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Original research article

Protective effects of various dosage of Curcumin against morphine induced apoptosis and oxidative stress in rat isolated hippocampus

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ABSTRACT

Background: During recent years, the defensive role of Curcumin against oxidative stress and apoptosis has been experimentally documented. Long term consumption of morphine induces apoptosis and oxidative stress which may cause serious damage to brain cells. To investigate whether Curcumin could protect rat's hippocampus against morphine induced destruction, we assessed isolated hippocampus cells for oxidative stress, anti oxidant factor and apoptotic factor activities.

Methods: For this, 40 adult male rats were taken and randomly allocated to one of the five groups. Groups 1 and 2 received morphine (45 mg/kg) and normal saline (0.2 ml/rat) respectively for four weeks. Groups 3, 4 and 5 concurrently were treated with morphine (45 mg/kg, *sc*) and Curcumin (10, 20 and 40 mg/kg) for four weeks.

Results: The results showed that morphine significantly increased lipid peroxidation, mitochondrial GSH level, concentration of Bax; caspase-3 and caspase-9 activities while decreasing Bcl-2 concentration. Further, a significant decrease in superoxide dismutase and glutathione peroxidase activity was also observed. Various dosage of Curcumin attenuated these effects by significantly lowering lipid peroxidation, GSSG level, Bax concentration, caspase-3 and caspase-9 activities, while increasing superoxide dismutase and glutathione peroxidase activity, GSH level and Bcl-2 concentration.

Conclusions: These findings have demonstrated that Curcumin can act as an antioxidant and antiapoptotic agent against damage induced by morphine dependence.

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Introduction

Herbal and natural compounds with medicinal properties are considered widely as main sources of new medications with potential therapeutic properties. In recent years, these natural medicinal compounds, their flavonoids and fractions have been used as supplementary therapeutics to cure neurodegenerative diseases [1,2]. Usage of herbal medicine for management of oxidative stress and attenuation of apoptosis, has been proposed recently and many studies have been carried out for evaluating such protective effects of these medications [3,4]. Recent studies have demonstrated that chronic treatment with morphine and other opiates cause apoptosis and induce oxidative stress damage to brain cells by activating some neuroinflammatory and neuronal apoptotic pathways [5–7]. Some results have also indicated neurotoxicity caused by morphine dependence in parts of brain like hippocampus and amygdale [8,9]. *In vivo* experimental studies have demonstrated that apoptotic proteins such as caspases-3,9 and 8 and DNA fragmentation may be involved in bringing about morphine-induced apoptosis leading to developmental and functional changes in brain [10,11]. Chronic morphine induction has been seen to increase immunodensity of Fas ligand receptor and Bax protein, while decreasing Bcl-2 protein activity in hippocampus [12–15]. Mitochondria can be an active player in mediating opioids and other drug-induce neurotoxicity, neuronal apoptosis and oxidative stress. Combinations of cocaine and heroin (morphine being its metabolite) can potentiate the release of

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cytochrome c resulting in decrease mitochondrial viability [14]. Morphine in an increasing doses manner cause hippocampus neuron nucleuses degeneration such as CA1, CA2 and CA3 [16].

Curcumin (diferuloylmethane) is the most abundant component of turmeric, which is extracted from rhizomes of the plant *Curcuma longa*. This non-nutritive yellow pigment is an established nutraceutical dietary phenol and thus of important medicinal and pharmacological value [3]. Curcumin has antioxidant, anti-inflammatory, antiapoptotic, anticancer, immunomodulatory and neuroprotective properties [1]. Curcumin has been proposed as a putative agent in the treatment of some neurodegenerative disorders [3]. Recent studies indicate that mechanism of action of Curcumin is inhibition of Ca^{2+} entry and PKC activity. This compound exerts antioxidant and anti-inflammatory effects by inhibiting NF-κB and thereby suppressing major proinflammatory cytokines like IL-6 and TNF-α [17]. Furthermore, a synergistic effect of Curcumin on superoxide dismutase and catalase activities in striatum and mid brain regions is also well-defined [18]. Recent finding suggests that Curcumin can attenuate oxidative stress and mitochondrial dysfunction by increasing the protein levels of UCP2 (The uncoupling protein 2 that plays an important role in inhibiting oxidative stress and apoptosis) and inhibiting oxidative stress [19]. This study investigates the *in vivo* role of various dosage of Curcumin in offering protection to the rat hippocampus cell against morphine-induced oxidative stress and apoptosis.

