VITAMIN C PROTECTS AGAINST SODIUM FLUORIDE INDUCED NEURODEGENERATION IN BRAIN OF RATS

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Abstract - This study reports the protective mechanism of vitamin C against NaF induced neurodegeneration. NaF enhance the production of free radicals which oxidizes membrane lipids and alter several antioxidants activity thus initiate apoptotic pathway in neural cells and resulted in neurodegeneration. The present study includes four groups of rats with different experimental exposure with a period of 60 days. The first group of individuals served as control, received normal water and rat feed. The second group of animals were intoxicated with 20ppm of sodium fluoride through drinking water. The 3rd and 4th were treated with 40mgkg^{-1} body weight and 80 mgkg^{-1} body weight (respectively) along with 20 ppm of fluoride. After 60 days of experimental period histopathological (H and E stain and biochemical parameters such as lipid peroxidase (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and reduced & oxidized glutathione (GSH & GSSG) levels were assessed in the brain tissue.Fluoride exposed animals showed significant decreased in the activity of CAT, SOD, GPx and levels of GSH with increased LPO and GSSG levels. Results also support altered cell morphology (Hippocampus).Vitamin C, free radical scavenger, reverted the aforesaid alterations thus reduce the effect of fluoride on brain.

Key words - Hippocampus, sodium fluoride, LPO, Vitamin C, Neurodegeneration

I. INTRODUCTION

Chronic exposure to high fluoride (>1 ppm) levels leads to it accumulation in the hard tissues such as bones and teeth and to a lesser extent in the soft tissues. In later stages of fluorosis soft tissues including brain, liver, kidney and heart are affected. Fluoride ingestion at high amount known to cross blood brain barrier and accumulate in the brain [1].Chronic F toxicity can alter neuronal and cerebrovascular tissue, abnormal behavior patterns and metabolic lesions in the brain of experimental animals [2]. Compelling evidence provide that F disturbs the CNS functions by several mechanisms. Of particular concern, F can generate free radicals and increased lipid peroxidation (LPO) in the brain, especially in the cerebral cortex and hippocampus [3]. Some of the neurotoxic effects of F in rats include oxidative stress, alterations in antioxidant enzyme levels and histopathological changes in brain cells of rats with decreased learning and memory abilities [4]. The biochemical milieu in the brain was altered by F exposure [5]. The hippocampus is more sensitive and important target site for F neurotoxicity. CA3 area of hippocampus plays a vital role in long term memory. Earlier studies have shown that F can alter synaptic interface structure and membrane fluidity [6] in the CA3 area of the hippocampus, thus affecting the transmission of neural information.F accumulation in the neurons and neuroglia leads to morphological changes mainly in the hippocampus [7]. However, its amelioration is not found in literature, particularly with treatment of vitamin C. Vitamin C or ascorbic acid is a water soluble vitamin, acts as an antioxidant, present in citrus fruits, vegetables and strawberries. Vitamin C is a potential antioxidant; it can able to neutralize free radicals, and is widely distribute throughout the body. It plays a critical role against oxidative stress [8]. Treatment with ascorbic acid against sciatic nerve injury in rats showed neuroprotection [9]. Several studies evidenced as ascorbic acid had neuroprotective properties against ischemia and excitotoxicity [10]. Many enzymes required vitamin C as a co- factor and efficient antioxidant, protecting cells against free radical mediated damage [11]. Vitamin C acts as a free radical scavenger and reduce the LPO level [12]. Ascorbic acid acts as a neuromodulator in normal brain functioning and serves as a neuroprotectant in various conditions of oxidative stress [13]. Vitamin C concentration in the brain is properly regulated,in the normal brain it is in high concentration, and is a part of the intracellular antioxidant network and as such is an important neuroprotective constituent [11]. High intake of vitamin E & vitamin C had been found to be associated with lower risk of Alzheimer's disease (Engelhartet al., 2002).

The present study is aimed to evaluate sodium fluoride induced oxidative stress, free radical generation thus leading to neurodegeneration and its ameliorationwith antioxidant, vitamin C treatment.

II. MATERIAL & METHODS

Adult male wistar rats (3 months old & weight approximately 250 to 280 gr) were used to all the experiments. The animals were kept in standard laboratory conditions with 12h light/dark period at a temperature of 22±2^{o}C as per the guidelines given by the CPCSEA at Department of Zoology, Osmania University, Hyderabad, India. And animals were

supplied with dry rat food and drinking water ad libitum.

