOVA) utilizing computerized statistical program (InStat). $P<0.001$ was considered statistically significant.

RESULTS

Results are shown in Tables 1 to 3 and Figures 1 to 2. Brain homogenates were analysed separately for NOS isoform expression. The data obtained indicated that nNOS protein expression in the brain homogenates increased in animals with iron overload compared to control ($P < 0.001$, Figure 1) and decreased in iron overload group treated with curcumin.

A protein band that cross-reacted with an antiserum specific to inducible NOS was also present in the brain homogenates, so the level of this protein within the homogenate was increased in animals with iron overload compared to control ($P < 0.001$ Figure 2) and decreased in iron overload group treated with curcumin. Endothelial

Table 1. Brain serotonin, dopamine, nitric oxide (NO) of rats fed on the test diet for 10 weeks.

	Normal	Iron overload	Curcumin	Curcumin and Iron
5-HT (µg/g)	30.6 ± 0.60	14.7 ± 0.47*	33.3 ± 1.9	29.2 ± 2.90**
DOPA (ng/g)	450.6 ± 22.5	212.7 ± 1.3*	445.9 ± 12.2	413.1 ± 14.9**
NO (µmol/g)	3.57 ± 0.25	12.3 ± 0.40*	4.09 ± 0.19	4.69 ± 0.25**

Values are expressed as means±S.E; *Significantly different from normal group at *P* < 0.001; **Significantly different from iron overload group (control) at *P < 0.001*.

Table 2. Serum iron, ferritin, peroxidation value (MDA), GSH and SOD of rats fed on the test diet for 10 weeks.

	Normal	Iron overload	Curcumin	Curcumin and Iron
GSH (mg/l)	40.6 ± 0.60	14.7 ± 0.47*	45.9 ± 1.10	43.1 ± 0.95**
SOD (U/ml)	17.4 ± 1.13	6.9 ± 0.46*	19.3 ± 0.79	17.8 ± 0.99**
MDA (nmol/l)	60.1 ± 1.7	111.5 ± 3.7*	67.0 ± 1.8	70.5 ± 2.9**
Iron (µmol/l)	30.6 ± 0.60	120.7 ± 2.54*	31.9 ± 1.62	40.2 ±1.54**
S. Ferritin (µg/l)	130.6 ± 0.56	262.7 ± 4.68*	131.9 ± 1.62	137.2 ± 1.78**
MAO (ulU/ml)	60.7 ± 2.54	159.5 ± 2.93*	61.2 ± 3.21	65.2 ± 2.87**

Values are expressed as means±S.E; *Significantly different from normal group at P < 0.001; **Significantly different from iron overload group (control) at P < 0.001.

Table 3. nNOS and iNOS protein expression of rats fed on the test diet for 10 weeks.

Groups	nNOS protein expression pmol/min/mg protein	iNOS protein expression pmol/min/mg protein
Normal	2.77 ± 0.117	2.63 ± 0.146
Iron overload	7.03 ± 0.248*	8.11 ± 0.263*
Curcumin	2.91 ± 0.133	3.00 ± 0.100
Curcumin & Iron	3.07 ± 0.147**	3.23 ± 0.186**

Values are expressed as means±S.E; *Significantly different from normal group at P < 0.001; **Significantly different from iron overload group (control) at P < 0.001.

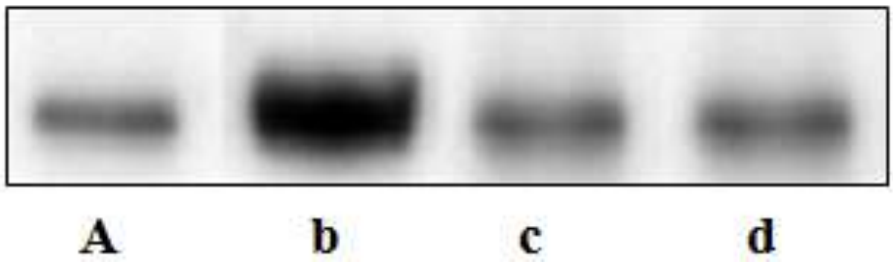

Figure 1. Western blot analysis of neuronal NOS (nNOS) expression in rat's brain homogenates: Normal (a), iron overload (b), curcumin (c) and iron treated curcumin groups (d).

NOS protein expression was absent in both controls, animals with iron overload, curcumin and iron treated curcumin groups (data not shown) Table 3.

DISCUSSION

In the present study the intake of iron in the diet was

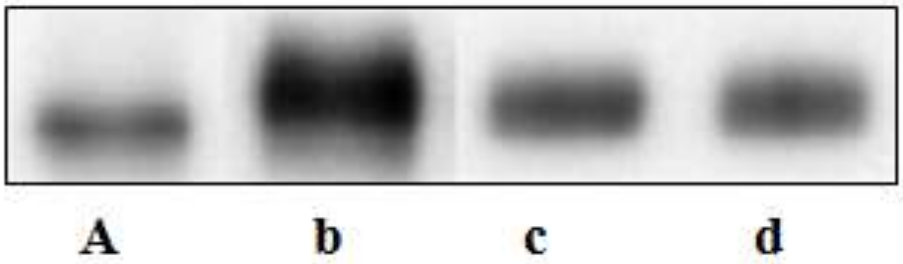

Figure 2. Western blot analysis of inducible NOS (iNOS) expression in rat's brain homogenates: Normal (a) iron overload (b), curcumin (c) and iron treated curcumin groups (d).

significantly stimulated MAO leading to brain serotonin and dopamine as reported by others (Nowakowska and Chodera, 1997). In addition, there was a significant increase MDA level indicating an increased oxidative stress. Increased iron was known to induce lipid peroxidation (oxidative stress) (Velez-Pardo et al., 1997). Thus, elevated MDA contents observed in the present study was the consequence of excess iron in brain.

