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Ameliorative effects of oleanolic acid on fluoride induced metabolic and oxidative dysfunctions in rat brain: Experimental and biochemical studies

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ABSTRACT

Beneficial effects of oleanolic acid on fluoride-induced oxidative stress and certain metabolic dysfunctions were studied in four regions of rat brain. Male Wistar rats were treated with sodium fluoride at a dose of 20 mg/kg b.w./day (orally) for 30 days. Results indicate marked reduction in acidic, basic and neutral protein contents due to fluoride toxicity in cerebrum, cerebellum, pons and medulla. DNA, RNA contents significantly decreased in those regions after fluoride exposure. Activities of proteolytic enzymes (such as cathepsin, trypsin and pronase) were inhibited by fluoride, whereas transaminase enzyme (GOT and GPT) activities increased significantly in brain tissue. Fluoride appreciably elevated brain malondialdehyde level, free amino acid nitrogen, NO content and free ·OH radical generation. Additionally, fluoride perturbed GSH content and markedly reduced SOD, GPx, GR and CAT activities in brain tissues. Oral supplementation of oleanolic acid (a plant triterpenoid), at a dose of 5 mg/kg b.w./day for last 14 days of fluoride treatment appreciably ameliorated fluoride-induced alteration of brain metabolic functions. Appreciable counteractive effects of oleanolic acid against fluoride-induced changes in protein and nucleic acid contents, proteolytic enzyme activities and other oxidative stress parameters indicate that oleanolic acid has potential antioxidative effects against fluoride-induced oxidative brain damage.

1. Introduction

Fluoride, an essential trace element is widely distributed in nature as its compounds or free ions. Excessive intake of fluoride through drinking water, food or other sources causes adverse health effects such as fluorosis in mammals and other toxic effects on cultured tissues (He and Chen, 2006; Gao et al., 2009a,b). Pollution of ground water by fluoride has been identified as one of the major problems in many developing countries (Jhala et al., 2008). Other than drinking water, there are other sources of fluoride like food contaminated with high concentration of fluoride and dental products like toothpaste, mouth rinses, drugs, fluoride dust and fumes from industrial belt (Hassan et al., 2009). Fluoride at mild doses has remarkable prophylactic effects as inhibiting dental caries while at higher doses it causes dental and skeletal fluorosis (Shanthakumari et al., 2004). However, harmful effects of excess fluoride intake are observed in soft tissues like liver, kidney and brain (Monsour and Kruger, 1985). Toxic effects of any environmental pollutant depend on the dose and duration of its exposure as well as the susceptibility of the tissue to that particular toxicant. Brain is highly susceptible to oxidative stress because of presence of more unsaturated fatty acids, high oxygen utilization, high iron content, and decreased activities of detoxifying enzymes (Bharath et al., 2002). Chronic fluorosis impairs the brain development in young rats (Guan, 1986), disturbs learning and memory processes (Chioca et al., 2008) and shows other neurological disorders such as paralysis of limbs, vertigo, spasticity in extremities in human beings (Waldbott et al., 1978).

Fluoride has potential effects on cellular oxygen metabolism and can promote the production of reactive oxygen species (ROS) (Inkielewicz and Krechniak, 2004). Enhanced production of ROS results in impaired cellular antioxidant defence system, inducing oxidative stress. Previous studies reveal that fluoride induces cellular oxidative damage by altering the cellular thiol status and the activities of some antioxidant enzymes like superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) (Mittal and Flora, 2006, 2007; Basha et al., 2011). ROS is implicated in pathogenesis

