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Vitamin C and E Supplementation Can Mitigate NaF Mediated Hematological and Hepatic Disorders in Adult Wistar Rats

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Abstract

The study aimed to investigate the effect of combined vitamin E and C, on hematological and hepatic systems of adult male Wistar rats with fluoride toxicity. Eighteen rats were randomly divided into three groups such as control, treated (given NaF, 20 mg/kg bw/rat/day), and supplemented (NaF and vitamin E-200 mg/kg bw + C- 400 mg/kg bw). Altered hematological parameters like erythrocyte count, leukocyte count and hemoglobin concentration were seen in NaF groups. Estimation of osmotic fragility of erythrocyte showed a marked increase fragile erythrocytes in fluoride-treated groups compared to control and supplemented groups. Total thiol concentration was also decreased due fluoride fluoride-induced oxidative stress and it was protected in supplementary groups. The oxidative imbalance induced by NaF in liver was evaluated by estimating the activity of antioxidant enzyme superoxide dismutase and catalase and with estimation of lipid peroxidation and protein oxidation product malondialdehyde and protein carbonyl. All enzymatic and non-enzymatic markers of oxidative imbalance were significantly altered (increased/decreased) due to fluoride toxicity, which was again observed to be protected in the supplemented group. The histopathological analysis of the liver also proved the mitigatory effect of the combined vitamin E+C on fluoride-induced hepatic insult.

Keywords: Fluoride, hematology, liver, oxidative stress, vitamin E+C

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Introduction

Fluoride is the 13th most copious element in the earth, scattered all over the world needed for human beings. Chemically it is very electronegative derive from fluorine gas which is one of the halogen gas. Fluoride exists in the nature in combination with other element (Jadhav et al., 2015). Ground water is one of the important sources of fluoride mainly the fluoride bearing element like amphiboles, topaz, fluorite, apatite and micas, igneous and metamorphic rocks (Edmunds & Smedley, 1996). Fluoride is released from waste material of different industries like flux, steel, glass, aluminum, nickel and copper production causes the water pollution (Li et al., 1988). Fluoride is widely used to fluoridate the water; medicine production and production of many dentifrices like toothpaste, mouth washes and also drugs etc. (Shulman & Wells, 1997). Human beings are affected by fluoride comes from these sources. WHO decided the permissible level of fluoride upto 1.5 ppm. Solubility of fluoride depends on calcium content in ground water. Fluoride play biphasic role in human body depending on its consumption amount. In the bone it is act as an anabolic agent and causes the proliferation of osteoblastic cell (bone forming cell) (Pak et al., 1995). In teeth fluoride causes the induction of remineralization process and fluoride turn into converted fluoroapatite which has the higher dissolution rate by acid (Aoba & Fejerskov, 2002). Above 1.5 ppm fluoride causes the fluorosis of hard and soft tissues like dental fluorosis, skeletal fluorosis and non-skeletal fluorosis respectively like Cardiotoxicity, neurotoxicity, hepatotoxicity and

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hematotoxicity, renal toxicity, spleen toxicity reproductive toxicity (Pal et al., 2022). Due to worldwide distribution fluoride causes fluorosis of such country like Mexico, Iran, Bulgaria, USA, Kenya, China, Ethiopia, Argentina, Korea and Africa (D. Sharma et al., 2017). India is moderately affected by fluoride and 17th states are affected by fluorosis like dental, skeletal and non skeletal fluorosis. 6 million children of this are severely affected by fluorosis among the affected 62 million people (Ali et al., 2016). Shortt et al in 1937 first reported the fluorosis in India, prakasam district of Andhra Pradesh. Rajasthan, Gujarat and Andhra Pradesh are roughly affected by fluoride whereas West Bengal is moderately affected fluoride (P. Sharma et al., 2018). Fluoride level is very high in Birbhum district of West Bengal, about 20.40 ppm (Batabyal & Gupta, 2017). From ground water; fluoridated tablet, water, drug, cosmetics; processed food with fluoride human beings are affected. Its metabolism involved three aspect 1st is the gastric absorption from stomach (20-25%) via pH dependent manner and in proximal intestine (70-75%) via simple and facilitated diffusion; 2nd is the is the distribution of absorbed fluoride from plasma into the calcified tissue bone and teeth and less amount to soft tissue and 3rd is the urinary excretion which also depends on urinary pH (Barbier et al., 2010).

Liver is responsible for maintain normal body metabolic homeostasis by metabolism and biotransformation of xenobiotics compound. So therefore liver is very susceptible to affected by fluoride followed by kidney (Thangapandiyan S, 2013). Excess ingestion of fluoride causes the alteration of structural and hepatic functional parameter

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manifestation like degenerations and inflammation of hepatocytes. Fluoride causes histopathological changes of liver such as dilation of sinusoids, cellular hyperplasia etc. (Shashi & Thapar, 2001). Fluoride causes oxidative stress by inducing the generation of excess free radicals which decreases the antioxidant enzyme parameter such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) etc.; increases the amount of lipid peroxidation product such as malondialdehyde (MDA) and reactive ketone and aldehyde due to carbonylation of protein (Guo et al., 2003). Various studies revealed that high concentration of fluoride is also responsible for alteration of hematological parameter in rats (Agha et al., 2012), rabbits (Atmaca et al., 2014), and humans (Ersoy et al., 2010). Ultimately it is known that fluoride shows its unfavorable effect due high amount of concentration. The main objective of this study is to find out the protection against fluoride induced toxicity. Though various study proved that vitamin C and vitamin E can defense against oxidative stress induced damage of liver and blood hematology (Abdel-Azeem et al., 2013). Only one study used the combined vitamin E and C against the endometrial damage of rat induced by fluoride (Guney et al., 2007). For this study we used vitamin C (V-C) and vitamin E (V-E) as a supplementary diet to combat the free radical induced alterations of different hematological and hepatological parameter. Various studies reported that they are non-enzymatic antioxidants (Lobo et al., 2010).