III. EXPERIMENTAL DESIGN

Animals were allowed to acclimatize for 10 days before conducting experiments. At the time of doses animals were randomly categorized into four groups and five individuals for each.The animals assigned into the following treatment groups:

1. Group I: Control rats received normal water.
2. Group II: Fluoride (20ppm) through orally.
3. Group III: Fluoride (20ppm) + Vitamin C (40 $mgkg^{-1}$ body weight).
4. Group IV: Fluoride (20ppm) + Vitamin C (80 $mgkg^{-1}$ body weight).

These groups were maintained for 60 days and used for experimental analysis.

Chemicals: Pyragallol, tiobarbituric acid (TBA), reduced glutathione (GSH), N-ethylmaleimide (NEM), NADPH, DTNB chemicals were purchased from Sigma chemicals and other chemicals were obtained from local chemical laboratories.

Antioxidant enzyme assays: SOD, CAT, GPx are three primary enzymes, involved in direct elimination of active ROS, while GSH and other enzymes are secondary, help in detoxification of ROS by decreasing peroxide level [15].

1. **Super oxide dismutase (SOD):**The SOD activity was assessed based onthe ability of the enzyme to inhibit O_2^- depend auto-oxidation of Pyrogallol (1,2,3-benzenetriol). One unit of SOD activity is the amount of the enzyme that inhibit the rate of auto-oxidation of pyrogallol. SOD activity in the brain tissue is measured by the method of Murkland and Murkland[16].The change in absorbance of samples was recorded at420nm.
2. **Lipid per-oxidation (LPO):**Lipid per oxidation is estimated by the method of Garcia et al.,[17].An increased concentration of end products of lipid peroxidation is the evidence for the involvement of free radicals in human disease. MDA has been widely used for biomarker of lipid peroxidation which reacts with thiobarbituric acid (TBA). The reactivity of TBA toward MDA to yield an intensely colored chromogen fluorescent red adductand record the absorbance at 532 nm.
3. **Catalase:** The catalase is estimated by the method of Brannan et al.,[18].Catalase breaks down hydrogen peroxide, H_2O_2, which is a byproduct of metabolism. Hydrogen peroxide is toxic if it accumulates in a cell.The assay is based on the disappearance of H_2O_2 in the presence of the enzyme. Peroxidase reduced the H_2O_2 to give a red colored compound and absorbance measured at 505 nm.
4. **Glutathione (GSH):** Glutathione is the important central redox agent of most aerobic organisms. As a substrate for glutathione S- transferase, this agent reacts with a number of chemical species such as free radicals, peroxides, lipid peroxides and heavy metals. GSH act as an antioxidant, a free radical scavenger and a detoxifying agent. The glutathione content in the brain tissue was estimated by the method of Hissin and Hilf [19]. GSH react with a fluorescent reagent O-pthaldehyde to yield a fluorescent complex and measured spectrophotometrically at wavelength of 350nm and 420nm.
5. **Oxidized glutathione (GSSG):**Determination of glutathione disulfide (GSSG) in the brain tissue by the method of Hissin and Hilf[19]. GSH in the sample is reacts with N-ethylmaleimide (NEM) to form GS-NEM. This is followed by the back – extraction of excess NEM from the sample with dichloromethane. GSSG concentration is measured by spectrophotometry by GSH recycling method, which is based on the principle of conversion of GSSG to GSH by glutathione reductase and NADPH and reaction with DTNB. Fluorescence at emission 420nm was recorded by excitation at 350nm.
6. **Glutathione per oxidase (GPx):** To estimate glutathione peroxidase levels in brain sample Rotrucket al.,[20] method is used. GPx is found in the cytoplasm and mitochondria, which acts in concert with GSH to reduce H_2O_2 to H_2O. In this process, GSH is oxidized to produce GSSG. Read the absorbance at 420nm and GPx activity is reported as unit/mg protein.

IV. HISTOLOGY OF BRAIN

A. Hematoxylin and Eosin (H&E) staining: To stain the slides modified method of Lillie and Fullmer[21] is used. Hydrate the brain sections in alcohol series (100%, 90%, 80%, and 70%) and water.Stained with Harris hematoxylin for up to2 min and washed with water for remove excess stain. Then immerse in 1% Eosin-Y solution. Dehydrate slides in alcohol in the following order: 70%, 80%, 90% and 100% ethanol for 1 min for 3 times and clear in xylene. The stained sections were examined by using Olympus microscope (40X).

