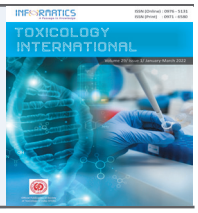
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Effect of *Ginkgo biloba* extract on Histomorphometric changes of Hippocampus against Fluoride Toxicity in Wistar Rats

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Abstract

Prolonged ingestion of fluoride leads to the pathogenesis known as fluorosis. Fluoride exposure leads to neuro degenerative changes such as reduction in the neuronal cell size and number. Exposure to fluoride showed neurodegenerative changes like shrunken neurons, increased in folding of the nuclear membrane, mitochondrial alterations, and dilated rough endoplasmic reticulum cisternae and clusters of vesicles near the Golgi bodies. Present study is to explore the histomorphometric changes of the hippocampus of fluoride induced brain and treated with *Ginkgo biloba* Extract (GBE). Thirty number (30) adult male Wistar rats were randomly separated into 5 Groups (n=6). Group1 (Control) supplemented with water, 2 to 5 Groups were supplemented with 100ppm of sodium fluoride for 30 days, while the Groups 3, 4, and 5 were *Ginkgo biloba* extract treated at 50mg/kg, 100mg/kg and 200mg/kg body weight for 15 days, after sodium fluoride treatment, results showed that, 200mg/kg GBE provided effective and complete protection against fluoride toxicity. From the present study concluded that 50, 100 and 200 mg/kg GBE was significantly attenuated the fluoride induced toxicity in a dose depending manner. Present histomorphometrical study showed delayed neuronal death in the CA1 and CA3 regions of hippocampus of fluoride group that was significantly attenuated by GBE (50,100 and 200 mg/kg) in a dose dependant manner.

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1. Introduction

Fluoride has been described as vital nutrient and physiologically essential for the normal growth and development of human beings. Fluoride consumption in optimal amounts through drinking water can protect against the development of dental caries without staining the teeth[1]. According to World Health Organization 1.5 ppm of fluoride in drinking water is safe. The chief resource of fluoride to the human body is mainly by drinking water[2]. Fluoride exposure leads to dental and skeletal fluorosis. Excessive fluoride exposure may result in central nervous system dysfunction[3]. Fluoride exposure causes neurodegenerative diseases like Alzheimer's, Huntington's and Parkinson's by damaging the brain mitochondrial cells[4]. Fluoride can penetrate readily into the brain and gets accumulated in the tissues producing neurotoxic effects[5]. Chronic exposure of fluoride through drinking water resulted in damage hippocampus of rats and also formation of beta-amyloid plaques[6]. Histomorphometrical observation of rat brain after the administration of high fluoride along with low iodine showed many pyknosed nuclei and decreased Nissl substance which could be due to lipid peroxidation. The brains of animals exposed to low iodine and high fluoride showed features of apoptosis[7].

Ginkgo biloba is commonly cultivated as a Chinese medicinal plant. It has neuroprotective, antioxidant, free radical scavenging, anti-apoptotic and memory enhancing properties. *Ginkgo biloba* belongs to ginkgoacece family[8] and its leaf extract composed of flavone glycosides, 24% (quercetin, kaempferol, isorhamnetin) and terpene lactones, 6% (ginkgolides and bilobalide). *Ginkgo biloba* Extract (GBE) has neuromodulatory and cognitive enhancement property that can influence on neurotransmission and anti apoptotic functions. The present study was carried out to ascertain if *Ginkgo biloba* extract could exert neuroprotective potentials against fluoride induced toxicity in rat model.

2. Materials and Methods

2.1 Chemicals

Sodium fluoride NaF (Madras fluorine Private Ltd: Batch. No.038P011, India), *Ginkgo biloba* leaf extract (Kshipra Biotech Ltd, Batch. No.140101, India). All other reagents and chemicals were used in the present study were in good analytical grade.

