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

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Abstract

Introduction: Fluoride toxicity causes irreversible damage to soft tissues like brain, liver, heart and lung. Fluoride toxicity and hypoxia exposure might alter hepatic 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% O₂), group IV (NaF; 20 mg/kg b.wt/day; ip), group V (NaF + hypoxia, 10% O₂), group VI (L-ascorbic acid + hypoxia, 10% O₂), group VII (L-ascorbic acid + NaF) and group VIII (L-ascorbic acid + NaF + hypoxia, 10% O₂). The treatments were carried for 21 days. Gravimetry, serum oxidant 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, hepatic somatic index, altered serum SOD, MDA, vitamin C, vitamin E, nitric oxide and hepatic vitamin C in 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 and histopathology of liver in rats treated with hypoxia, NaF and hypoxia with NaF indicate 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.

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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.

Introduction

Fluoride toxicity causes irreversible damage to soft tissues like brain, liver, heart and lung¹. Fluoride

can cross cell membranes by simple diffusion and enter soft tissues. Fluoride induces necrosis and apoptosis². 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 intoxication³. Hence the present study was schemed to assess the role of l-ascorbic acid as antioxidant on NaF and hypoxia or in combination on hepato cellular oxidative stress in male albino rats.

Materials and Method

Healthy adult male albino Wistar rats weighing about 150 -180 grams were procured from animal house. Acclimatized for one week to the laboratory conditions at 21-25°C 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% O₂), group IV (NaF; 20 mg/kg b. wt/day; ip), group V (NaF + Hypoxia, 10% O₂), group VI (l-ascorbic acid + hypoxia, 10% O₂), group VII (l-ascorbic acid + NaF) and group VIII (l-ascorbic acid + NaF + hypoxia, 10% O₂). 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% nitrogen to induce chronic normobaric hypoxia for 21 days. Temperature was maintained at 22-27°C⁴.

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: Serum superoxide 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 dislocation at the end of 21 days experimental protocol. The liver was carefully collected, isolated immediately and fixed in freshly prepared 10 % formalin 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 as mean ± 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 (group IV), 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) (1 st 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) (21 st 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 ^c	<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 in rats 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 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

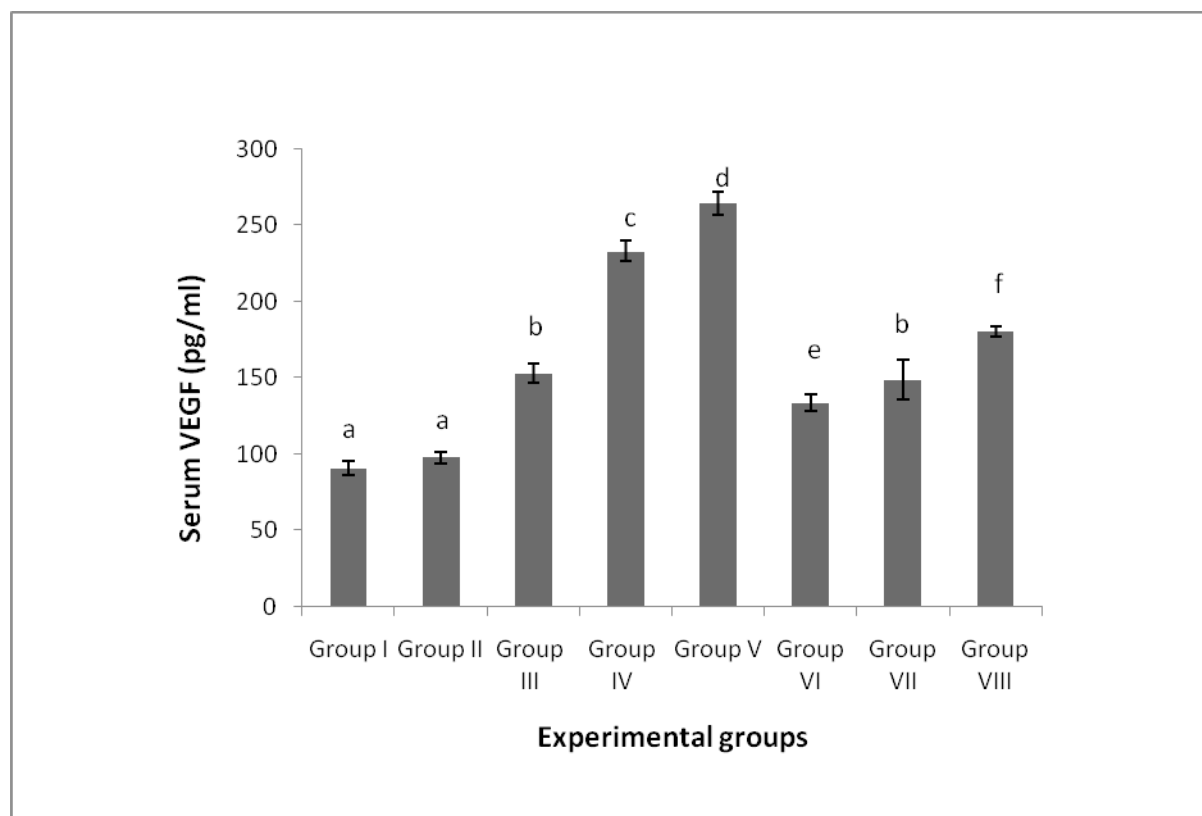
Table-3 shows significant decrease in serum vitamin C, hepatic vitamin C and serum vitamin E concentration in rats exposed to hypoxia(group III), NaF (group IV), hypoxia and NaF (group V) as compared to respective controls. However simultaneous supplementation with l-ascorbic acid in group VI (l-ascorbic 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 ^c	<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), 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

Fig-1 shows significant increase in serum VEGF concentration in rats 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 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 \pm 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.

