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Ellagic acid attenuates arsenic induced neuro-inflammation and mitochondrial dysfunction associated apoptosis

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ABSTRACT

Arsenic, being a global pollutant needs a potential remedy which could fight against its associated toxicities. Ellagic acid (EA) is a known agent for its anti-inflammatory, antioxidant and antiapoptotic effects, and it is commonly found in fruits. The present study is designed to determine protective efficacy of EA against arsenic induced toxicity with special mention to inflammation and mitochondrial dysfunction in hippocampi of wistar rats. Rats were pre-treated with EA (20 and 40 mg/kg b.wt; p.o. for 11 days) along with arsenic (10 mg/kg; p.o. for 8 days). Total reactive oxygen species level and mitochondrial membrane potential were analyzed using flow cytometry. Protein and mRNA expression of apoptotic and inflammatory markers were also evaluated in rat hippocampus. Our results show that arsenic exposure increased total ROS generation and DNA fragmentation, decreased mitochondrial membrane potential alongwith an increase in expression of pro-apoptotic and inflammatory markers. suggesting that EA complementation downregulated total ROS generation dose dependently. Apoptotic markers, BAX and Bcl_2 as well as inflammatory markers, IL-1β, TNFα, INFγ got altered significantly on its administration. Moreover, it also attenuated effects on mitochondrial membrane potential. Based on our findings, EA might substantiate to be a budding therapeutic candidate against arsenic induced neurotoxicity.

1. Introduction

Arsenic, a toxic metalloid broadly distributed in soil and water bodies due to its natural existence and anthropogenic sources [1,2]. It is suggested that inorganic forms of arsenic are more toxic than organic forms. Numerous experimental studies have been carried out to investigate morphological, physiological, pharmacological and neurochemical effects following its exposure. Arsenic effortlessly crosses blood brain barrier and its concentration builds up in brain parts to exert its neurotoxic effects [3–5].

Arsenic has been reported to modify levels of biogenic amines and affects the behavioral and neurochemical functions in developing and adult rat brains [6,7]. Experimental studies on rodents have revealed learning and memory insufficiency with alteration in motor behavior [8,9]. Exposure to sodium arsenate/arsenite through drinking water decreased AChE activity in brain of rats and also inhibited synthesis and release of acetylcholine in brain slices [6,10]. Interestingly, decrease in AChE activity following arsenic and gallium arsenite treatment was correlated with impairment in learning and memory in rats [11]. Subchronic exposure of sodium arsenite augmented dopamine and 5-hydroxyindoleacetic acid (5-HIAA) levels in mid brain and cortex [4].

Oxidative stress is suggested to be a likely mechanism involved in arsenic neurotoxicity [1,12,13]. Arsenic enhances generation of free radicals including hydroxyl radicals, superoxide anions, dimethyl arsenic peroxy radical, dimethyl arsenic radical, nitric oxide. It also impairs the antioxidant system in brain and other biological tissues thereby increasing the oxidative stress [14–16]. Arsenic has high affinity towards GSH which boosts its oxidative stress vulnerability by creating an imbalance in the cellular redox status [17–19].

Arsenic is a major health concern according to WHO which has recommended chelation therapy alongwith antioxidants supplementation to treat related intoxications [20]. Thus, it is extremely necessary that new compounds are screened for management of arsenic neurotoxicity and other related dysfunctions.

Ellagic acid, EA (2,3,7,8-tetrahydroxybenzopyrano [5,4,3-cde] benzopyran-5-10-dione) is a phenolic compound and an excretion product of many plant species. It is generally present in fruits and nuts including blueberries, blackberries, raspberries, strawberries and walnuts [21–23]. It has a variety of beneficial effects including anti-oxidant, anti-inflammatory, anti-fibrotic and anti-cancer properties

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[24–26]. EA's protective effect has been attributed to its superoxide and hydroxide scavenging action. Further, EA has also been reported to inhibit lipid peroxidation and 8-OhdG formation both in vitro and in vivo [27–30].

In view of its potential therapeutic value, EA may have the propensity for the management of arsenicosis and related neurotoxicological implications, though the molecular mechanism pertaining to its anti-oxidative efficacy is largely unknown. It is in this context that we carried out the present work to study the protective role of EA in arsenic-induced neurotoxicity, genotoxicity, inflammation and mitochondrial dysfunction with special reference to ROS generation.

2. Materials and methods

2.1. Chemicals and reagents

DCFDA, Rhodamine 123, TRI® reagent, corn oil and sodium arsenate were purchased from Sigma–Aldrich Chemicals Pvt. Ltd (MO, USA). EA was purchased from TCI, Japan. M-MLV Reverse Transcriptase (AM2043), Hibernate®, B27®, Glutamax-I, 2X Mastermix, RNase inhibitor, dNTP mix and Random primers were purchased from Thermo Fisher (CA, USA). CBA kits for TNFα (Cat no. 558309) and INF-γ (Cat no. 558305) were purchased from BD Biosciences, USA. Ac-DEVD-pNA substrate was bought from Santa Cruz, USA.

2.2. Animals

Wistar rats (200 ± 25 g) were used for the current study. Animals were procured from Central Animal House facility of Jawaharlal Nehru Medical College, Aligarh Muslim University and housed individually in cages maintained under suitable conditions with temperature (25 ± 1 °C), humidity (60 ± 10%) in a well ventilated room with 12-h light and dark cycle. Rats were provided ad-libitum diet and water. All animal experimental procedures were approved by the Institutional Animal Ethics Committee and carried out as per CPCSEA guidelines and the doses and schedules of EA and arsenic were based on the pilot studies and previously published reports [1,31–33].