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of various disorders including neurological, metabolic and cellular dysfunctions. Oxidative damage of nuclear and mitochondrial DNA in human brain is supposedly involved in mild cognitive impairments that establishes relevance of integrity of brain DNA to brain function (Scott and Pandita, 2006). Fluoride is known to be neurotoxic and thus impairs brain functions (Chouhan and Flora, 2008) by oxidative stress mediated damage of brain tissues (Bharti and Srivastava, 2009; Narayanaswamy and Piler, 2010; Nabavi et al., 2012a; Mansour and Tawfik, 2012) or by alteration in neurotransmitter level (Pereira et al., 2011). Fluoride influences metabolic status of mammalian brain as evidenced by significant dose-dependent reduction in content of acidic, basic, neutral, and total protein contents in the cerebral hemisphere, cerebellum and medulla oblongata regions of mice brain after oral administration of NaF at the dose of 6 and 12 mg/kg body weight/day for 30 days (Trivedi et al., 2007). Fluoride toxicity along with low iodine content alters expression of some proteins which are mainly related to cellular signalling and protein metabolism (Ge et al., 2011). Sharma et al. (2008) reported that fluoride initiates an endoplasmic reticulum stress response in ameloblasts that interferes with enamel protein synthesis causing dental fluorosis.

Oleanolic acid, a pentacyclic natural triterpenoid, distributed in many plants and foods. It has certain pharmacological effects (Liu, 1995) as well as antioxidant activity (Yin et al., 2007 and Gao et al., 2009a,b). Multiple biological activities of oleanolic acid have been studied (Dubey et al., 2013). It is well known for its hepatoprotective effects against chemical-induced acute liver injury as well as chronic liver fibrosis and cirrhosis (Liu, 1995). It is used alone or in combination with other hepatoprotective ingredients as oral medications (Pollier and Goossens, 2012). Induction of metallothionein could be an important mechanism for the generalized beneficial effects of this triterpenoid against toxic insults (Liu et al., 1998). Hoskeri et al. (2012) reported *in vivo* prophylactic effects of oleanolic acid isolated from chloroform extract of *Flaveria trinervia* against ethanol-induced liver toxicity in rats. Studies of Yin et al. (2007) revealed that this compound exhibited several non-enzymatic antioxidant activities such as superoxide anion scavenging activity, metal ion chelating effect, xanthine oxidase inhibitory effect, reducing power and lipid oxidation depressing action. These findings partially explained the antioxidative potentiality of oleanolic acid.

The aim of the present study is to evaluate the protective role of oleanolic acid against fluoride-induced oxidative and metabolic dysfunctions in four discrete brain regions (cerebrum, cerebellum, pons and medulla) of rats and to elucidate the possible mechanism involved therein.

2. Materials and methods

2.1. Chemicals

Sodium fluoride (NaF, molecular weight 41.99) procured from Qualigen (India), chemicals like, TCA, PCA, RNA, NADPH. Na_2, boric acid, sucrose, diethylether, GSH, 5,5′,DTNB, NADH, thiobarbituric acid, haemoglobin, EDTA, haematoxylin, leucine, BSA, H_2O_2, methanol, ethanol, DMSO, glutathione reductase, H_2SO_4, HCl, NaOH etc. of analytical grade were purchased from Merck (India), SRL (India), Sigma–Aldrich (India) and ultrapure water by Millipore was used throughout the experiment to avoid metal contamination in preparation of reagents. Oleanolic acid was dissolved in water immediately before use.

2.2. Extraction and isolation of oleanolic acid from plant material

Oleanolic acid was isolated from the aerial parts of *Neanotis wightiana*, a commonly used medicinal plant of Tripura, collected from Kalsi (Jolaibari), South Tripura in March 2008 and identified by Prof. B.K. Datta, taxonomist, Department of Botany, Tripura University. A Voucher specimen (#BD/02/08) has been deposited in the National Herbarium, Botanical Survey of India, Botanical Garden, Howrah 711 103. Oleanolic acid was isolated from this plant due to traditional claim of local people of Tripura about the therapeutic potential of *N. wightiana* in brain and liver disorders.

2.2.1. Extraction method

Fresh air-dried aerial parts of *N. wightiana* (3.0 kg) were extracted with MeOH (10 L × 3, 1 week each) at room temperature. The MeOH extract was concentrated under reduced pressure in *vacuo* to a semi solid mass (400 g). The residue (350 g) was suspended in 125 mL water and extracted with hexane, chloroform, ethyl acetate and *n*-BuOH (three times each, 200 mL), successively (Das et al., 2013).