2.2 Animals

Thirty male adult Wistar rats, weighing (120 to 160gm) of body weight were obtained from Center for Laboratory and Animal Research (CLAR), Saveetha Institute of Medical and Technical Sciences, Chennai, India. Animals were housed in polypropylene cages and supplied with regular pellet diet and filtered water *ad libitum*. Rats were maintained in normal light and dark cycle at room temperature of 22–24° C. The present animal experiment conducted at institute was accepted by the Institutional Animal Ethical Committee, Reg. No (SU/CLAR/RD/019/2016) and the animal lab work involving rats were strictly followed the rules and guidelines of CPCSEA as per regulated by Government of India.

2.3 Experimental Design

Animals were separated into 5 experimental groups and every group had six animals (n=6). Group 1 control(C): Received water *ad libitum* throughout experimental period (45 days) Group II (Fluoride): Received water consists 100 ppm of fluoride for a period of 30 days *ad libitum* and supplemented normal diet and water till 45 days, Group III (Fluoride+50mg GBE): Provided 100ppm of fluoride water for 30 days *ad libitum* followed by *Ginkgo biloba* extract (50mg/kg b.w) for 15 days, Group IV (Fluoride+100mg GBE): Provided 100 ppm of fluoride water for 30 days *ad libitum* followed by *Ginkgo biloba* extract (100mg/kg b.w) for 15 days. Group V (Fluoride+200 mg GBE): Provided 100 ppm of fluoride water for 30 days and *ad libitum* followed by *Ginkgo biloba* extract (200mg/kg b.w) for 15 days. After 45 days of experiment, brain samples were taken to count the number of viable neurons in the CA1 and CA3 regions of hippocampus.

2.4 Dose Selection

Two hundred and twenty one mg of Sodium fluoride was melt in one liter of filtered water to obtain 100 ppm of fluoride[9]. Fluoride was supplemented to animals through drinking water (*ad libitum*) in water feeding bottles. *Ginkgo biloba* extract was administered at doses of 50, 100, 200 mg/kg body weight orally to the rats by fixing the oral gavage needle to the syringe.

2.5 Brain Tissue Processing Procedure

The rats were sacrificed under isoflurane anesthesia then brains were collected by performing craniotomy. The collected brain tissue was harvested and placed in buffered formalin solution. After proper fixation, the brain tissue was washed thoroughly in running water overnight. Then brain was dehydrated by following the ascending grades of alcohol (50, 60, 70, 80, 90 and 100%) each for 60 minutes. After that the brain tissues were cleared with two changes of xylene each with 30 minutes. Embedding was done with two changes of molten paraffin for 30 min to remove the xylene. Then they were mounted in L blocks with molten paraffin and solidified. The solidified blocks were trimmed to small size and sectioned them by using rotary microtome (Inco India) into 3 to 5-micron thickness.

2.6 Cresyl Violet Staining Procedure

Preparation of Cresyl violet solution (0.1%): 100mg of cresyl violet stain powder was dissolved in 100ml of distilled water with the help of magnetic stirrer cum heater. 0.5 ml of 10% acetic acid was added to adjust the pH between 3.5-3.8. The solution was filtered before use. The sections were stained as follows: Xylene-10 min (two changes each of five min), Descending grades of alcohol (100%, 90% and 70%) for two min each, Distilled water-15 min, 0.1 % cresyl violet stain-30 min at 600 C, Cool to room temperature, Ascending grades of alcohol (90% and absolute alcohol) for one-two min each, Xylene–two min each, Mount with Distrene Polystyrene Xylene (DPX)

2.7 Light Microscopic Examination

The stained slides were examined under 10X and 40X with light microscope; the CA1, CA3 regions of hippocampus. Proper stained slides without artifacts in the regions of interest were considered for counting the neurons.