B. Congo red: Congo red stain is used for the detection of amyloid in neurons. The tissue thickness of sections is 5 microns and stained with Congo red and counter stained with Gill's haematoxylin[22].

Statistical analysis: The experimental data exposed to ANOVA and t-test to compare the statistical difference between groups in all possible combinations and the significant is P<0.05.

V. RESULTS

1. Anti-oxidant defense system:

1.1 Lipid peroxidation: Lipid peroxidation (LPO) (malondialdehyde; MDA) in the fluoride treated rats found to be significantly (P*<0.05) higher, whereas, revertedto normal by treatment with Vitamin C against NaF(P<0.05).

1.2 Super oxide dismutase: Superoxide dismutase (SOD) levels weresignificantly(P<0.05) decreased in fluoride treated groups as compared to control and their levels reverted in NaF + vit C administered groups.

1.3 Catalase: In fluoride administered rats CAT activity was decreased as compare to control and reversed CAT activity (P<0.05) was observed in vitamin C treated towards NaF fed group of rats.

1.4 Reduced GSH: Fluoride administered rats resulted in significantly(P<0.05) decreased GSHlevels as compared control group. The results showed normal GSH amount in NaF + vitamin C received rats.

1.5 Oxidized Glutathione: NaF administered rats resulted in significantly increase (P<0.05) in oxidized glutathione (GSSG) levels as compared control group. The data showed (Table 1) reversed GSSG levels on vitamin C treatment towards NaF toxicity.

1.6 Glutathione peroxidase: Glutathione peroxidase (GPx) levels were increased in fluoride intoxicated rats and their levels obviously decreased in NaF+vitamin C treated rats

Groups → / Antioxidant marker ↓	Control	Fluoride	F+Vitamin C (40 mg/ kg Bw)	F+Viatamin C (80 mg/ kg Bw
LPO	0.68±0.008	0.78±0.008	0.72±0.007	0.66±0.008
SOD	0.60±0.04	0.45±0.05	0.57±0.06	0.59±0.06
CAT	0.38±0.008	0.21±0.008	0.31±0.018	0.36±0.008
GSH	0.81±0.007	0.73±0.007	0.79±0.007	0.82±0.008
GSSG	0.80±0.008	0.90±0.008	0.85±0.008	0.79±0.005
GSH-Px	0.85±0.013	0.65±0.008	0.74±0.008	0.83±0.008

Table 1.Protective effects of vitamin C on brain tissue antioxidants of rats exposed to NaF. The data presented as mean±SE. The significance of data is P<0.05 (n=5).Units: LPO, µmol of MDA/gr. weight of tissue; SOD, Units/mg protein; CAT, µ mole/min/mg of tissue;GSH, µg/mg protein; GSSG, µg/mg protein; GSH-Px, µMol of GSH consumed/min/mg protein.

2. Histology of brain: To study brain architecture i.e. cell density, cell morphology and cell communications H & E stain has performed.

2.1 H and E stain: The normal structure of cells with round shape was observed in control rats and NaF +vit C treated rats, as shown in Figure 1. A remarkable change was observed in the brain of the rats exposed to NaF i.e., cells with irregular in shape with darkly stained cytoplasm and nucleus.

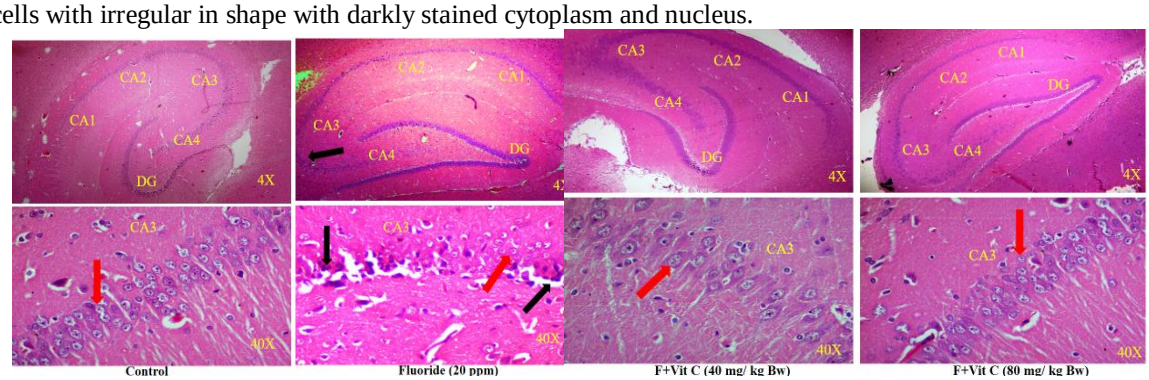


Fig. 1 H & E stained brain hippocampal region (4X) with particular interest on CA3 region (40X) of rats exposed NaF and Vitamin C.Black color arrow marks showing the altered cells in terms of size and shape and red color arrow mark showing the normal cells with round regular shape and normal size (Lawrence digital microscope).

2.2 Congo red stain: Congo red stained sections in control and as well as NaF + vit C treated rat brain revealed normal cells without any protein aggregations. Examination of NaF treated rats showing aggregations which may of amyloid proteins or which induce to development of plaques.

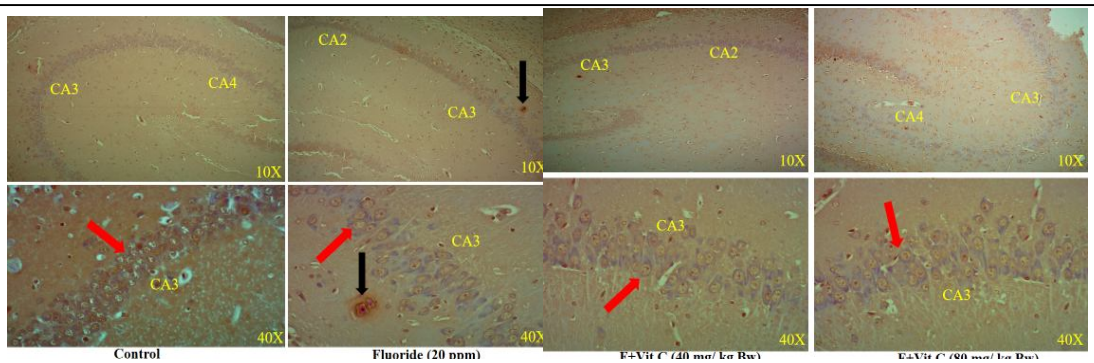


Fig. 2Congo red stained brain hippocampal CA3 region with 10X and 40X. Black color arrow marks showing the amyloid like aggregations in NaF treated rats and red color arrow mark showing the normal morphology without any such type of aggregations (Lawrence digital microscope).

DISCUSSION

Brain tissue is highly vulnerable to ROS mediated oxidative damage because of high content of polyunsaturated lipids, has high iron content [23]. Fluoride increases LPO levels and alter the antioxidant defense mechanism. Earlier research reports found increased levels of malondialdehyde (MDA) formation which is the marker of the extent of lipid peroxidation. The present data also agree with increased LPO in F treated rats (14.70%) as compared to control and its levels were significantly reverted in vitamin C administered rats.F induced neurodegeneration is accompanied with tissue catalase activity [24]. The present results reported the activity of CAT is reduced significantlyin fluoride treated groups as compared to control and reversed CAT activity was observed in NaF+vit C (both 40 and 80 mg/ kg Bw), but 80 mg/kg treated rats produced better results over 40 mg/ kg Bw.Significant depletion in GSH levels (9.87%) in the brain of NaF intoxicated rats was observed in the present study as well as in earlier studies [25] indicates that fluoride depends upon GSH for detoxification. Restored GSH levels were found onvit C treatment towards NaF toxicity. This is due to the fact that intracellular GSH is an important factor in the cytotoxic effect of a large number of compounds due to its reducing action. Decreased SOD (about 25%), GPx (23.52%) activities and increased oxidized GSH (GSSG) (11.11%) levels were noticed in NaF treated group of animals as compared to control group of rats and reversed in their activities (SOD &GPx) were observed onvit C treatment. And also found restored GSSG levels in vit C administered animals compared to fluoride received ones.Ascorbic acid is a potential reducing reagent known to help in the activation of several enzymes and to act as an antioxidant for detoxifying various toxic substances. In rats, NaF caused a significant decline in total ascorbic acid and GSH levels in the brain, thus indicating increased stress in the animals leading to rapid utilization of ascorbic acid. This suggests that stored ascorbic acid is rapidly oxidized under fluoride-induced stress and converted into its de-hydro form [25]. Vitamin C is considered to the first line natural antioxidant defense and a powerful inhibitor of LPO which is the main neurodegeneratingmechanism on fluoride exposure [26]. Rats with chronic fluoride exposure showed a number of histopathological changes in the brain including demyelination, a decrease in the number of Purkinje cells, thickening and disappearance of dendrites, swelling of mitochondria and dilation of endoplasmic reticulum in neurons [27]. Fluoride intoxication critically damage pyramidal cells in the all regions of hippocampus (CA1 to CA4) and granule cells in dentate gyrus [28]. Histopathological studies (H & E stain) clearly showed the occurrence of neuron degenerative changes such as alterations in the shape and size in NaF treated rats whereas normal round shape cells were observed in control and vit C treated groups.The neuroprotective effects of vitamin C is due to its antioxidant activity; vitamin C treatment reduce oxidative stress induced by chronic fluoride intoxication. Free radical caused oxidative damage of cell membrane is prevented by vitamin C treatment. Vitamin C also reduce the excitotoxicity thereby prevent cell death [29]. A decrease in LPO, an increase CAT activity in the brain on treatment with plant extract and vitamin C were the reason for their neuroprotection [30]. Antioxidants are the most effective tools to eliminate free radicals which cause oxidative stress,thusvit C prevent free radical induced neurodegeneration.