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Methods

Chemicals

Sodium fluoride (NaF) of 99% purity and thiobarbituric acid (TBA) were purchased from Sigma Chemical Company. Hydrogen peroxide, EDTA, NADPH, NAD, glutathione reductase, trichloroacetic acid and 2, 4-dinitrophenylhydrazine were procured from SRL, Mumbai, India. Vitamin C and vitamin E were purchased from Merck, Mumbai, India and Himedia, Mumbai, India respectively. Other chemicals were of analytical grade.

Experimental animal model

Eighteen adult male Wistar rats (b.wt. 120±10 g) were procured from registered breeder (Saha Enterprise, Kolkata) and were maintained in standard laboratory condition (14:00 h dark: 10:00 h light, 25±2 $^{\circ}$C) having free access to food and water. All animals were fed with standard laboratory diet composed of 71% carbohydrate, 18% protein (consist of 6.32% wheat flour, 4.38% barley meal and 10% of casein), 7% fat along with 4% salt mixture and vitamins (Biswas & Kumar Mukhopadhyay, 2020). Animal experimentations were done as per the ethical guidelines of Animal Ethics Committee of our University (Sanction No. PU/IAEC/PM/20).

Experimental design

Following acclimatization period of one week rats were randomly divided into three groups (n=6). Group I rats were given vehicle only. Group II rats were gavaged with NaF at a dose of 20 mg/kg/day. Group III rats were given same dose of NaF along with VC (200 mg/kg/day) & VE (400 mg/kg/day) orally. NaF was prepared by dissolving 1.5 mg

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of NaF in 250 μL distilled water for 100 gm weight of animal per day (Pal & Mukhopadhyay, 2021). Vitamin C and E were dissolved in distilled water and olive oil respectively (Mondal et al., 2016). After completion of 30 consecutive days of treatment schedule, rats from each group were euthanized on next morning under overnight fast. Primarily blood was collected and liver was dissected out and weighed. Plasma and serum were isolated from using standard protocol and kept in -20 $^{\circ}$C for further studies. Some portion of liver was kept in Buffered formol solution for histopathological analysis. For biochemical studies liver tissues were stored in -80 $^{\circ}$C.

Histology of Liver

Paraffin blocks were prepared from buffered formol fixed liver and thin tissue sections were cut using a well precision microtome (Optex brand, Pink Pearl Corporation, Osaka, Japan). H/E staining was done and photomicrographs were taken using both 100X and 400X magnification of a microscope (Zeiss, Thornwood, NY, USA).

Blood Sample collection

From plasma total antioxidant and total oxidant status of blood were measured. From serum hepatic functional parameter were also measured. Hematological parameters were also measured from heparinized blood.

Assessment of RBC and WBC count and estimation of hemoglobin concentration

From the collected blood total count of erythrocyte and leukocyte were done and also the concentration of hemoglobin was estimated. An automated cell counter machine (Beckman Coulter, France) was used for this total count and estimation.

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Assessment of plasma total antioxidant status (TAS) and total oxidant status (TOS)

From the isolated plasma the concentration of total antioxidant status was evaluated based on suppression of radical $ABTS^+$ [2, 2-azinobis-(3-ethylbenzothiozoline-6-sulfonic acid)] which absorb the 734 nm wavelength visible light and calculate the result with the trolox standard curve. The stock solution of 7 mM $ABTS^+$ was prepared by adding 2.45 mM potassium persulphate with 0.01 M phosphate buffer (pH- 7.4) and incubate the solution for 12-16 hrs in dark. The final working solution $ABTS^+$ was prepared just before use by diluting the stock solution with 0.01M PBS and adjusting the OD at 734 of the solution in this range 0.700±0.05 (Ai). Then 10 µl samples were to the adjusted $ABTS^+$ solution and incubate it into measurement chamber for 6 minutes. Then measure the absorbance at 734 nm (Af) and transformed it into mM trolox equivalent (REF). TOS was evaluated by using Erel methods (REF). The oxidants present in the plasma sample causes the oxidation of ferrous-o- dianisidine complex to ferric ion which was enhanced by addition of glycerol. This ferric ion formed a colored complex with xylenol orange in presence of acidic medium. The result was converted to µM H_2O_2 equivalent standard curve. For this assay reagent 1 (R1) containing xylenol orange (150µM), NaCl (140 mM), glycerol (1.35 M) and 25 mM H_2SO_4 were added with Millipore water. Reagent 2 (R2) containing ferrous ion (5 mM), O- Dianisidine dihydrochloride (10 mM) and H_2SO_4 (25 mM) were added with Millipore water and adjusted the pH 1.75. Then 900µL of R1 and 140 µL samples were added and measured the OD at both 560 nm and 800 nm and subtracting OD from 560 to 800 nm termed as

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Ai. Then 44 µL of R2 added to this solution, incubate 4 minutes, measured the OD and subtracting the OD from 560 nm to 800 nm termed as Af. Then final of value of TOS was obtained as follows ΔA= (Af – Ai) from the H_2O_2 standard graph. The oxidative stress index (OSI) was evaluated by the following formula OSI= [(TOS, µM H2O2 equivalent) ×100/ (TAS, µM Trolox equivalent)]. The unit of OSI is arbitrary unit (AU) (Re et al., 1999).