2.8 Quantification of Cells

Ten brain sections from the each rat for the each area were selected for counting the cells. Number of viable neurons in the CA1 and CA3 regions was counted with 400X magnification across 250µ length with the aid of ocular micrometer. All the slides were properly coded before the counting to avoid any manual bias. The results were expressed as number of viable cells per unit length of the field (Number of cells/250µ).

2.9 Statistical Analysis

The values were expressed as mean ± SEM. The data was analyzed by one-way analysis of variance (ANOVA) followed by Student Newman Keuls's multiple comparison test (Sigma Plot 13 Software Inc., USA). P value ($P<0.05$) was considered as statistical significant.

3. Results and Discussion

Quantitative analysis of cresyl violet stained viable neurons (normal cell membrane, nucleus and not darkly stained) in CA1 and CA3 region of hippocampus were counted manually under the light microscope (400X) from all the treatment groups. The viable cell counts were expressed as the number of cells per unit length of the cell field (cells/250 μ m). The fluoride groups showed less number mean of viable neurons in CA1 and CA3 region of hippocampus compared to control. It was found to be statistically significant ($P<0.001$). GBE 50, GBE 100, GBE 200 mg/kg groups showed more number mean of viable neurons in CA1 and CA3 region of hippocampus compared to fluoride and it was found to be statistically significant. GBE 200 mg/kg showed significantly higher mean number of viable neurons in CA1 and CA3 regions of hippocampus compared with other doses such as 50 mg and100 mg GBE. Results showed that, 200 mg/kg GBE provided effective protection than 50 and 100 mg/kg GBE in fluoride toxicity (Figure 1). Histomorphological studies showed pyramidal neurons, Pyknotic neurons, neural degeneration in the CA1 and CA3 regions of hippocampus. Delayed neuronal death in the CA1 and CA3 regions are hippocampus of fluoride group that was significantly attenuated by GBE (50,100 and 200mg/kg) in a dose dependent manner (Figure 2 and 3). On examination of Cresyl violet stained brain sections of rats concluded, the number of viable neurons were significantly very less in fluoride group. This condition was mitigated by *Ginkgo biloba* extract in dose dependent manner.

Present study focused on histomorphometrical changes associated with fluoride toxicity and this toxicity was ameliorated by different doses of *Ginkgo biloba*

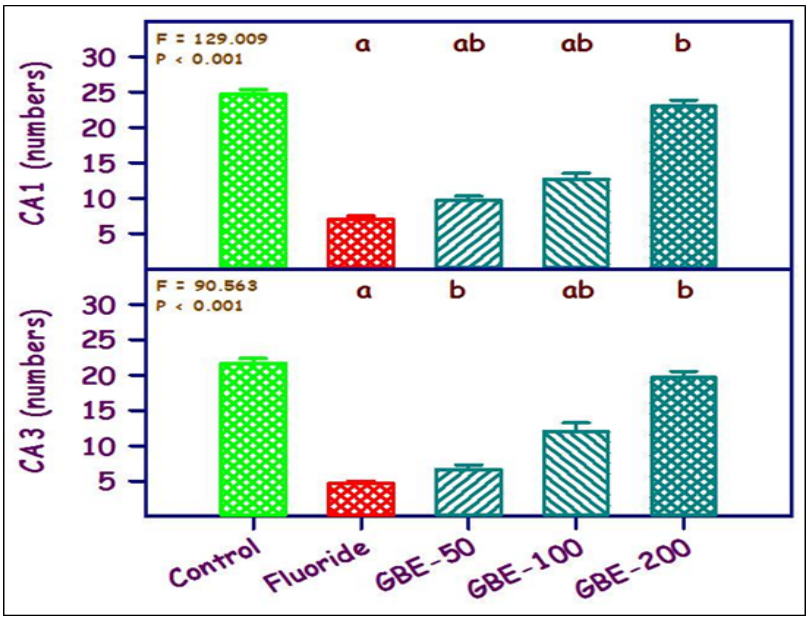


Figure 1. Effect of GBE treatment on mean number of viable Pyramidal cells of CA1 and CA3 region of hippocampus in fluoride induced toxicity (GBE-50, GBE-100 and GBE-200 are the doses (mg/kg). The values are mean ± SE (n=6 each). a – Significantly different from the control group, b - Significantly different from the fluoride group.