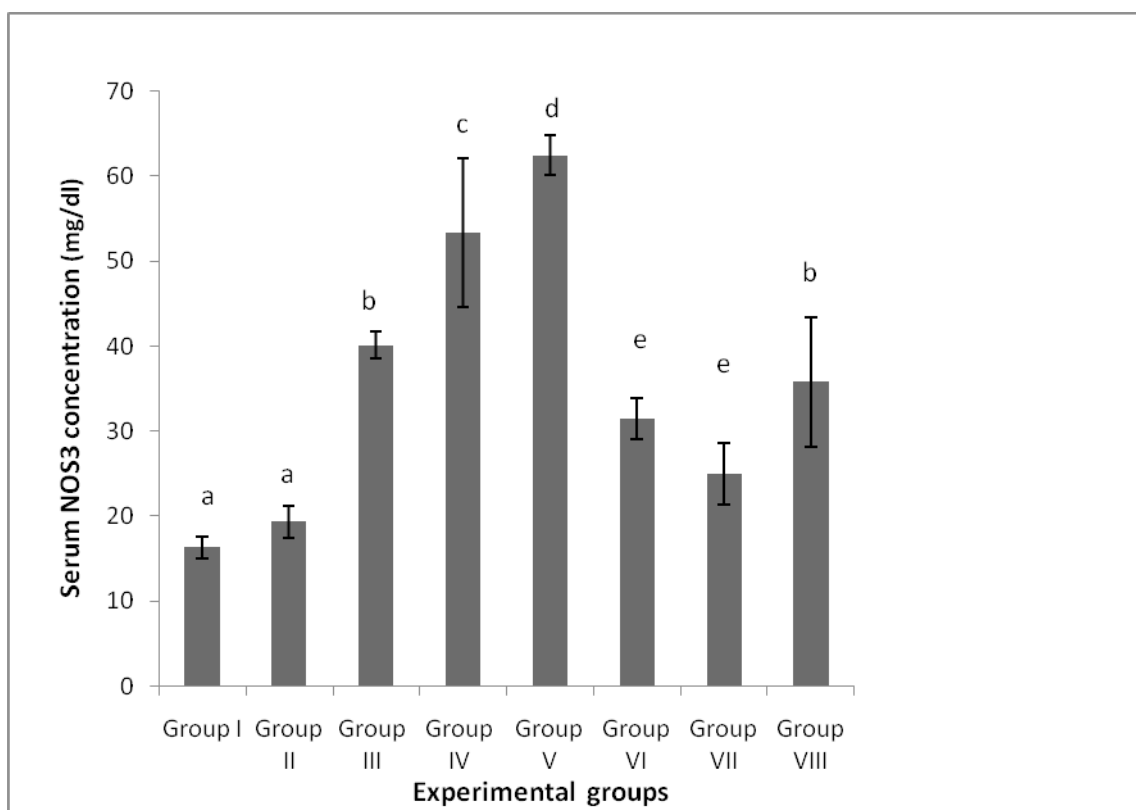


Fig. 2

[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 \pm SD, $p \leq 0.05$ is significant, values with different superscripts a,b, c,d,e,f are significantly different from each other

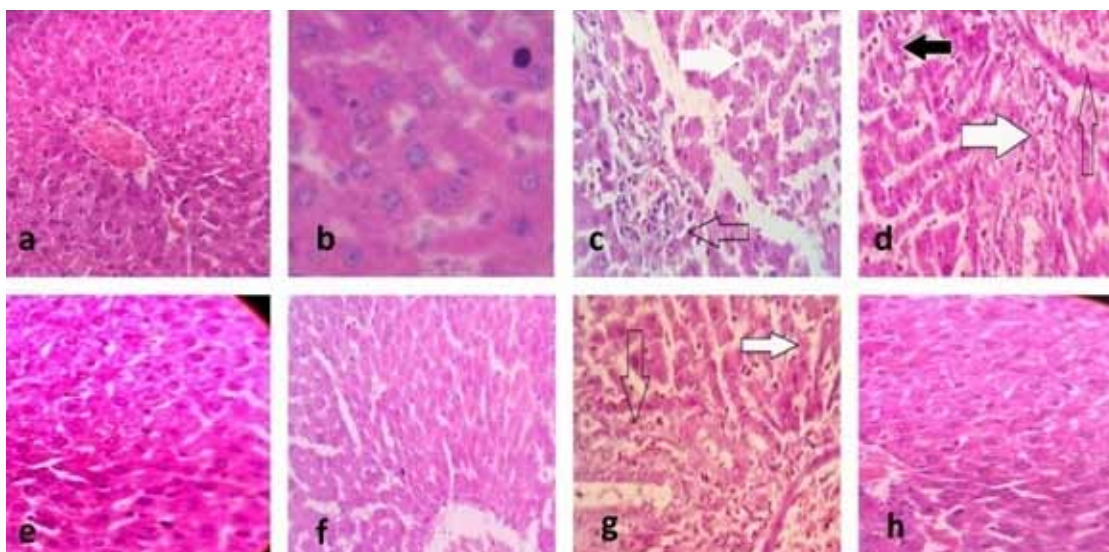
**Fig. 3**

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 nitrosative stress 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 hepatic tissues.

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 proteins and lipids⁵. 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 breakdown⁶.

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 superoxide, peroxy nitrite and hydroxyl radicals. MDA is an indicator of lipid peroxidation and an important biomarker of oxidative stress⁷. Increase in nitrite concentration in sodium 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 dysfunction¹⁰. 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 NOS3 genes 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 in hypoxia 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^{14,15}. 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).

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