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Prophylactic neuroprotective efficiency of co-administration of *Ginkgo biloba* and *Trifolium pretense* against sodium arsenite-induced neurotoxicity and dementia in different regions of brain and spinal cord of rats

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ABSTRACT

The present study was carried out to evaluate the potential protective role of co-administration of *Ginkgo biloba*, *Trifolium pretense*against sodium arsenite-induced neurotoxicity in different parts of brain (Cerebral cortex, Hippocampus, striatum and Hind brain) and in the spinal cord of rats. Sodium arsenite caused impairment in the acquisition and learning in all the behavioral tasks and caused significant increase in tumor necrosis factor-α,thiobarbituric acid-reactive substances andlipid profile, while caused significant decrease in glutathione, total thiol content, total antioxidant capacity, acetylcholinesterase, monoamine oxidase and ATPases activities. These results were confirmed by histopathological, fluorescence and scanning electron microscopy examination of different regions of brain. From these results sodium arsenite-induced neurodegenerative disorder in different regions of brain and spinal cord and this could be mediated through modifying the intracellular brain ions homeostasis, cholinergic dysfunction and oxidative damage. The presence of *Ginkgo biloba and/orTrifolium pretense* with sodium arsenite minimized its neurological damages. It was pronounced that using *Ginkgo biloba* and *Trifolium pretense* in combination was more effective as protective agents compared to use eachone of them alone.

1. Introduction

Arsenic is a naturally occurring toxic metalloid, which has two forms: organic and inorganic. Inorganic arsenic (iAs) is mainly released in the environment as trivalent arsenite (As^{III}) or pentavalent arsenate (As^{V}) and affecting millions of people worldwide (Pastoret et al., 2012). Toxicity of arsenic is exerted by the reactive oxygen species generation and depletion of antioxidant cell defenses resulting in antioxidant-pro-oxidant disruption (Pachauri et al., 2013). Arsenic-induced neurotoxicity occurs through a postulated mechanism which majorly involves oxidative stress with increased reactive oxygen species, lipid peroxides along with decrease in the activities of antioxidant enzymes as well as arsenic exposure associated with wide range of neurological complications in humans such as impaired memory, Alzheimer's disease, poor concentration and Parkinson's disease (Mundey et al., 2013). Our previous study showed that sodium arsenite-induced biochemical perturbations, oxidative damage and lipid peroxidation, and reduced antioxidant enzymes and content of sulfhydryl groups in plasma, brain, liver, testes, kidney and lung(Yousef et al., 2008; El-Demerdash et al., 2009).

Ginkgo biloba is an old unique tree with no living relatives which has been flourished during the Mesozoic era since 150 million years ago. *Ginkgo biloba* extract has a long history of medical use for treating conditions of cerebral dysfunction associated with brain

Abbreviations: EGB, *Ginkgo biloba*, *T. pretense*, *Trifolium pretense*; TBARS, thiobarbituric acid reactive substances; GSH, reduced glutathione; TAC, total antioxidant capacity; AChE, acetylcholinesterase; MAO, monoamine oxidase; TNF-α, tumor necrosis factor; Chol/HDL ratio, atherogenic rate; ApoB, apolipoprotein B; SPs, Senile plaques; Aβ, amyloid β; NFTs, neurofibrillary tangles; p-tau, hyperphosphorylated tau; APP, β-amyloid precursor; ROS, reactive oxygen species; β-actin, beta actin; LPO, lipid peroxidation; IL-6, Interleukin, interleukin-6; AD, Alzheimer's disease.

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aging and neurodegenerative dementia, regard to its constituents from flavone glycosides (quercetin, kaempferol and isorhamnetin) and terpene lactones (ginkgolides and bilobalide) (Solfrizzi and Panza, 2014). Mechanisms which may be involved in *Ginkgobiloba* extract-induced neuroprotection are modulation of ion homeostasis, glucocorticoid level, Aβaggregation and synthesis of growth factors(Ahlemeyer and Krieglstein, 2003). Also, Stoll et al. (1996) reported that *ginkgobiloba*extract (EGb 761) independently improves changes in passive avoidance learning and brain membrane fluidity in the aging mouse.

Isoflavonal phytoestrogens are plant-derived particles that structurally mimicking the endogenous estrogens which are comprising of diphenolic chemical structure. It can directly bind to estrogenic receptors (ER) in order to regulate expression of gene mediated by estrogen response element (Occhiuto et al., 2007). Red clover (*Trifolium pretense*) extract contains high levels of isoflavones (genistein, biochanin A, formononetin, pratensein, calycosin, daidzein and irilone). *In vitro* studies have demonstrated that estrogens protect dopaminergic neurons against inflammation-induced neurotoxicity (Liu et al., 2005).