Serotonin binding proteins (SBP) located in brain extract are involved in storage, protection and/or transport of serotonin as well as catecholamines. Such binding is increased by Fe^{2+}, but not by Fe^{3+}. It was believed that Fe^{2+} binds first to SH^- group of SBP. Monoamines also form coordination bonds with trapped iron leading to potential change in SBP functions. These findings show iron-induced oxidative stress adversely influencing neurotransmitters which may lead to neurodegeneration (Jimenez et al., 1993).

Others have demonstrated that direct injection of ferrous ammonium sulfate into cerebral spinal fluid of experimental rats induced 2-fold increase in iron content of brain cortex synaptosomes (Nagy et al., 1985). This may demonstrate the iron potential to cross an additional obstacle (blood-brain-barrier) to enter brain cells. However, the exact mechanism of iron intake and export from the brain is not fully understood (Ponka, 2004).

Generally metals can oxidize monoamines either directly or through oxygen free radicals produced by iron. Many studies have proposed that iron induces lipid peroxidation (Velez-Pardo et al., 1998) and demonstrated in confirmation that Fe^{2+} behaves like oxidants (sodium percodate) and superoxide radicals. Where, this study showed an increase in serum iron and ferritin in iron overload group.

Dopamine biosynthesis may be also affected due to its exposure to mild oxidizing conditions leading to its partial oxidation. Dopamine-quinones covalently modify cysteinyl residues in tryptophan hydroxylase (TPH; the rate-limiting enzyme in serotonin), leading to loss of its catalytic activity (Kuhn and Arthur, 1998).

However, serotonin and melatonin can inhibit reactive oxygen species (ROS) production, MDA, carbamyl ion and mitochondria oxidation of thiols in addition to degradation of 2-deoxyribose (Park et al., 2002). This may conclude the protective role of serotonin on iron mediated neuronal damage.

Accordingly disturbances in neurotransmitters levels like serotonin and dopamine and their oxidation metabolites may be associated with neurodegenerative diseases (Burke et al., 2004). Thus, the obtained changes results from excess iron in brain may dispose the brain to developing neurodegenerative disorders such as Parkinson's and Alzheimer's diseases. Iron levels increase with the severity of neuropathological changes in Parkinson's disease (PD), presumably due to increased transport through the blood-brain barrier in late stages of parkinsonism (Mario et al., 2004).

Abnormal amounts of iron in the brain have been demonstrated in a number of age-related neurodegenerative disorders including Alzheimer Disease (AD) (Bishop et al., 2002) and Parkinson Disease (PD) (Kaur and Andersen, 2004).

The injection of ferrous ammonium sulfate into the cerebral spinal fluid of rats induced 2-fold increase in iron content of brain cortex synaptosomes (Nagy et al., 1985). This may be attributed to the fact that iron must cross an additional obstacle (blood-brain-barrier to enter brain cells). The exact mechanism of iron intake and export from the brain is not fully understood (Ponka, 2004).

Iron-overload group demonstrated the depletion of GSH and SOD joined with an increase in oxidized glutathione (GSSG), NO and lipid peroxidation in accordance with previous reports, where iron-overload can potentiate various forms of cell injury (Iancu and Shiloh, 2005) with oxidative stress through the formation of hydroxyl radicals (Buss et al., 2002) and lipid peroxidation (Ozyurt et al., 2006). In particular, the generation of reactive oxygen species (ROS) can result in reversible and irreversible cell and tissue damage (Bouwstra, 2008; She et al., 2002).

Both nNOS and iNOS expression in brain were increased in group with iron overload but these were decreased by curcumin treatment, while eNOS were not affected in both groups. This might be explained in the

increase of NO in iron overload group and decreasing of it by curcumin treatment.

Dietary curcumin lowered lipid peroxidation and GSSG by enhancing the activities of antioxidant enzymes in conformity with previous studies (Arun and Nalini, 2002). It exerted beneficial effect in preventing oxidative stress in rats (Suryanarayana et al., 2007). Dietary antioxidants have preventative effects on oxidative stress (Lau et al., 2005). So, the present study indicated an increase in SOD and GSH in iron overload group that received curcumin.

Curcumin can stabilize lysosomal membrane, cause uncoupling of oxidative phosphorylation and has strong oxygen radical scavenging activity, that is responsible for its antiinflammatory property (Kohli, 2005). It was shown that iron chelators, antioxidants and MAO-B inhibitors have efficacy in a variety of cellular and animal models of CNS injury (Zheng et al., 2005).

Conclusion

The present study concluded that curcumin displayed effective neuroprotective potency. Curcumin was the most effective in inhibiting iron-dependent lipid peroxidation in rat brain homogenates, this may be due to its ability to iron chelation and its antioxidant actions. Also, it has MAO inhibitory effects concerned with increasing of serotonin and dopamine levels.

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