The ethyl acetate soluble extract (20.2 g) was column chromatographed through silica gel and eluted with stepwise gradient of $CHCl_3$/EtOAc (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 20:80, 10:90 each 500 ml). The fraction eluted with $CHCl_3$/EtOAc 70:30 gave a gummy residue, which on further column chromatographed through silica gel gave oleanolic acid (=3β-hydroxyolean-12-ene-28-oic acid).

2.3. Animals and treatments

Male rats of Wister strain ($N = 24$), weighing 140–150 g were chosen for the present experiment. Animals were first acclimatized for 7 days at 12 h light/dark cycle in polypropylene cages at 25 ± 2 °C. Rats were allowed to take standard 18% protein (casein) diet throughout the experiment and water ad libitum. Food intake capacity of each group of animals was recorded periodically. All animals received humane care as per CPCSEA guidelines. Animal ethical committee of Tripura University approved the protocols for the experiments before experimentation. Animals were randomized into four groups of six animals each and treated according to the following schedule.

Group I – Control animals (received the vehicle only).
Group II – Fluoride (as sodium fluoride at a dose of 20 mg/kg b.w./day orally for 30 days).
Group III – Fluoride (as sodium fluoride at 20 mg/kg b.w./day orally for 30 days) + oleanolic acid (OA) (5 mg/kg b.w./day orally for last 14 days of NaF treatment).
Group IV – Fluoride (as sodium fluoride 20 mg/kg b.w./day orally for 30 days) + vitamin C (20 mg/kg b.w./day orally for last 14 days of NaF treatment).

After treatment, all animals were sacrificed under light ether anaesthesia. Whole brain was removed, washed and perfused with normal saline to remove residual blood and then blotted dry. All the extraneous materials were removed before weighing. The cerebral hemisphere, cerebellum, pons and medulla regions of brain were dissected carefully, blotted free of blood, weighed to the nearest mg and utilized for study. All the following parts of brain were kept at −20 °C until biochemical analysis was performed.

2.4. Body weight and organo-somatic index

The body weight of each animal of each group was taken onward the commencement day of treatment and also noted periodically until sacrifice to observe the changes of body weight in different groups. The organ weight (whole brain) of respective group of animals was also recorded after sacrifice of animals. From these, the organo-somatic index (OSI) of brain was calculated (Krishnaiah and Reddy, 2007).

$$\text{Organo-somatic index} = \frac{\text{weight(g) of the organ}}{\text{Day 30 total body weight(g)}} \times 100$$

2.5. Biochemical assays

2.5.1. Estimation of RNA and DNA from rat brain

RNA and DNA were isolated from 5% tissue homogenates (in 0.1 M phosphate buffer, pH 7.4) of different regional tissues of rat brain (cerebrum, cerebellum, pons and medulla) by the method of Stroev and Makarova (1989), except that DNA was extracted with 0.8 M PCA at 70 °C. The RNA and DNA were measured in the respective extracts by UV-absorption at 270 and 290 nm respectively on Dynamica double beam UV–VIS spectrophotometer (model Halo DB-20).

2.5.2. Tissue protein content

The acidic, basic, neutral, and total proteins were extracted separately by the method of Shashi et al. (1992) and Trivedi et al. (2006). The tissue was homogenized in ice-cold 10% TCA to precipitate proteins. The homogenates were incubated at 70 °C for 20 min, then cooled, and centrifuged. The residue left was washed with ethanol to remove lipids and nucleic acids and was taken as total protein. The residue was mixed with known volume of 0.2 M HCl and incubated at 100 °C for 30 min and centrifuged. The resulting supernatant was taken as the extract of basic protein. The residue was then treated with a known volume of 0.1 M NaOH and kept overnight at room temperature and centrifuged. The supernatant served as the aliquot of acidic protein. Neutral protein was calculated by subtracting the sum of basic and acidic proteins from total proteins. Determination of acidic, basic, neutral, and total proteins was done spectrophotometrically by the method of Lowry et al. (1951) using bovine serum albumin as standard.