2. Material and methods

2.1. Chemicals

All reagents and chemicals used were of analytical grade. Arsenic in the form of $Na_2HAsO_4.7H_2O$ anhydrous (decomposes with water) was purchased from Lobachemie for laboratory reagents and fine chemicals (India), *Ginkgo biloba* was obtained commercially from Nature's Bounty, Inc. (USA), *Trifolium pretense* (Red clover) was purchased from Nature's Way, Inc. (USA). Total antioxidant capacity (TAC) kit was purchased from Biodiagnostic Co. (Cairo, Egypt), cholesterol and triacylglycerol were assessed by enzymatic kits (Spinreact, Spain), while cholesterol HDL precipitating reagent was from Biosystems for reagents and instruments (Spain). Proteostat®Aggresome detection kit for flow cytometry and fluorescence microscopy was purchased from Enzo life sciences, Switzerland. Maxime RT-PCR premix kit was purchased from Intron biotechnology, South Korea. TNF-α primer was obtained from Vivantis international company, Malaysia. B-actin primer was obtained from Biolegio, Nijmegen "the Netherlands". Other chemicals were of the highest purity commercially available.

Sodium arsenite (Na_2HAsO_4) was dissolved in distilled water and the dose was 5 mg/kg BW according to the study of Nagaraja and Desiraju (1994). *Ginkgo biloba* (EGB) was dissolved in saline and the dose was 100 mg/kg BW according to the study of Can et al. (2003). *Trifolium pretense* (*T. pretense*) was dissolved in saline and the dose was and the dose was 250 mg/kg BW according to the study of El-Gendy (2012).

2.2. Animals and treatment

Adult male *Wistar* albino rats weighting 145–160 g were used in the present experiments. Animals were obtained from Faculty of medicine, Alexandria University, Alexandria, Egypt. The local committee approved the design of the experiments, and the protocol conforms to the guidelines of the National Institutes of Health (NIH). Animals were housed in a stainless steel wire cages and kept on basal diet and given food and water *ad libitum*. After two weeks of acclimation, animals were divided into five groups as follows: control group, groups 2, 3, 4 and 5 were orally treated with sodium arsenite (Na_2HAsO_4), *Ginkgo biloba* (EGB) plus sodium arsenite, *Trifolium pretense* (*T. pretense*) plus sodium arsenite and EGB plus *T. pretense* and sodium arsenite, respectively. Rats were orally administered their respective doses every day for 12 weeks.

2.3. Behavioral studies

2.3.1. Morris water maze

A long-term memory test for 6 rats in each group was carried out as described previously using a Morris water maze (MWM) with minor modifications (Von Engelhardt et al., 2008). Briefly, an invisible escape platform (hexagonal stone) was placed at a fixed location equidistant from the side wall in the middle of the pool and submerged 1.5 cm below the water surface. Two main parameters were measured in the MWM task, the first was the swimming strategies which were categorized into three general learning strategies (Table 1). Spatial strategy (spatial direct, spatial indirect, focal correct), Systematic strategy (scanning, random, focal incorrect) or Looping strategy (chaining, peripheral looping) (Pop et al., 2013) and the second is the latency time taken by each animal to locate the platform. Testing in the maze lasted eight days. The first three days were acquisition training with an invisible platform (spatial learning). Days 4–6 were reversal training, again with an invisible platform by moving the platform to the south west direction. A trial began by placing the rat in the water facing the wall of the pool at one of the starting points. If the animal failed to escape within 60 s, it was manually guided to the platform and allowed to stay there for 15 s until the start of the next trial. In the seventh day, a probe trial was conducted with removal of the escape platform and the same rats were allowed to swim freely for 120 s. In the eighth day, 24 h after the probe test, trials were conducted using the visible platform (cued testing), the same rats were allowed to undergo 4 trials to reach the visible platform and the duration of each trial was 60 s. The behavior of animals was monitored by using video camera.

2.3.2. Passive avoidance test

Rats were subjected to passive avoidance test to assess learning and memory deficits as described by Tota et al. (2009). Briefly, 6 rats from each group were placed into a wooden rectangular chamber divided into 2 compartments. One compartment is lighted by an overhead stimulus light and the other is black so as to remain dark. The two compartments are separated by a wooden door and each has a grid floor placed through which a low intensity foot shock (0.5 mA) was given for 10 s. The duration of a trial was 270 s. The 1st trial was for acquisition. Retention was tested from the 2nd trial onwards, 24 h after each trial. The transfer latency time refers to the transfer of animal from one compartment to another which has been recorded in seconds. The criterion for learning was taken as an increase in the transfer latency time on retention (2nd or subsequent) trials as compared to acquisition (1st) trial. The shock was not delivered in the retention trials to avoid reacquisition.

2.3.3. Novel object recognition

An object recognition test was performed to assess learning and memory. During the sampling phase, rats were removed from their home cages and placed in the object recognition box, which contained two identical objects (A1 and A2) fixed to the floor. After 3 min, the rat was removed from the object recognition box and returned to its home cage. Objects A1 and A2 were also then removed from the box. Twenty minutes were allowed to elapse before the start of the 3 min choice phase. During the choice phase, rats were singly removed from their home cages and returned to the object recognition box, where two new objects (A3 and B) were fixed to the floor. Object A3 was identical to objects A1 and A2, whereas object B differed from objects A1, A2, and A3. A videotape recording was made of the sample and choice phases, and two raters independently rated the time each rat explored each of the objects during the sample and choice phases. The percentage time spent exploring the new object during the choice phase corrected