Assessment of hepatic functional parameter

After 30 days of treatment blood was collected from all the groups without anticoagulant (non-heparinized vial). Serum was collected, kept it into room temperature for 30 mins, into freeze for 30 mins and then centrifuged at 3000 rpm for 10 mins. After centrifugation serum was collected for estimation of such liver function parameter like aspartate aminotransferase (AST or SGOT), alanine aminotransferase (ALT or SGPT) and alkaline phosphates (ALP). AST assay was done by using MBK GOT (AST) kit (2,4-DNPH Assay). ALT assay was done by using MBK GPT (ALT) kit (2,4-DNPH Assay). For ALP assay MBK ALP KIT was used (Kind and King's Assay).

Determination of total thiol

Thiol is carbon bounded sulphydril group act as an important antioxidant pool, present cell membrane and helps in protection of membrane damage by free radicals. Therefore the altered total thiol concentration due to fluoride intoxication was estimated by using following protocol. For this assay 0.25 M Tris-EDTA buffer (0.25 M Tris Hcl buffer containing 20mM EDTA) and 10 mM DTNB solution (diluted in Tris EDTA buffer

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solution, pH- 8.2) were prepared. DTNB is light sensitive reagent and preparation was done in dark. After the preparation of all reagents 200µl serum added with 600 µl Tris EDTA buffer and mixed well. Then 40 µl 10 mM DTNB and 3.16 ml absolute methanol added to this solution. This solution then incubates for 30 minutes at room temperature and centrifuged at 3000 rpm for 10 minutes. Absorbance was measured by collecting the supernatant at 412 nm visible wavelength. The result was expressed as µM.

Estimation of hepatic oxidative stress

Extent of NaF mediated oxidative stress and its recovery (if any) was measured from liver tissues by assessment of different oxidative stress parameters.

Assessment of SOD activity

Superoxide dismutase inhibits the auto-oxidation of hematoxylene to hematein. Liver was collected from the entire group and tissue sample was prepared by using ice cold homogenizing phosphate buffer (0.1 M, pH- 7.2). Tissue concentration was 50 mg/ml. This homogenizing sample was then centrifuged at 10,000g for 10 min at 4ºC. Spectrophotometric evaluation of enzyme activity was done by using 0.05M phosphate buffer (0.1M EDTA) and hematoxylene. Hematoxylene was prepared by addition of 0.01 M phosphate buffer. The enzyme activity was measured by using JASCO v530 Easton, MD at 560 nm and the result expressed as units/mg of tissue (Concepcion Navarro et al., 1993).

Assessment of CAT activity

The activity of catalase enzyme evaluated from H_2O production from H_2O_2. For this

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assay 50 mg tissue was collected from all the groups and homogenized the tissue in ice cold Tris-Hcl buffer (0.05 M, pH- 7.0) and homogenate was centrifuged 10,000 g for 15 min at 4ºC. H_2O_2 solution was prepared by addition 400 µl 30% H_2O_2 from stock solution with 10 ml distilled water. Then in cuvette 0.5 ml prepared H_2O_2 and 2.5 ml D.W were added and take the reading at 240 nm within 20 sec. Then 40 µl supernatant was added and keep inside in spectrophotometer chamber and reading was taken within 30 sec interval (60, 90, 120, 150, 180,210 sec). The result was expressed in mm H_2O_2 / mg of tissue / min and all the process was performed in dark (Beers Jr. & Sizer, 1952).

Assessment of MDA production

Malondialdehyde (MDA) is one important product of the lipid peroxidation. This assay measures the MDA level in the sample. MDA react with thiobarbituric acid (TBA) to form MDA-TBA adduct which was quantified by using spectrophotometer. 0.1 M ice cold phosphate buffer (pH- 7.4) was used for tissue homogenization at 50mg tissue/ml buffer concentration. Sup was collected by centrifugation at 10,000 g for 10 min at 4-8ºC of homogenizing sample. TBA-TCA reagent was prepared by addition of 0.932 gm TBA and 15 gm TCA with 0.25 N HCl and make up the volume up to 100 ml with the addition of 25 ml 95% methanol. Then blank sample sup sample were prepared and boiled at 100ºC for 10 min and cooled the sample in room temperature. Then both the sample was centrifuged (4,000 g for 10 min) and sup was collected and spectrophotometric measurements was done at 535 nm wavelength. The calculation was done by using following formula $OD \times 10^4 / 1.56 \times 100$ mg tissue and unit is nM/mg of

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tissue (Ohkawa et al., 1979)

Assessment of protein carbonylation (PC)

This assay was performed to measure carbonyl product of protein oxidation (reactive ketones and aldehydes) generated due to the action of free radical on protein. 0.05 M phosphate buffer at 50 mg/ ml tissue concentration was used for homogenization and homogenate was centrifuged at 2500 rpm for10 min. Then supernatant was collected and measure the protein content by using lowery kit. The sample was taken at 1 mg protein concentration make up the volume up to 4 ml with 0.5 Mm 2,4- Dinitrophenylhydrazine (DNPH) and 1hour incubation was done at 37ºC. Then 1.25 ml 20% TCA was added kept in ice box for 10 min and after that bring the solution in normal room temperature. Then centrifuged the solution (3000 rpm, 5 min), discard the sup, collected the pellet, again added 1 ml 20 % TCA, added 1 ml ethanol: acetate, centrifuged at 3000 rpm for 5 min, discarded the sup and 1 ml gunanidine hydrochloride was added to the pellet. After that OD was taken at 280 nm and 370 nm against water putting as a blank and result expressed as μM/mg protein (Levine et al., 1994).

Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM). One-way ANOVA followed by Tukey's HSD post hoc analysis was performed to check any statistical difference between the parameters of the studied groups and $p<0.05$ was considered as lowest significant. Statistical analysis was carried out using the statistical

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program packages SPSS version 20.0 (SPSS Inc., Chicago, USA).

Results

Effect of fluoride on gravimetry of liver

Fluoride caused a significant decrease in the weight of liver ($p<0.01$) when compared to control group and after supplementation of Vitamin E+C the near normal values ($p<0.001$) were obtained (Fig.1).

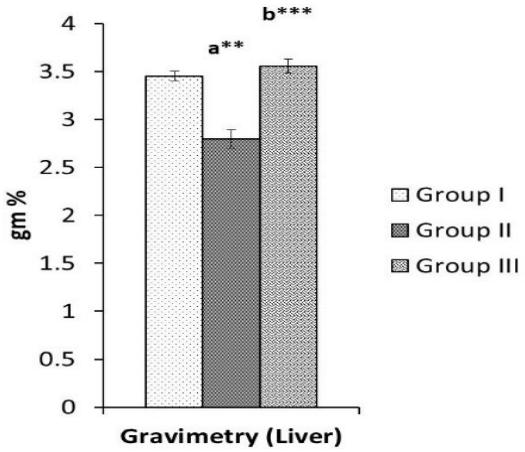

Fig.1- Bar diagram depicting the effect of Vitamin E+C on gravimetry of liver across the three groups as indicated. The data are presented as Mean ± SEM, (n=6). [a] designates control verses treated and supplement; [b] designates treated verses supplement. Level of significance; **($p<0.01$), ***($p<0.001$).

Effect of fluoride on histopathological alterations of hepatic tissue

The normal histoarchitecture of liver in control rats was exhibited in Fig. 2-A (10X) & 2D (40X). Fluoride caused increased sinusoidal space and degeneration of hepatic chords resulting in cytoplasmic vacuolation, increased diameter of central vein and presence of free nuclei [Fig. 2B (10X) & 2E (40X)]. Vitamin E+C supplementation retained the

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normal structural features as similar to that of control [Fig. 2C (10X) & 2F (40X)].

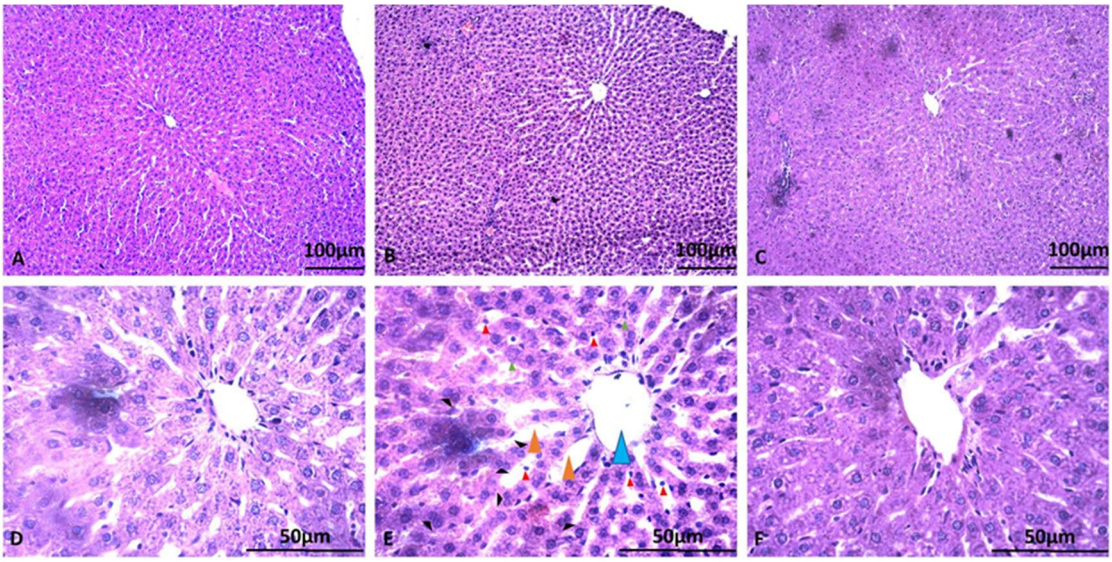

Fig. 2- Photomicrographs of H/E stained hepatic tissue under Vitamin E+C supplementation across all the groups. In control group, hepatic tissue showed all normal features (A-100x, D-400x). In fluoride treated group displayed free nuclei, picnotic nucleus (red arrow head), cytoplasmic vacuolation, increased sinusoidal space (orange arrow head), karryorhexis (green arrow head),bi-nucleation(black arrow head) and increased diameter of central vein (blue arrow head) (B-100x,E-400x). Restoration of normal features were observed except the diameter of central vein in vitamin E+C supplementation group (C-100x, F- 400x).

Effect of fluoride in RBC, WBC level and hemoglobin concentration

Exposure to fluoride caused a significant decrease in the RBC count ($p<0.001$), WBC count ($p<0.001$) and hemoglobin concentration ($p<0.001$) when compared to control group (Fig.3 A, B & C). However, after supplementation of Vitamin E+C the near normal values were obtained in all cases ($p<0.001$ in all cases) in comparison to treated group but difference still persisted in hemoglobin concentration ($p<0.01$) with regard to control group (Fig.3 A, B & C).

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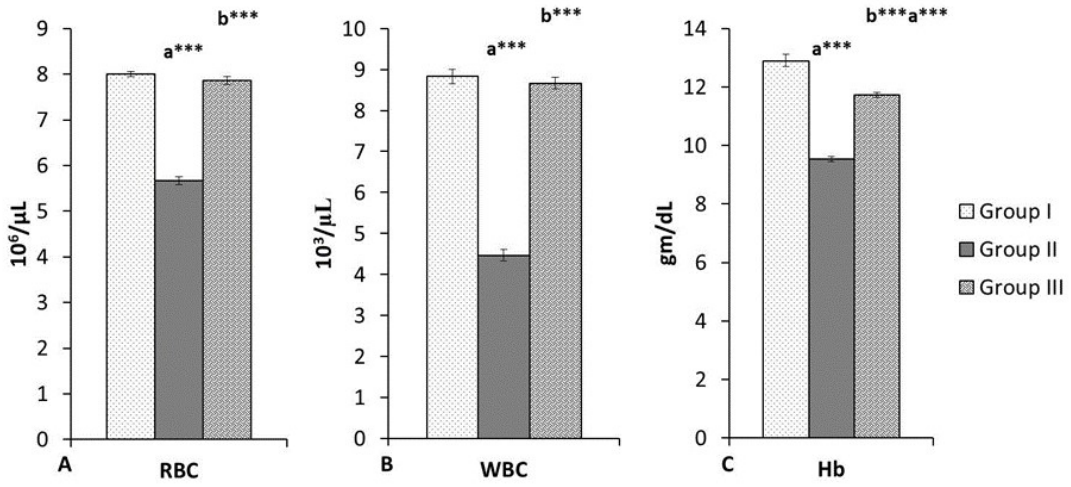

Fig.3- Bar diagram illustrating the effect of Vitamin E+C on RBC counts (A), WBC counts (B) and hemoglobin concentration (C) across the three groups as indicated. The data are presented as Mean ± SEM, (n=6). [a] designates control verses treated and supplement; [b] designates treated verses supplement. Level of significance; ***($p<0.001$).

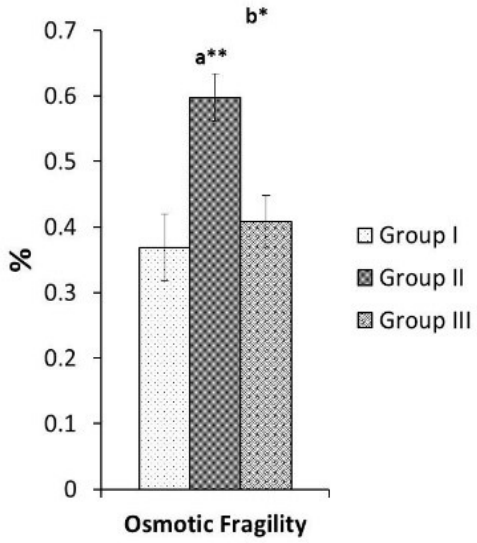

Fig.4- Bar diagram describing the effect of Vitamin E+C on osmotic fragility of erythrocytes among control and experimental groups. The data are presented as Mean ± SEM, (n=6). [a] designates control verses treated and supplement; [b] designates treated verses supplement. Level of significance; *($p<0.05$), **($p<0.01$).

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Effect of fluoride in erythrocyte osmotic fragility

The percentage of hemolysis of erythrocytes was significantly increased ($p<0.01$) in fluoride treated group with respect to control group. Supplementation of Vitamin E+C significantly decreased ($p<0.05$) the hemolysis of erythrocytes with respect to treated group to near normal level (Fig.4).

Effect of fluoride on plasma redox status

ROS generation due to fluoride toxicity altered the redox balance of blood as indicated by a significant increase of TOS ($p<0.01$) in plasma of treated group as compared to control group. Supplementation of Vitamin E+C significantly restored ($p<0.05$) the alteration with respect to treated group to near normal condition (Fig.5 A). Similarly, decrease of plasma TAS was noted ($p<0.01$) after fluoride treatment in comparison to control which after supplementation reached the normal value ($p<0.001$) (Fig.5 B). The OSI was markedly increased ($p<0.01$) in fluoride treated rats compared to that of controls. Vitamin E+C supplementation resulted in significant decrease ($p<0.01$) of OSI level attaining normal value (Fig. 5 C).

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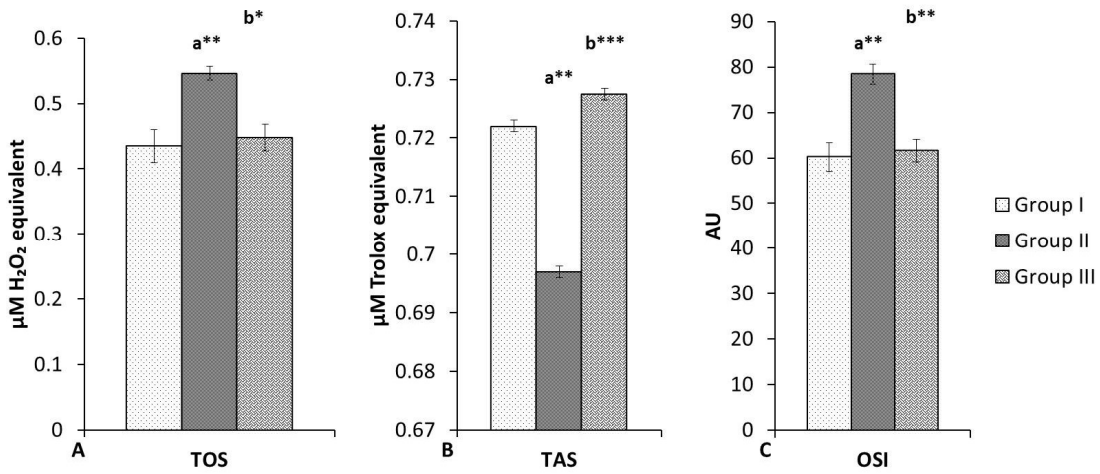

Fig.5- Bar diagram representing the effect of Vitamin E+C on plasma TOS (A), TAS (B) and OSI (C) levels between the control and experimental groups. The data are presented as Mean ± SEM, (n=6). [a] designates control verses treated and supplement; [b] designates treated verses supplement. Level of significance; *($p<0.05$), **($p<0.01$), ***($p<0.001$).

Effect of fluoride on serum total thiol

Similarly, decrease of serum total thiol was noted ($p<0.01$) after fluoride treatment in comparison to control which after supplementation reached the normal value ($p<0.01$) (Fig.6).

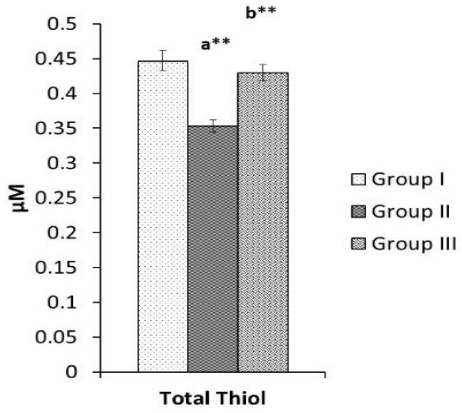

Fig.6- Bar diagram signifying the effect of Vitamin E+C on serum total thiol level across the three groups as specified. The data are presented as Mean ± SEM, (n=6). [a] designates control verses treated and supplement; [b] designates treated verses supplement. Level of significance; **($p<0.01$).

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Treatment with fluoride caused significant increase of serum SGPT and SGOT levels ($p< 0.01$ and $p<0.001$) when compared to control. The level was normalized ($p<0.01$ and $p< 0.001$) in Vitamin E+C supplemented group even though difference persisted in SGOT level ($p<0.001$) with control value (Fig. 7 A & B). Similarly activity of serum ALP enzyme was decreased ($p<0.001$) in fluoride treated group with respect to control group. Vitamin E+C co-administration caused the enzyme activity to achieve near normal value ($p<0.001$) (Fig.7 C).

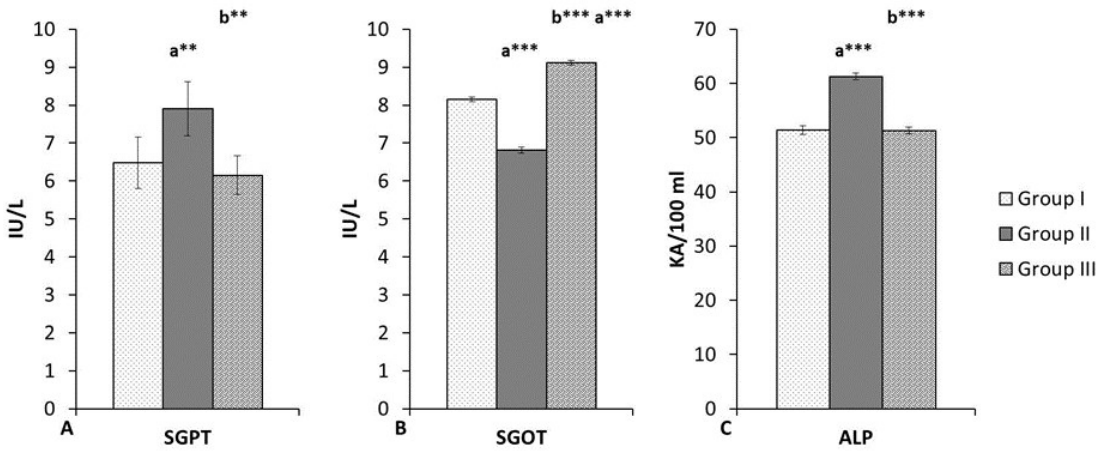

Fig.7- Bar diagram showing the effect of Vitamin E+C on serum SGPT (A), SGOT (B) and ALP (C) levels among the control and experimental groups. The data are presented as Mean ± SEM, (n=6). [a] designates control verses treated and supplement; [b] designates treated verses supplement. Level of significance; **($p<0.01$), ***($p<0.001$).

Effect of fluoride on oxidative stress markers of liver

Fluoride toxicity markedly decreased the activity of SOD and Catalase ($p<0.001$ in both cases) indicating increased oxidative stress when compared to control. The activity level was found to be near normal levels ($p<0.01$ and $p<0.001$) in Vitamin E+C supplemented

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group (Fig. 8 A & B). Fluoride exposure resulted in lipid peroxidation and protein oxidation which was evident from the increase in hepatic MDA ($p<0.001$) and PC ($p<0.001$) levels in comparison to control group. Vitamin E+C supplementation decreased the MDA ($p<0.001$) and PC ($p<0.001$) levels respectively to attain the near normal values (Fig. 8 C & D).

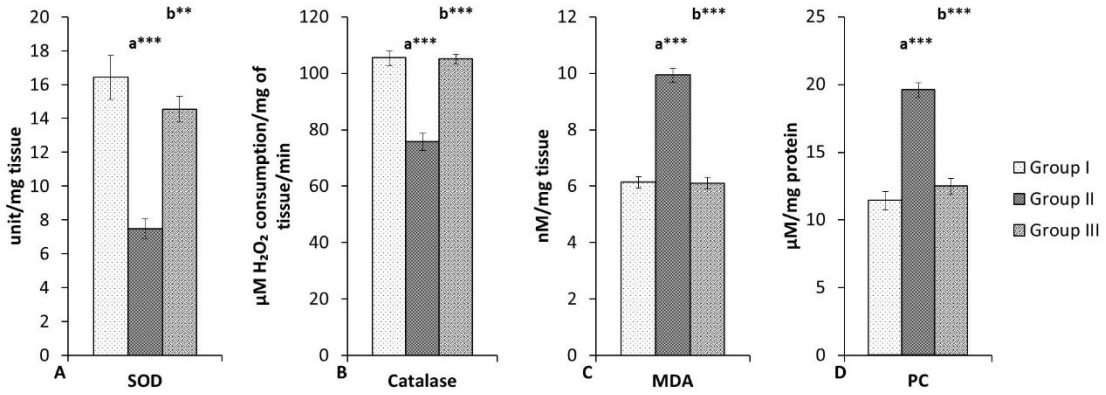

Fig.8- Bar diagram presenting the effect of Vitamin E+C on hepatic SOD activity (A), catalase activity (B), hepatic MDA levels (C) and PC levels (D) respectively across the three groups as indicated. The data are presented as Mean ± SEM, (n=6). [a] designates control verses treated and supplement; [b] designates treated verses supplement. Level of significance; **($p<0.01$), ***($p<0.001$).

Discussion

Fluoride present all over the world and acknowledged it as an important environmental toxicant. Upon chronic exposure it causes adverse health effect to human. Majority of the people are affected with fluorosis from fluoride containing ground water and other fluoride contaminated sources. Fluoride can easily permeate the cell membrane that's why it shows its drastic effect when it is consumed at very high dose. The present study

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concerned with the using of combined vitamin E and C (vitamin E+C) as a supplementary diet to evaluate the hematoprotective and hepatoprotective special effects against the sodium fluoride (NaF) induced toxicity in adult male Wistar rats. The gravimetrical analysis of rat body weight and liver organ weight showed that there was no significance difference of body weight in all the control, treated and supplemented group but in case of liver weight there was significance changes of the weight with the control and treated; treated and supplement and no significant difference found in between control and supplemented group. Some other studies reported that there was significant decrease of body and liver weight of mice in NaF treated group (Vani & Reddy, 2000). One study reported that liver weight was significantly increased in the NaF treated group in mammals (Srivastava et al., 1989). The present study shown that there was decrease in hematological parameter such as decrease in erythrocyte count, leukocyte count and hemoglobin concentration in NaF treated group as compared with control and supplemented groups. Similar report was also presented by various studies that decrease in WBC count and RBC count but in case HGB some studies reported that there were no significance differences in both control and treated group (Khan et al., 2013). From this study it was observed that NaF induced altered parameters were protected in the supplemented group receiving combined vitamin E+C. The reason behind this decreasing no of WBC count probably due to the deleterious effect of fluoride on bone marrow and hematopoietic organ and decrease RBC count may be associated with inhibitory effect of fluoride on erythropoiesis (Khan et al., 2013). The osmotic

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fragility of erythrocyte shows a significant increase in treated group which was protected in supplementary group (vitamin E+C). Present study also evaluated the total thiol concentration of erythrocyte membrane from isolated serum and observed that decreased thiol concentration due to fluoride toxicity and ameliorated by using of combined vitamin E+C. In cell membrane it acts as an antioxidant pool and in presence of any oxidative radicals it donates the hydrogen atom and forms a disulphide bridge. Therefore excess free radicals causes loosening of the integrity of erythrocyte membrane and RBC become fragile; life span of RBC was also shortened. All these altered hematological parameter are protected in supplemented group. Some studies reported that osmotic fragility of erythrocyte depends on the thiol concentration of plasma membrane. Present study was also evaluated the total antioxidant and total oxidant status from plasma. It was seen that TAS values was decreased and TOS value was increased which indicate that oxidative stress occur due to fluoride induced toxicity. This alteration was also protected in vitamin E+C supplemented group. Oxidative stress index (OSI) was evaluated from the ratio of TOS and TAS which showed significance increase of oxidative stress in treated compare to control and supplemented group proved that oxidative stress was protected.

Liver play a vital role in maintaining the normal body metabolic homeostasis by metabolism. Liver is responsible for biotransformation (the process by which liver can detoxify the toxic element or producing less toxic product) of xenobiotic compounds. The present study evaluated the result of some hepatic functional parameter such AST, ALT, ALP and observed that increased activity of these entire enzyme in serum due to

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fluoride intoxication. This study showed that this alteration was protected in vitamin E+C supplemented group. Similar findings were also reported by some studies (Hassan & Yousef, 2009). Some studies revealed that decreased activity of alkaline phosphatase enzyme in fluoride treated groups as because fluoride inhibits this enzyme (Nabavi et al., 2012). These enzymes released into the blood stream when the liver is damaged and from this study due to increased activity of these enzymes in fluoride treated groups proved that fluoride can cause the hepatocellular injury and releasing the enzyme into the blood stream. These are important indicators of liver dysfunction used as biomarkers in clinical findings (Bogin et al., 1976). Present study showed the histoarchitecture changes of liver sections stained with normal hematoxylene and eosin in the fluoride treated groups compared with the control. Hepatic abnormalities like cellular necrosis, binucleated nucleus, disintegration of hepatic cords, increased sinusoidal space, increased diameter of central vein etc were also found in the treated groups. Whereas in supplementary group the protection is very les compared to control but in compared to treated there is protection because of there is less no of binucleated cell, hepatocellular necrosis etc. Bouaziz et al reported the same architectural changes with that of control and treated. Some studies also reported that high concentration of fluoride causes degeneration and necrosis of liver and kidney cell of rabbits (Shashi et al., 2002). To evaluate NaF induced hepatotoxicity done by oxidative stress some biochemical assay were done such as the estimation of antioxidant enzyme activity like SOD and CAT. Here the result shows that decrease activity of both this antioxidant enzyme in fluoride treated group compared to

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the control but when compared with supplementary group there is protection and preventing the reduction of antioxidant enzyme activity. Shanta kumari et al., shown that there is decrease activity of this antioxidant enzyme of fluoride treated rats (Shanthakumari et al., 2004). Patel and Chinoy showed the similar effect on fluoride treated ovary of mice. Another study reported that there was decrease in the activity of SOD and CAT antioxidant enzyme in mice brain and gastrocnemius muscle (Apel & Hirt, 2004). Assay non enzymatic parameters were done such as MDA (lipid peroxidation product) and protein carbonyl (protein oxidation product). The result of this non enzymatic parameter showed the increase production of MDA and protein oxidation product in fluoride treated groups, whereas protection was found in vitamin E+C group. Similar result was reported by some studies and showed the increase lipid peroxidation of rat kidney due to chronic exposure of fluoride (Krechniak & Inkielewicz, 2005).

From different literature survey it is known that fluoride causes fluoride causes the oxidative stress by induction ROS generation. Our present study was also reported the enhanced hepatocellular ROS production in NaF treated group and this ROS generation was also protected in vitamin E+C group. As fluoride induce ROS generation causes oxidative imbalance between the free radical generation and antioxidant enzyme. Therefore decrease in the activity of antioxidant enzyme inducing the oxidative stress causes the hepatotoxicity of rats and mice (Chattopadhyay et al., 2011). The function of antioxidant enzyme is to conversion toxic free radical to non toxic compound. Super oxide dismutase causes the reduction of superoxide anion to hydrogen peroxide (H_2O_2)

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and this H_2O_2 is then reduced to H_2O and molecular oxygen by the help of another antioxidant enzyme CAT present in mitochondria. Superoxide dismutase is off three types such as CuZn SOD (cytoplasm), Mn SOD (mitochondria), and Ec SOD (extracellular) (Bartsch & Nair, 2000). By this way the antioxidant enzyme scavenges the free radical. Therefore free radical disrupt the oxidative balance causes the damaging DNA, protein oxidation and lipid peroxidation. Lipid peroxidation is the process where free radicals acts on unsaturated lipid present on membrane attack the C=C of lipid and produces the lipid peroxyl radicals. This lipid peroxyl radical in presence of another lipid generate lipid hydroperoxide and lipid peroxyl radicals (Girotti, 1998). Protein oxidation is metal catalyzed, free radical induced oxidation of protein. The metals like Fe^{2+}, Cu^{2+} binds to the active site of amino acid of protein which is further attacked by H_2O_2 and other free radical and produces reactive aldehyde and ketones (Aldini et al., 2010).

So to prevent fluoride induced excess free radical generation mediated damage of lipid and proteins vitamin E and vitamin C were used combindly as non enzymatic antioxidant source. Vitamin E is lipid soluble and prevents lipid peroxidation process by free radicals. It works in hydrophobic phase of cell membrane. α-tocopherol prevents the membrane damage by donating its hydrogen atom from OH- tocopherol to free radicals and lipid peroxyl radicals and converted itself α-tocopheryl radical whereas vitamin C is work in aqueous phase of both intracellular and extracellular medium donates its hydrogen atom to those α-tocopheryl radical to form normal α-tocopherol (Lobo et al., 2010).

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References

Abdel-Azeem, A. S., Hegazy, A. M., Ibrahim, K. S., Farrag, A. R. H and El-Sayed, E. M. (2013): Hepatoprotective, antioxidant, and ameliorative effects of ginger *(Zingiber officinale roscoe*) and vitamin e in acetaminophen treated rats, Diet. Suppl. 10(3):195-209.

Agha, F. E., El-Badry, M. O., Hassan, D. A. A and Abd Elraouf, A. (2012): Role of vitamin E in combination with methionine and L-carnosine against sodium fluoride-induced hematological, biochemical, DNA damage, histological and immunohistochemical changes in pancreas of albino rats, Life. Sci. 9(2):1260-1275.

Aldini, G., Vistoli, G., Regazzoni, L., Benfatto, M. C., Bettinelli, I and Carini, M. (2010): Edaravone inhibits protein carbonylation by a direct carbonyl-scavenging mechanism: Focus on reactivity, selectivity, and reaction mechanisms, Antioxid. Redox. Signal. 12(3):381-392.

Ali, S., Thakur, S. K., Sarkar, A and Shekhar, S. (2016): Worldwide contamination of water by fluoride, Environ. Chem. Lett. 14: 291–231.

Aoba, T and Fejerskov, O. (2002): Dental fluorosis: Chemistry and biology, Crit. Rev. Oral. Biol. Med. 13: 155-170.

Apel, K and Hirt, H. (2004): Reactive oxygen species: Metabolism, oxidative stress, and signal transduction, Annu. Rev. Plant. Biol. 55: 373-399.

Volume 27 June 2021
Indian Journal of Biological Sciences
(A Peer Reviewed Journal)
ISSN 0972-8503
http://dx.doi.org/10.62424/ IJBS.2021.27.01.01

Atmaca, N., Yldrm, E., Güner, B., Kabakç, R and Bilmen, F. S. (2014): Effect of resveratrol on hematological and biochemical alterations in rats exposed to fluoride, Biomed. Res. Int. 2014: 698628.

Barbier, O., Arreola-Mendoza, L and Del Razo, L. M. (2010): Molecular mechanisms of fluoride toxicity, Chem. Biol. Interact. 188(2):319-333.

Bartsch, H and Nair, J. (2000): Ultrasensitive and specific detection methods for exocylic DNA adducts: Markers for lipid peroxidation and oxidative stress, Toxicology.153(1-3): 105-114.

Batabyal, A. K and Gupta, S. (2017): Fluoride-contaminated groundwater of Birbhum district, West Bengal, India: Interpretation of drinking and irrigation suitability and major geochemical processes using principal component analysis, Environ. Monit. Assess. 189(8): 369.

Beers Jr., R. F and Sizer, I. W. (1952): A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase, J. Biol. Chem. 195(1): 133–140.

Biswas, S and Kumar Mukhopadhyay, P. (2020): Casein- and pea-enriched high-protein diet can take care of the reprotoxic effects of arsenic in male rats. Andrologia. 52(5): e13560.

Bogin, E., Abrams, M., Avidar, Y and Israeli, B. (1976): Effect of fluoride on enzymes from serum, liver, kidney, skeletal and heart muscles of mice, Fluoride. Q. