L-ascorbic Acid Supplementation Ameliorates Sodium Fluoride Induced Oxidative and Nitrosative Hepatic Damage in Hypoxic Rats

Jaya Simha Reddy[1], R. Chandramouli Reddy[2], Raju H. Taklikar[3], Kusal K. Das[2]

[1]PhD Scholar, [2]Junior Research Scientist, [2]Professor, Laboratory of Vascular Physiology and Medicine, Department of Physiology, Shri B.M. Patil Medical college,Hospital & Research Centre, BLDE (Deemed to be University), Vijayapur-586103, Karnataka, India, [3]Department of Physiology, Navodaya Medical College, Hospital & Research Centre, Raichur-584103, Karnataka, India

Abstract

Introduction: Fluoride toxicity causes irreversible damage to soft tissues like brain, liver, heart and lung**.** Fluoridetoxicity and hypoxiaexposure might alterhepatic oxygen sensing cell signaling mechanisms and a supplementation of antioxidant like l-ascorbic acid might have protective role.

Aim: To investigate the exposure of sodium fluoride or hypoxia alone or in combination with or without administration of L- ascorbic acid on biochemical and transcriptional pathways in hepato toxicity **.**

Materials and Method: Male albino rats were divided into 8 groups (n= 6/group), group I(control), group II (l-ascorbic acid, 50 mg/100g. b.wt), group III (hypoxia, 10% O_2), group IV (NaF;20 mg/kg b.wt/day; ip), group V (NaF + hypoxia, 10% O_2), group VI (l–ascorbic acid + hypoxia, 10% O_2), group VII (l-ascorbic acid + NaF) and group VIII (l-ascorbic acid + NaF + hypoxia, 10% O_2). The treatments were carried for 21 days. Gravimetry, serumoxidant and antioxidant status were assessed by using spectrophotometer, serum levels of vascular endothelial growth factor (VEGF) and nitric oxide synthase 3(NOS3) was done by ELISA technique. Histopathological evaluations of hepatic tissue were done. ANOVA followed by "Tukey" test were done for analysis of data in between the groups.

Results: Gravimetry and biochemical evaluation showed significant decrease in body weight hepatosomatic index, altered serum SOD, MDA, vitamin C, vitamin E, nitric oxide and hepatic vitamin Cin rats treated with hypoxia (group III), NaF (group IV) and NaF with hypoxia (group V). In case of l-ascorbic acid supplementation in group VI, VII and VIII showed remarkable improvement. Alteration in serum VEGF, NOS3 andhistopathology of liver in rats treated with hypoxia, NaFand hypoxia with NaFindicate hepatic dysfunction by oxidative and nitrosative stress, whereas l-ascorbic acid supplementation were found to be beneficial against fluoride and hypoxia induced alteration of hepatic function.

Conclusion: Supplementation of L-ascorbic acid is salubrious to combat both sodium fluoride and hypoxia induced oxidative & Nitrosative stress.

Keywords: *L –ascorbic acid, sodium fluoride, hypoxia,oxidative stress, Nitrosative stress.*

Corresponding Author:
Dr . Kusal K. Das
Professor, Laboratory of Vascular Physiology and Medicine, Department of Physiology, Shri B. M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Bangaramma Sajjan Campus, B.M. Patil Road, Vijayapura-586103, Karnataka, India
e-mail: kusaldas@gmail.com
Telephone: +91-9513341100

Introduction

Fluoride toxicity causes irreversible damage to soft tissues like brain, liver, heart and lung[1]. Fluoride

can cross cell membranes by simple diffusion and enter soft tissues. Fluoride induces necrosis and apoptosis[2]. Fluoridetoxicity and hypoxiaexposure alterhepatic oxygen sensing cell signaling transduction pathways. Weather fluoride induced toxicity and low oxygen microenvironment act through similar or different mechanisms is not known. Antioxidant supplementation is useful in fluorosis management and fluoride intoxication[3]. Hence the present study was schemed toassess therole of l-ascorbic acid as antioxidant on NaF and hypoxia or in combinationonhepato cellular oxidative stress in male albino rats.

Materials and Method

Healthy adult male albino Wistar rats weighing about 150 -180 gramswere procured from animal house. Acclimatized for one week to the laboratory conditions at 21-25ºC and fed with laboratory stock diet and water *ad libitum*..

Experimental Groups: Male albino rats were divided into 8 groups (n= 6 in each group), group I(control), group II (l-ascorbic acid,50 mg/100g. b.wt), group III (hypoxia, 10%O_2), group IV (NaF; 20 mg/ kg b .wt/day; ip), group V (NaF + Hypoxia, 10% O_2), group VI (l-ascorbic acid + hypoxia, 10% O_2), group VII (l-ascorbic acid + NaF) and group VIII(l-ascorbic acid + NaF + hypoxia, 10% O_2). The interventions were carried for 21 days.

NaF was injected intra peritoneally at a dose of 20mg/ kg body weight/day for 21 days [4]. L-ascorbic acid was administered orally by using force feeding needle with syringe for 21 days (50mg/100g body weight).

Exposure of Rats to hypoxia: Acrylic chamber was used to induce hypoxia. Rats in cage were kept in chamber and given mixture of 10% oxygen and 90% nitrogento inducechronic normobaric hypoxia for 21 days. Temperature was maintained at 22-27°C [4].

Gravimetry: Animals of all groups were weighed on the starting day of protocol and on the 21st day i.e. on the day of sacrifice using digital weighing balance. Percentage of body weight gain was determined. Liver was weighed after sacrifices at the end of experiment and further hepato somatic index was determined by using the following formula:

Hepato somatic index = Weight of heart × 100/Body weight

Sample Collection: At the end of 21st day all animals were kept for an overnight fast and blood was collected in plain tubes by doing retro-orbital puncture. These blood samples were centrifuged and serum was separated.

Oxidant and antioxidant parameters: Serumsuperoxide dismutase (SOD), malondialdehyde (MDA), nitric oxide was determined. Serum nitric oxide synthase 3 (NOS3) and vascular endothelial growth factor(VEGF) was estimated by ELISA kit. Serum vitamin C,vitamin E and hepatic vitamin C levels was estimated.

Histopathology Procedure: Animals of all groups were sacrificed by cervical dislocationat the end of 21 days experimental protocol. The liver was carefully collected, isolated immediately and fixed in freshly prepared 10 % formal in for 24 hours and thin sections were taken. Histopathological evaluations were done to identify changes in liver for treatment groups and compared with control. CPCSEA guidelines were carefully followed during experiments.

Statistical Analysis: SPSS software version 16.0 was used. One-way ANOVA followed by "Tukey" test were done to find out intergroup significant differences. All values were represented asmean ± SD. $p \leq 0.05$ considered as statistically significant.

Results

Table-1 shows significant decrease in % of body weight gain, liver weight and hepatosomatic index of rats exposed to hypoxia (group III), NaF (groupIV), hypoxia and NaF (group V) as compared to control (group I) at the end of 21st day. However simultaneous treatment with l-ascorbic acid showed greater % of body weight gain, liver weight and hepatosomatic index in group VI (l-ascorbic acid + hypoxia), group VII (l-ascorbic acid + NaF) and group VIII (l-ascorbic acid + NaF + hypoxia).

Table-1

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Group VIII	P Value
Initial body weight (gm) (1st day)	158.9 ±5.31[a]	155.33 ±6.6[a]	155.53 ±1.75[a]	155.33 ±6.11[a]	155.93 ±2.6[a]	154.47 ±4.01[a]	156.53 ±4.6[a]	155.67 ±5.4[a]	0.9693
Final body weight (gm) (21st day)	208.33 ±3.1[a]	214.33 ±3.7[a]	180.67 ±7.5[b]	169.67 ±8.6[c]	163.03 ±3.2[c]	195.33 ±5.5[d]	193 ±2.6[d]	186.67 ±2.8[e]	<0.0001*
Percent of body weight gain	31.17 ±3.2[a]	38.11 ±4.9[a]	16.14 ±3.6[b]	9.19 ±1.3[c]	4.55 ±1.6[d]	25.93 ±1.4[e]	23.33 ±2.1[e]	20.62 ±2.4[e]	<0.0001*
Liver weight (gm)	7.66 ±0.14[a]	8.08 ±0.22[a]	5.30 ±0.2[b]	4.54 ±0.32[c]	3.46 ±0.41[c]	6.37 ±0.18[d]	6.01 ±0.1[d]	6.27 ±0.28[d]	<0.0001*
Hepato-somatic index	3.67 ±0.03[a]	3.77 ±0.08[a]	2.94 ±0.22[b]	2.68 ±0.2[b]	2.12 ±0.27[b]	3.27 ±0.18[a]	3.12 ±0.04[a]	3.36 ±0.19[a]	<0.0001*

[Table-1]: Effect of l- ascorbic acid supplementation on sodium fluoride and hypoxia induced changes in gravimetry. Group I(control), group II (l-ascorbic acid), group III(hypoxia), group IV (NaF), group V (hypoxia + NaF), group VI (l-ascorbic acid +hypoxia), group VII (l- ascorbic acid +NaF) and group VIII (l-ascorbic acid +hypoxia + NaF), values expressed as Mean ± SD, $p \leq 0.05$ is significant, values with different superscripts a,b, c,d,e are significantly different from each other

Table-2 shows significant increase in serum MDA and serum nitrite concentration inrats exposed to hypoxia(group III), NaF (groupIV), hypoxia and NaF (group V) as compared to respective controls. However simultaneous supplementation with l-ascorbic acid in group VI (l-ascorbic acid +hypoxia), group VII (l-ascorbic acid + NaF) and group VIII (l-ascorbic acid + NaF + hypoxia) rats showed significant improvements of serum MDA and serum nitrite.

Table-2

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Group VIII	P Value
Serum MDA	5.51 ±2.15[a]	7.39 ±2.15[a]	21.73 ±7.18[b]	23.37 ±4.81[b]	27.01 ±5.1[c]	12.26 ±3.02[d]	15.44 ±5.09[e]	16.36 ±2.03[e]	<0.0001*
Serum nitrite	35.07 ±5.05[a]	36.27 ±10.68[a]	86.14 ±8.74[b]	92.61 ±3.28[b]	111.2 ±3.46[c]	72.35 ±4.33[d]	59.77 ±5.98[e]	49.77 ±2.91[f]	<0.0001*

[Table-2]: Effect of l- ascorbic acid supplementation on sodium fluoride and hypoxia induced changes in oxidative status. Group I(control), group II(l-ascorbic acid), group III(hypoxia), group IV(NaF), group V (hypoxia + NaF), group VI (l-ascorbic acid +hypoxia), group VII (l- ascorbic acid +NaF) and group VIII (l-ascorbic acid +hypoxia + NaF), values expressed as Mean ± SD, $p \leq 0.05$ is significant, values with different superscripts a,b, c,d,e are significantly different from each other

Table-3 shows significant decrease inserum vitamin C,hepatic vitamin C and serum vitamin E concentration inrats exposed to hypoxia(group III), NaF (group IV), hypoxia and NaF (group V) as compared to respective controls. However simultaneous supplementation with l-ascorbic acid in group VI (l-ascorbic acid +hypoxia), group VII (l-ascorbic acid + NaF) and group VIII (l-ascorbic acid + NaF + hypoxia) rats showed significant improvements of serum vitamin C, hepatic vitamin C and serum vitamin E concentration.

Table-3

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Group VIII	P Value
Serum SOD units/ml	15.03 ±0.98[a]	16.38 ±0.68[a]	7.17 ±0.99[b]	5.24 ±0.98[c]	2.59 ±0.61[d]	11.12 ±1.62[e]	8.29 ±1.02[b]	11.68 ±1.82[e]	<0.0001*
Serum vitamin C mg/dl	3.64 ±0.67[a]	5.15 ±0.2[b]	3.19 ±0.17[a]	2.81 ±0.16[c]	1.91 ±0.15[d]	3.22 ±0.27[a]	3.21 ±0.19[a]	3.47 ±0.58[a]	<0.0001*
Hepatic vitamin C µg/g	204.27 ±6.07[a]	230.93 ±9.16[b]	145.67 ±6.17[c]	115.81 ±15.92[d]	64.97 ±16.2[e]	153.71 ±10.46[c]	169.27 ±5.24[f]	183.37 ±6.38[g]	<0.0001*
Serum vitamin E mg/dl	4.48 ±0.5[a]	4.61 ±0.43[a]	3.52 ±0.25[b]	2.13 ±0.75[c]	1.06 ±0.25[d]	3.98 ±0.04[b]	3.82 ±0.11[b]	3.62 ±0.58[b]	<0.0001*

[Table-3]: Effect of l- ascorbic acid supplementation on sodium fluoride and hypoxia induced changes in antioxidant status.Group I(control), group II(l-ascorbic acid), group III(hypoxia), group IV(NaF), group V (hypoxia + NaF), groupVI (l-ascorbic acid +hypoxia), groupVII(l- ascorbic acid +NaF) and group VIII (l-ascorbic acid +hypoxia + NaF), values expressed as Mean ± SD, $p \leq 0.05$ is significant, values with different superscripts a,b, c,d,e are significantly different from each other

Fig-1shows significant increase in serum VEGF concentration inrats exposed to hypoxia (group III), NaF (groupIV), hypoxia and NaF (group V) as compared to respective controls. However simultaneous supplementation with l-ascorbic acid in group VI (l-ascorbic acid +hypoxia), group VII (l-ascorbic acid + NaF) and group VIII (l-ascorbic acid + NaF + hypoxia) rats showed significant improvements in serum VEGF concentration.

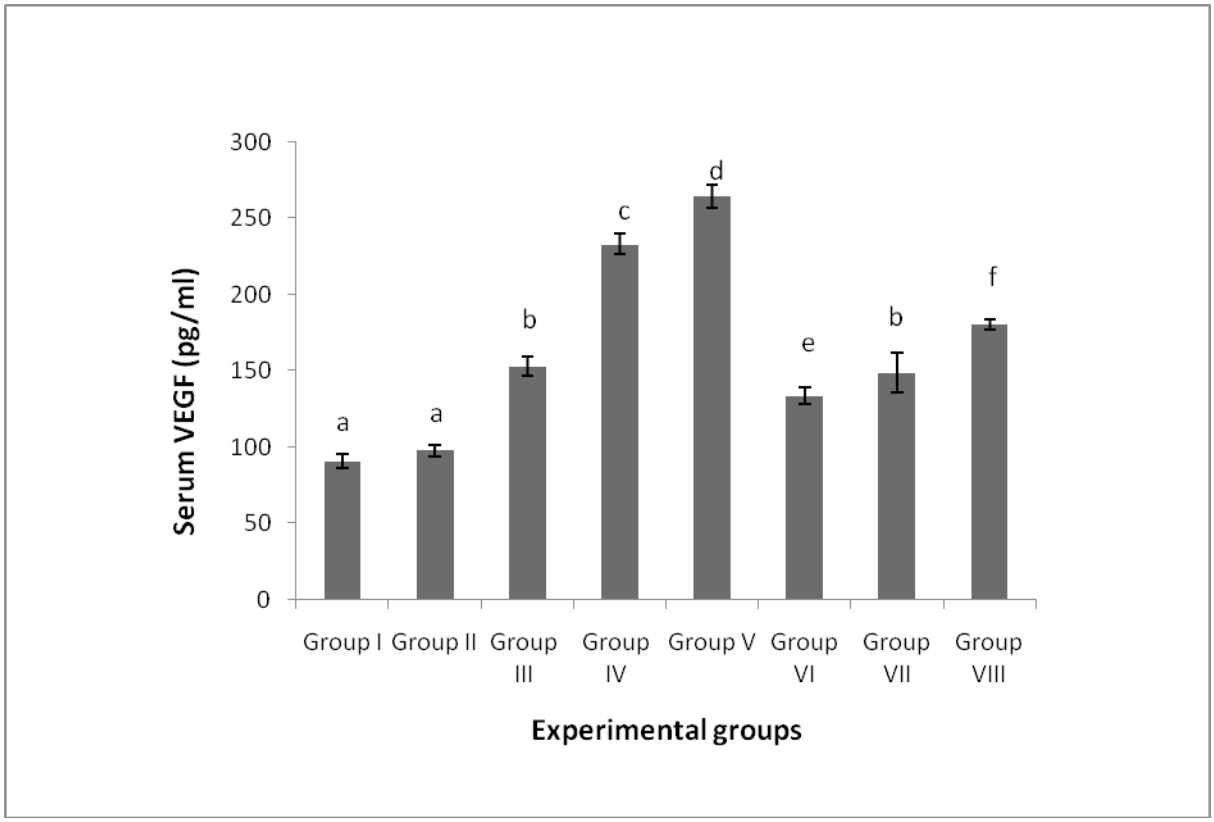

Fig. 1

[FIG-1]: Effect of l- ascorbic acid supplementation on sodium fluoride and hypoxia induced changes on serum VEGF level. Group I(control), group II(l-ascorbic acid), group III (hypoxia), group IV (NaF), group V (hypoxia + NaF), group VI (l-ascorbic acid + hypoxia), groupVII(l- ascorbic acid +NaF) and group VIII (l-ascorbic acid + hypoxia + NaF), values expressed as Mean ± SD, $p \leq 0.05$ is significant, values with different superscripts a,b, c,d,e,f are significantly different from each other

Fig-2 shows significant increase in serum NOS3 concentrations inrats exposed to hypoxia (group III), NaF (group IV), hypoxia and NaF (group V) as compared to respective controls. However simultaneous supplementation with l-ascorbic acid in group VI (l-ascorbic acid + hypoxia), group VII (l-ascorbic acid + NaF) and group VIII (l-ascorbic acid + NaF + hypoxia) rats showed significant improvements in serum NOS3 concentrations.