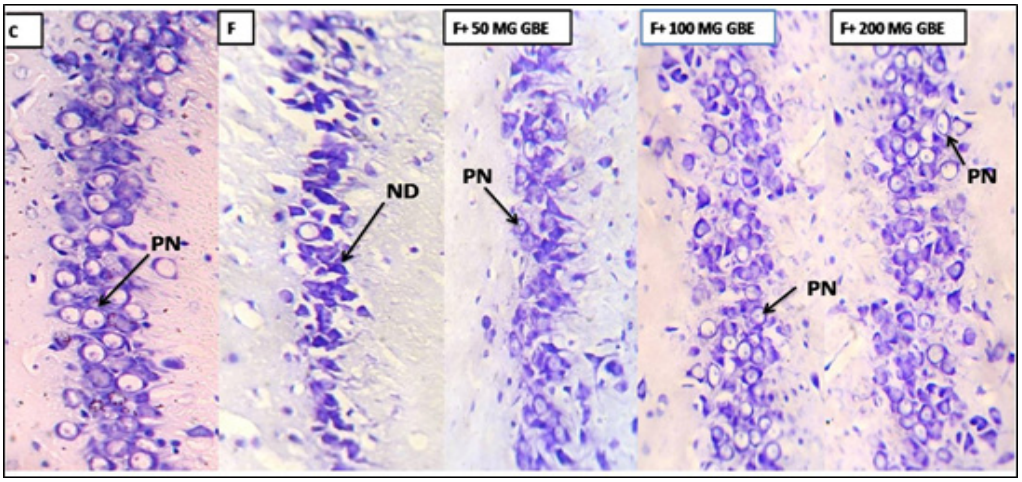


Figure 2. Effect of GBE treatment on mean number of viable Pyramidal cells of CA1 region of hippocampus in fluoride intoxicated rats (Cresyl violet staining). (PN:Pyramidal Neuron, PY: Pyknotic Neuron, ND: Neural Degeneration). (C: Control, F: Fluoride, F+50MG GBE, F+100MG GBE, F+200MG GBE (GBE: Ginko Biloba Extract).

extracts treatment. Fluoride alters levels of free radicals and increases lipid peroxidation in the brain particularly in the cerebral cortex and hippocampus[10]. Fluoride neurotoxicity targets the CA3 area of hippocampus because it plays a vital role in maintaining the long term memory. Therefore, fluoride can alter synaptic interface structure and membrane fluidity[11]. Sodium fluoride administered adult female mice showed significant