CONCLUSION

In conclusion, fluoride produces free radicals in excess amount which leads to damage anti-oxidant defense system of brain. Increased free radicals and reduced activity of anti-oxidant defense system results in neural cell death thereby progression of neurodegeneration. Natural anti-oxidant such as vitamin C showed enhanced anti-oxidant defense system of body and reduction of free radical production helpful in treatment against fluoride induced neurodegeneration. However, both doses are produced positive results, the 80 mg/kg Bw treated

rats produced better results over 40 mg/kg Bwtreated rats.

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REFERENCES

[1] C. Krishnaiah,and P.K. Reddy,"Dose-dependent effects of fluoride on neurochemical milieu in the hippocampus and neocortex of rat brain",Fluoride,vol40(2), pp. 101–110, 2007.

[2] J.A. Varner,K.F. Jensen,W. Horvath,R.L. Isaacson,"Chronic administration of aluminum-fluoride or sodium-fluoride to rats in drinking water: Alterations in neuronal and cerebrovascular integrity",Brain Res,vol 16; 784(1 -2), pp. 284-298, 1998.

[3] Blaylock RL: Excitotoxicity: a possible central mechanism in fluoride neurotoxicity. Fluoride. 2004; 37(4):301–314 (Research Review).

[4] Ge YM, Ning HM, Wang SL, Wang JD: Comet assay of DNA damage in brain cells of adult rats exposed to high fluoride and low iodine. Fluoride. 2005;38: 209-14.

[5] Bhatnagar M, Rao P, Saxena A, Bhatnagar R, Meena P, Barbar S: Biochemical changes in brain and other tissues of young adult female mice from fluoride in their drinking water. Fluoride. 2006; 39(4):280-4.

[6] Zhu WJ, Zhang J, Zhang ZG: Effects of fluoride on synaptic membrane fluidity and PSD-95 expression level in rat hippocampus. Biol Trace Elem Res. 2011; 139:197-203.

[7] Raghu J, Raghuveer CV, Mallikarjuna Rao C, Somayaji SN, Prakash B: Neuroprotective effect of Ascorbic acid and Ginkgo biloba against Fluoride caused Neurotoxicity. IOSR IOSR-JESTFT. 2014; 30-36.

[8] Banu OY, Melike E: Effects of vitamin c on sodium fluoride-induce oxidative damage in sertoli cells. Fluoride. 2015; 48(3): 241-251.

[9] Shokouhi G, Hadidchi S, Ghorbanihaghjo A, Rahbani-Noubar M, Panahi S, Forouzanfar M, Rashtchizadeh N, Mesgariet M: Neuroprotective Effect of Ascorbic Acid in Experimental Blunt Sciatic Nerve injury in Rats. The Int J of Nutr and Wellness. 2005; 1(2).

[10] Stamford JA, Isaac D, Hicks CA, Ward MA, Osborne DJ, O'Neill MJ: Ascorbic acid is neuroprotective against global ischaemia in striatum but not hippocampus: histological and voltammetric data, Brain Research. 1999; 835(2):229-240.

[11] Concepcion SM, Manuel P, Antonio M, Antonio M: Protective effect of vitamin C against the ethanol mediated toxic effects on human brain glial cells. Journal of Nutritional Biochemistry. 2003; 14: 606–613.

[12] Upasani CD, Khera A and Balaraman R: Effect of lead with Vitamins E, C, or Spirulina on malondialdehyde: conjugated dienes and hydroperoxides in rats. Indian Journal of Experimental Biology. 2001; 39(1): 70–74.

[13] Eduardo CJ, Ravi SA, Harsharan SB, Klaus L, Kurt A, Eduardo M, Michael H, Bernd LF: Ascorbic acid enhances the inhibitory effect of aspirin on neuronal cyclooxygenase-2-mediated prostaglandin E2 production. Journal of Neuroimmunology. 2006; 174: 39 – 51.

[14] Engelhart MJ, Geerlings MI, Ruitenberg A, van Swieten JC, Hofman A, Witteman JC, Breteler MM. Dietary intake of antioxidants and risk of Alzheimer disease. JAMA. 2002; 287: 3223–9.

[15] Pratap RS, Shashwat S, Suman K:Free radicals and oxidative stress in neurodegenerative diseases: relevance of dietary antioxidants. JIACM. 2004; 5(3): 218-25.

[16] Murkland S. and Murkland G: Involvement of the superoxide anion radical in the autoxidation of pyragallol and a convenient assay for superoxide dismutase. Eur. J. Biochem. 1974; 47: 469-474.

[17] Garcia YJ, Rodríguez AJ,Nancy PM: Lipid peroxidation measurement by thiobarbituric acid assay in rat cerebellar slices. Journal of Neuroscience Methods. 2005; 144 (1): 127-135.

[18] Brannan TS, Maker HS, Raes IP: Regional distribution of catalase in the adult rat brain. Journal of Neurochemistry. 1981; 36(1): 307-309.

[19] Hissin PJ, Hilf R: A fluorometric method for determination of oxidized and reduced glutathione in tissues.Anal Biochem. 1976; 74(1): 214-26.

[20] Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG: Science 1973; 179:588-590.

[21] Lillie RD, Fullmer HM: Histopathologic technic and practical histochemistry. New York: McGraw-Hill. (1976).

[22] Elghetany MT, Saleem A, Barr K. The congo red stain revisited. Ann Clin Lab Sci. 1989; 19(3): 190-195.

[23] Anand P, Nehru B: Alterations in glutathione system in adult and pup rat brains following chronic aluminum exposure. Indian J Occup Environ Med. 2006; 10:128–32.

[24] Shanthakumari D, Srinivasalu S, Subramanian S: Effect of fluoride intoxication on lipid peroxidation and antioxidant status in experimental rats. Toxicology. 2004; 204:219–28.

[25] Trivedi MH, Verma RJ, Sangai NP, Chinoy NJ: Black tea extract mitigation of NaF-induced lipid peroxidation in different regions of mice brain. Fluoride. 2011; 44(4) 243–254.

[26] Parihar S, Choudhary A, Gaur S: Toxicity of fluoride in liver of Albino rats and mitigation after adopting artificial (vitamin C and D) and natural (Aloe vera) food supplementations. Int. J. Adv. In Res. and Tec.2013; 2:1-10.

[27] Gouri NP, Otilia J, Banji F, Banji D, Ragini M, Pavani B: Fluoride toxicity- a harsh reality. IRJP. 2011; 2 (4): 79-85.

[28] Bhatnagar M, Pratima Rao and Sushma J: Neurotoxicity of fluoride: Neurodegeneration in hippocampus of female mice. Indian Journal of Experimental Biology. 2002; 546-554.

[29] Peng Y, Kwok KH, Yang PH, Ng SS, Liu J, Wong OG, He ML, Kung HF, Lin MC: Ascorbic acid inhibits ROS production, NF-kB activation and prevents ethanol-induced growth retardation and microencephaly, Neuropharmacology. 2005; 48:426-434.

[30] Santos LF, Freitas RL, Xavier SM, Saldanha GB, Freitas RM: Neuroprotective actions of vitamin C related to decreased lipid peroxidation and increased catalase activity in adult rats after pilocarpine-induced seizures, Pharmacology Biochemistry and Behavior. 2008; 89(1): 1-5.

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