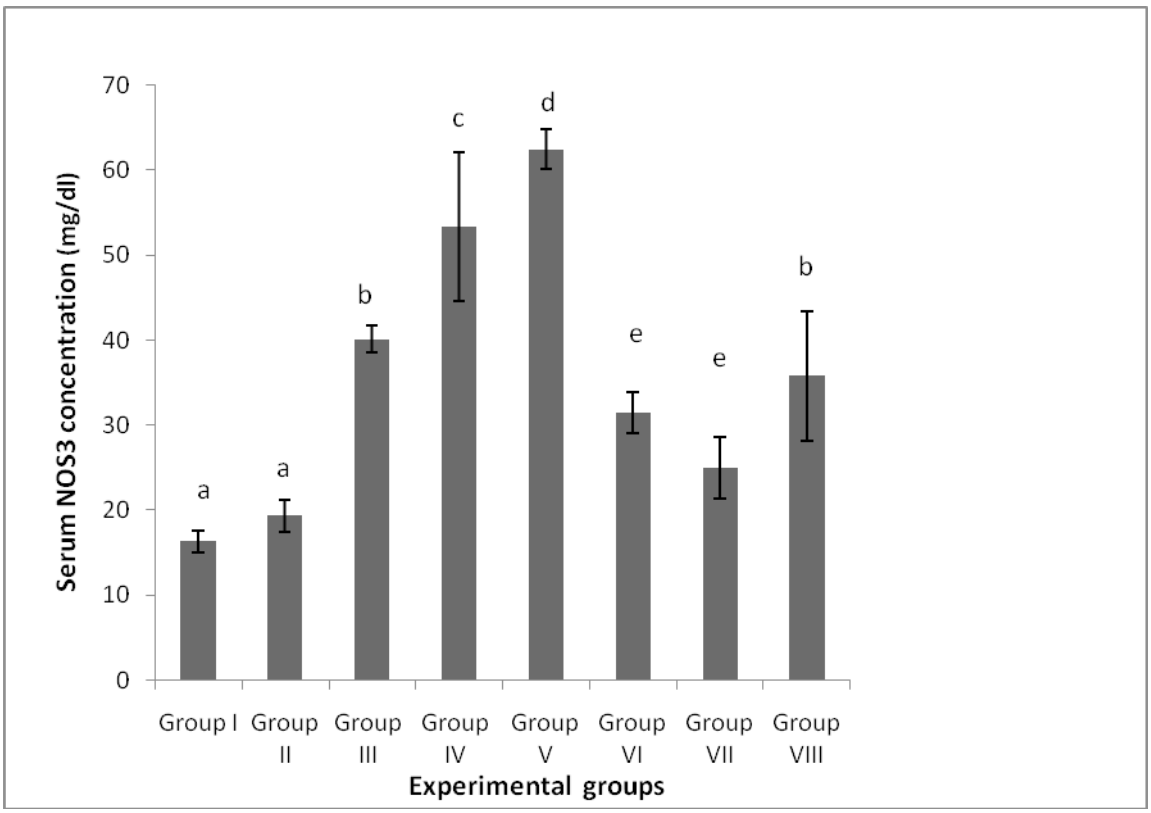

Fig. 2

[FIG-2]: Effect of l- ascorbic acid supplementation on sodium fluoride and hypoxia induced changes on serum NOS3 level. Group I(control), group II(l-ascorbic acid), group III(hypoxia), group IV(NaF), group V (hypoxia + NaF), groupVI (l-ascorbic acid +hypoxia), groupVII(l- ascorbic acid +NaF) and group VIII (l-ascorbic acid +hypoxia + NaF), values expressed as Mean ± SD, $p \leq 0.05$ is significant, values with different superscripts a,b, c,d,e,f are significantly different from each other

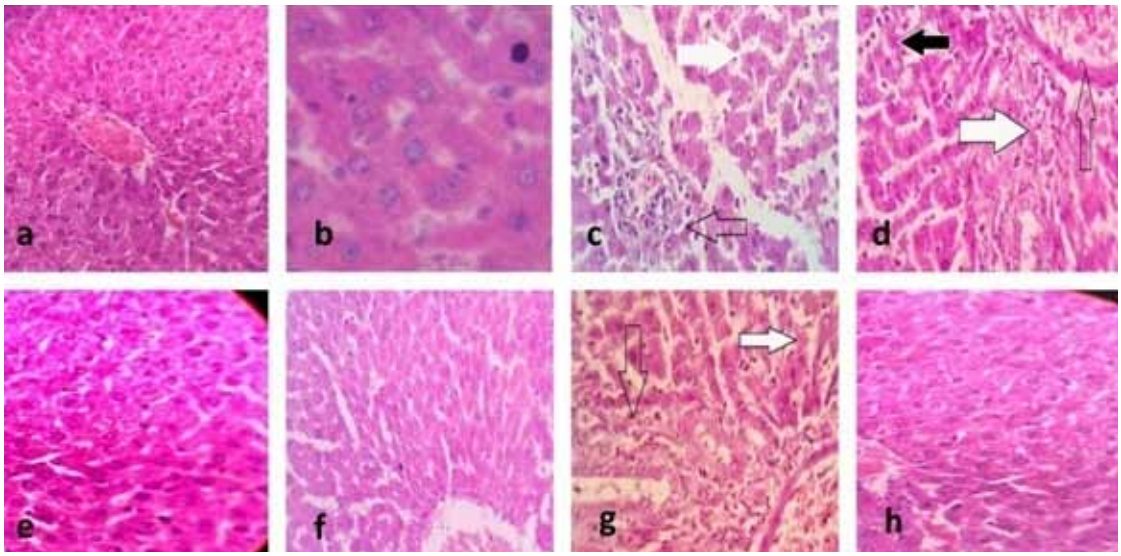

Fig. 3

Fig-3 (a-h) shows H & E stained sections of liver. Group I (control) and group II (l-ascorbic acid) showed normal architecture of liver (Fig- 3a & b). Group III (hypoxia) showed inflammation and sinusoidal congestion (Fig-3c), in group IV (NaF) showed focal necrosis, mononuclear infiltrate and central vein congestion (Fig-3d) and group V (hypoxia +NaF) also showed focal necrosis, mononuclear infiltrate and central vein congestion (Fig- 3e) whereas in case of l- ascorbic acid supplementation hepatic architecture appeared normal in group VI (l-ascorbic acid +hypoxia), group VII (l-ascorbic acid + NaF) and group VIII (l-ascorbic acid + NaF + hypoxia) rats (Fig-3f-h).

Discussion

Protective role of l-ascorbic acid on oxidative and nitrosativestress in hepatocytes were studied in hypoxia, sodium fluoride and in combination of hypoxia and sodium fluoride in experimental rats through evaluation of gravimetry, serum oxidant and antioxidant status along with histopathology of hepatictissues.

Gravimetry: Our study results shows decrease in body weight,change in % of body weight gain and hepatosomatic index in hypoxic and NaF administered rats or in combination of both. This decrease in body wt (%) and hepatosomatic index may be due to less food intake . NaF may cause inhibition of hunger centre in hypothalamus and fluoride also induces breakdown of tissue proteinsandlipids[5]. Supplementation with l-ascorbic acid showed % of body weight gain, liver weight gain and increased hepato somatic index due to protective actions of l-ascorbic acid in hypoxia and NaF induced alteration of tissue protein breakdown [6].

Oxidant and antioxidant status: In our study sodium fluoride induced and/or hypoxia exposed rats show increase in serum MDA and serum nitrite concentration due to oxidative stress. Oxidative stress is characterised by increase in reactive oxygen species (ROS) formation .ROS include free radicals like super oxide, peroxy nitrite and hydroxyl radicals. MDA is an indicator of lipid peroxidation and an important biomarker of oxidative stress[7]. Increase in nitrite concentration insodium fluoride induced and hypoxic rats may be due to over expression of NOS3 gene and increases production of nitric oxide(NO)[8,9]. our study results on supplementation of L-ascorbic acid on MDA and NO levels are indicative of preventing oxidative and nitrosative damage.

Our study also shows decrease in serum SOD, serum and hepatic vitamin C,serum vitamin E concentrations in sodium fluoride induced and/or hypoxia exposed rats. Sodium fluoride not only generates superoxide ions but also decreases enzymatic activity of SOD by binding to its active site .As a result SOD fails to detoxify hydroxyl ions. So inhibition of antioxidant enzyme SOD leads to oxidative damage at mitochondrial level and causes hepato cellular dysfunction[10]. Vitamin C combats superoxide in liver and also scavenges NO. Vitamin C also regenerates tetrahydrobiopterin (BH4). BH4 is important for NOS3 activity.Vitamin E with

lipid peroxyl molecule is oxidized to α -tocopheroxyl molecule. α-tocopheroxyl is recycled by L- ascorbic acid. Antioxidants like vitamin C and E protect against ROS damage[11,12]. On supplementation with L-ascorbic acid there was significant improvements in serum SOD, serum and hepatic vitamin C, serum vitamin E concentrations.

Serum NOS3 and VEGF concentrations: Our study results show that in sodium fluoride induced and/ or hypoxia exposed rats leads to increased serum VEGF and also NOS3 levels. Interestingly sodium fluoride toxicity mimics hypoxia like effects at hepatocellular level and increases ROS production. ROS induces directly NOS3 and VEGF gene expression. VEGF triggers hypoxic response and leads to upregulation of NOS3genes and enhances NO production .Increased NO levels itself down regulate VEGF expression.

Vitamin C supplementation has lead to decreased gene expression of NOS3 and VEGF in sodium fluoride induced and/or hypoxia exposed rats. This is due to decreased ROS production and NO levels to combat cellular hypoxia.

Histopathology of Hepatic tissues: Histological changes were observed inhypoxia exposed or NaF treated or in combination of hypoxia exposure and NaF treated rats. Sodium fluoride induced toxicity lead to disruption of hepatic architecture and lead to mononuclear infiltration and hepatic necrosis[1415]. In hypoxia exposure and NaF treated rats such changes have lead to hepato cellular damage.

Vitamin C supplementation prevented disruption of hepatic architecture and necrosis.

Conclusion

Both sodium fluoride and hypoxia enhances nitric oxide production by VEGF and NOS3 pathways leading to hepatocellular damage and apoptosis. The supplementation of L-ascorbic acid is salubrious to combat both sodium fluoride and hypoxia induced cell death in liver by oxidative & Nitrosative stress.

Conflict of Interest: None declared.

Source of Funding: Self

Ethical Clearance: The entire protocol for experiment was approved by Institutional Animal Ethical Committee bearing approval no (LCP/PG.Col/ IAEC/Oct-2015/66).

References

1. Raghu Jetti, Raghuveer, Mallikarjuna Rao, Somayaji, Prakash Babu. Neuroprotective effect of Ascorbic acid and Ginkgo biloba against Fluoride caused Neurotoxicity. Toxicol Food Technol 2001; 8:30-36.
2. Zhnag Z, Zhou B, Wang H, Wang F, Song Y, Liu S, et al.Maize purple plant pigment protects against fluoride-induced oxidative damage of liver and kidney in rats. Int. J. Environ. Res. Public Health 2014;11:1020-1033.
3. Raghu Jetti, vasudeva C, Raghuveer, Mallikarjuna Rao, Somayaji, Prakash Babu. The ameliorative effect of ascorbic acid and ginkgo biloba on learning and memory deficits associated with fluoride exposure. Interdiscip Toxicol 2013;6(4): 217–221.
4. Kusal K. Das, Jameel G. Jargar, Sikha Saha, Saeed M. Yendigeri and Shashi Bala Singh. Alpha-tocopherol supplementation prevents lead acetate and hypoxia-induced hepatic dysfunction. Indian JPharmacol 2015; 47(3):285–291.
5. Bouasla A, Bouasla I, Boumendjel A, Feki AE, Messarah M. Hepatoprotective role of gallic acid on sodium fluoride-induced liver injury in rats. Int. J. Pharm. Sci. Rev. Res 2014;29(2):14-18.
6. Shyamal Kanti Das, Chandan Roy, Sahina Khatun. The protective role of VIT- C(ascorbic acid) against Sodium fluoride (NaF) toxicity on reproductive system of adult male albino rat. WJPR2017;6(14):965-976.
7. Vasant RA, Narasimhacharya AVRL. Ameliorative effect of tamarind leaf on fluoride induced metabolic alterations. Environ Health Prev Med 2012; 17:484–493.
8. Hassan HA, Yousef MI. Mitigating effects of antioxidant properties of black berry juice on sodium fluoride induced hepatotoxicity and oxidative stress in rats.Food Chem Toxicol. 2009;47(9):2332-2337.
9. T. Nakamura and S A Lipton. Molecular mechanisms of nitrosative stress-mediated protein misfolding in neurodegenerative disease. Cell. Mol. Life. Sci 2007;64(13):1609-1620.
10. Djurasevic SF, Djordjevic J, Drenca T, Jasnic N and cvijic G. Influence of vitamin Csupplementation on the oxidative status of rat liver.Arch. Biol. Sci, 2008; 60(2); 169-173.

11. May JM, Harrison FE.Role of vitamin C in the function of the vascular endothelium. Antioxid Redox Signal. 2013 Dec 10; 19(17): 2068–2083.
12. Paul M. Vanhoutte. Nitric oxide: From good to bad . Ann Vasc Dis. 2018 Mar 25;11(1):41-51.
13. M Sireli, A Bulbul . The effect of acute fluoride poisioning on nitiric oxide and methemoglobin formation in the guinea pig, Turk j Vet Anim Sci. 2004;28:591-595
14. Krishnamoorthy P, Gowda NKS, Vallesha NC, Rajendran D, Maya, G. & Verma, S, et al. Effect of subchronic fluoride exposure on immune status and histopathology in rats and its amelioration. Research report Fluoride. 2015; 48:123-130.
15. Parihar S, Choudhary A, Gaur S. Toxicity of fluoride in liver of albino rat and mitigation after adopting artificial (vitamin C and D) and natural (Aloe vera) food supplementations. IJOART. 2013;2(2):1–11.
16. Samir D, Zine K. Preventive effect of zinc on nickel induced oxidative liver injury in rats. Afr.j. Biotechnol 2013;12(51):7112-7119.
17. Lee SJ, Kim SY, Min H. Effects of vitamin C and E supplementation on oxidative stress and liver toxicity in rats fed a low-fat ethanol diet. Nutr Res Pract2013;7(2):109-114.