Materials and methods

Animals

Forty male Wistar rats (weighing 200–250 g) about 8 weeks old, were obtained from Pasteur Institute of Iran (Tehran, Iran) and transferred to the lab. For 2 weeks, the animals were housed at room temperature (21–23 °C) with free access to standard food and water and maintained on 12 h day–night cycle. Our experimental protocol was in accordance with Guidelines for the Care and Use of Laboratory Animals published by National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Research Council of the Tehran University of Medical sciences.

Drug

Curcumin (Sigma–Aldrich Inc., St Louis, MO, USA) and morphine (Themad Co., Tehran, Iran) were purchased. All agents were freshly prepared right before the use. Morphine was solved in distilled water and Curcumin primary was dissolved in a small quantity of dimethyl sulfoxide (DMSO; 0.05%) and then reached to the desired volume.

Experimental design

The rats were randomly allocated to one of the five groups (each group 8). Group 1 (as positive control) received morphine (45 mg/kg, *sc*) for four weeks. Group 2 (as negative control) received normal saline (0.2 ml/rat) for four weeks. Groups 3, 4 and 5 concurrently received morphine (45 mg/kg, *sc*) and Curcumin with dosage of 10, 20 and 40 mg/kg, respectively for four weeks. Curcumin was administered *via ip* and morphine was injected as *sc*.

Mitochondrial preparation

All the animals were anaesthetized using thiopental (50 mg/kg, *ip*) and hippocampus tissues were isolated. As described previously, each hippocampus tissue was homogenized in cold homogenization buffer (25 mM 4-morpholinepropanesulfonic acid, pH 7.3, 400 mM sucrose, 4 mM MgCl 2 and 0.05 mM EGTA). Homogenate cells were centrifuged at 450 × *g* for 10 min and the supernatant was re-centrifuged at 12,000 × *g* for 10 min. Final sediment was re-suspended in homogenization buffer and stored in ice. Protein concentration was determined by Protein Dc assay kit (Bio-Rad) [20]. The homogenized cell solutions were analyzed for measurement of oxidative stress and apoptosis factors.

Determination of oxidative stress factors

Study of lipid peroxidation

Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as Malondialdehyde (MDA), natural bi-products of lipid peroxidation. For assessment of MDA, 100 μL of samples homogenization solution or MDA standard was added to separate micro centrifuge tubes. Next, 100 μL of SDS lysis solution was added to both sample and standard tubes and mixed thoroughly. After a 5-min incubation time at room temperature, 250 μL of TBA reagent was added to each of the sample and standard tube. All the tube was again incubated but this time, at 95 °C for 45–60 min. Following this, the tubes were centrifuged at 3000 rpm for 15 min and 300 μL of supernatant from each was transferred to another tube. 300 μL of n-butanol was added and after vortexing (1–2 min), the tubes were centrifuged for 5 min at 10,000 × *g*. Lastly, 200 μL of the both MDA standards and samples were transferred to a 96-well micro plate (compatible with a spectrophotometric plate reader) and absorbance was read at 532 nm. Results were expressed as nmol/mg of protein [21].

GSH and GSSG levels

Within the cells, glutathione exists in reduced (GSH) and oxidized (GSSG) states. In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form while less than 10% exists in the disulfide form (GSSG). For measurement of GSH and GSSG levels, 25 μL of the IX Glutathione Reductase solution was added to each well of a 96-well plate, followed by the addition of 25 μL of the IX NADPH solution. Next, 100 μL of the prepared standards glutathione solution or sample homogenization solution was added to each well and mixed thoroughly. In addition, 50 μL of the IX Chromogen was also added to each well and mixed. Immediately after this, the absorbance was read (405 nm) for each GSSG/GSH standard and sample. Finally, by inserting the sample absorbance in standard curve, amount of GSSG/GSH was determined and expressed as nmol/mg of protein [22].

Study of manganese superoxide dismutase (SOD) activity

SOD which catalyzes the dismutation of the superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. In order to determine the SOD activity, 20 μL of unknown sample solution was added to each sample and 2nd blank wells, and 20 μL of ddH_2O (double distilled water) was added to 1st and 3rd blank wells. Next, 200 μL of WST Working Solution (1 ml of water-soluble tetrazolium salt;WST dissolved in 19 ml of Buffer Solution) was added to each well and mixed. 20 μL of Dilution Buffer was added to 2nd and 3rd blank wells. Furthermore, 20 μL of enzyme working solution to was added to each sample and 1st blank well. After mixing thoroughly, the plates were incubated at 37 °C for 20 min and absorbance was read at 450 nm using a micro plate reader. As recommended by manufacturer, SOD activity was calculated using the following equation: SOD activity = {[(A blank 1 − A blank 3) − (A sample − A blank 2)]/(A blank 1 − A blank 3)} × 100. Data was reported as U/ml/mg protein [23].

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