2.3. Experimental design

Rats were randomly divided into four groups containing 12 animals each.

Group 1: Control, Vehicle only, [C]

Group 2: Arsenic as sodium arsenate (10 mg/kg; p.o in drinking water) for 8 days [As]

Group 3: EA1 (20 mg/kg, p.o) pre-treatment for 3 consecutive days followed by administration with Arsenic up to 11th day [As + EA1]

Group 4: EA2 (40 mg/kg; p.o) pre-treatment for 3 consecutive days followed by administration with Arsenic up to 11th day [As + EA2]

2.4. Tissue preparation for biochemical analysis

At the end of the experimental period, animals were anaesthetized using 400 mg/kg chloral hydrate and sacrificed by cervical dislocation. Brains were dissected on ice cold saline to remove blood and for harvesting hippocampus. Hippocampi were immediately homogenized in ice cold phosphate buffer (0.1 mM, pH 7.4). The homogenate was centrifuged at 10,000*g* for 20 min at 4 °C to get tissue supernatant, which was used for estimation of caspase 3 activity and bead based assay for pro-inflammatory cytokines.

2.5. Preparation of single cell suspension for flow cytometry and comet assay

Freshly isolated hippocampi were kept in ice cold Hibernate A® medium (supplemented by B27® serum free supplement and Glutamax-I). Single cell suspension was obtained by mechanical dissociation and trituration using a pipette and was passed through 70 µm cell strainer followed by centrifugation at 450*g* for 10 min at 4 °C. The supernatant was discarded and the pellet was resuspended in Hibernate A® and kept at 37 °C for incubations with respective fluorescent probes.

2.5.1. Assessment of total reactive oxygen species (ROS) generation

2′,7′-Dichlorofluorescin diacetate (DCFDA) is a cell-permeable non-fluorescent probe. DCFDA measures hydroxyl, peroxyl and other reactive oxygen species (ROS) levels within the cell. The cells were incubated with 20 µM DCFDA for 40 min at 37 °C in the dark. The incubation was terminated and cytometric acquisition was done using BD FACS ARIA II flow Cytometer and results were analyzed using FACS DIVA® analysis software.

2.5.2. Assessment of change in mitochondrial membrane potential (ΔYm)

Rhodamine 123 is a cationic fluorescent dye that gets distributed according to the negative membrane potential across the mitochondrial inner membrane in respiring mitochondria. Loss of potential results in loss of the dye and, therefore, the fluorescence intensity. It has been used to monitor mitochondrial function in living cells. The cells were incubated with 5 µM Rhodamine 123 for 30 min at 37 °C in the dark. The incubation was terminated and cytometric acquisition was done using BD FACS ARIA II flow Cytometer and results were analyzed using FACS DIVA® analysis software.

2.5.3. Assessment of DNA fragmentation by comet assay

DNA damage was assessed using the alkaline comet assay as described by Singh et al. (1988) with slight modifications [34]. Cell suspensions with 0.5% low melting point agarose (LMPA) were overlaid on slides precoated with a fine layer of 1% normal melting agarose (NMA). A third layer of 1% LMPA was poured and slides were immersed overnight at 4 °C in lysing solution containing (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 0.2 mM NaOH, 0.1% Triton X-100 and DMSO; pH 10) followed by an alkali unwinding solution for 20 min. Electrophoresis was performed at 25 V in 1X TBE buffer for 45 min. Slides were dried and stained immediately with propidium iodide (1X) solution. Photographs were obtained at 40X using *Nikon Eclipse Ci-L* fluorescence microscope.

2.6. RNA isolation, cDNA synthesis and reverse transcriptase PCR

RNA was isolated from hippocampi of rats of each group using TRI® Reagent (Sigma-Aldrich, MO, USA) as mentioned in the manufacturer guidelines. High quality 2 µg RNA was reverse transcribed into cDNA as per manufacturer guidelines. Reaction mixture containing denatured RNA, random primers, 10X reaction buffers, dNTP mix (10 mM each), RNase inhibitor and M-MLV RT enzyme was incubated at 42 °C. The resulting cDNA was used as a template for semi-quantitative PCR using TCS Biocycler-1000. Amplification was done using specific primers (1 µM). Primer sequences used were β-actin (Forward CAACCTTCTTG CAGCTCCTC; Reverse TTCTGACCCATACCCACCAT), BAX (Forward GCCTCCTTTCCTACTTCGGG; Reverse CTTTCCCCGTTCCCCATTCA), Bcl_2 (Forward CGACTTTGCAGAGATGTCCA; Reverse CATCCACAGAG CGATGTTGT), IL-1β (Forward TCAAGCAGAGCACAGACCTG; Reverse ACTGCCCATTCTCGACAAGG), TNFα (Forward GAATTGTGGCTCTGG GTCCA; Reverse TCCAGTGAGTTCCGAAAGCC), IFNγ (Forward TGTC ATCGAATCGCACCTGA; Reverse TCAGCACCGACTCCTTTTCC). PCR was programmed for 35 cycles; denaturation at 95 °C, annealing at 58 °C and renaturation at 72 °C. The amplicons were run on 1.7% agarose gel. β-actin was used as an internal control. Band intensity analysis was done using Image J software (version 1.50, NIH, USA).

2.7. Bead based immunoassay for TNFα and INFγ

Pro-inflammatory cytokines, TNFα and IFNγ in hippocampi of rats