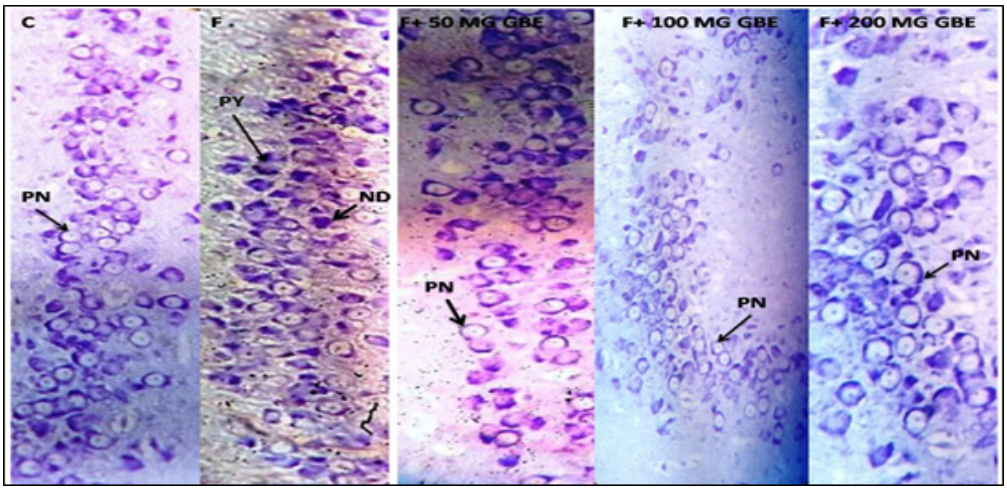


Figure 3. Effect of GBE treatment on mean number of viable Pyramidal cells of CA3 region of hippocampus in fluoride intoxicated rats (Cresyl violet staining). (PN: Pyramidal Neuron, PY: Pyknotic Neuron, ND: Neural Degeneration). (C: Control, F: Fluoride, F+50MG GBE, F+100MG GBE, F+200MG GBE (GBE: Ginko Biloba Extract).

number of degenerated nerve cell bodies in the CA3, CA4 and dentate gyrus areas of hippocampus[12]. Degenerative changes of neuronal cell bodies are characterized by dark cells, condensed nucleus, chromatolysis, and vacuolization and cell separation. It also included reduction in cell size and number of cell bodies.

Sodium fluoride administration in rats showed changes in phospholipid metabolism that might be the cause for neuronal degeneration[13]. 100ppm of fluoride causes considerable neuro-degenerative changes in the rat's hippocampus, amygdala, motor cortex, and cerebellum. These histomorphometrical changes indicates that, there is toxic effect after ingestion of high dose fluoride during the early developing stages of life on the growth, differentiation, and sub cellular organization of rats brain cells[14]. 30 or 100 ppm of fluoride exposure resulted in a decreased number of neurons in hippocampus and other parts of the brain leading to decreased learning and memory[15]. GBE showed protective role by reducing the neuronal damage after the transient occlusion of middle cerebral artery in rats[16].

Earlier study explored fluoride toxicity in rat hippocampus and investigated the neuronal density which revealed reduced neuronal density more distinct in the CA3 region of hippocampus[17]. The present study findings correlates with those studies by showing reduced neuronal density of viable neurons in CA3 region of hippocampus. *Ginkgo biloba* extract 150 mg/kg administered to rats for five days showed protection against mercury toxicity[18]. Previous studies conducted on histopathological changes in CA1 and CA3 region of hippocampus are similar to present study and proved that higher doses of GBE is more effective in treatment of fluoride toxicity in experimental rat model.

Toxic effects of fluoride can be reversed and ameliorated with supplementation of antioxidant rich nutrients. Bilobalide and quercetin constituents of *Ginkgo biloba* had significantly increased the brain derived neurotrophic factor level that enhanced neurogenesis in the hippocampal neurons[19]. *Ginkgo biloba* was found to increase the cell proliferation and neuroblast differentiation in the mouse dentate gyrus[20]. Flavonoid content of *Ginkgo biloba* extract has antioxidant property which protected the hippocampal neurons from β amyloid induced cell death[21]. *Ginkgo biloba* prevents the excitotoxicity induced cell death and offers neuro protection[22]. Prenatal exposure of GBE promotes cell survival in fetal hippocampus of rat by altering the hippocampal gene expression in gene cluster analysis[23].

4. Conclusion

Fluorosis, as a global public health problem, has received wide and deserving attention in recent years. 80% of

the diseases occurring in the world are appropriate to fluoride contaminated water and 65% of endemic fluorosis in the world is due to fluoride pollution. Present histomorphometrical study showed delayed neuronal death in the CA1 and CA3 regions of hippocampus of fluoride group that was significantly attenuated by GBE (50,100 and 200 mg/kg) in a dose dependant manner. This study investigated the therapeutic neuroprotective efficacy of GBE in experimental model of fluorosis by reversal of neuronal cell death in CA1 and CA3 region of hippocampus caused by fluoride toxicity.

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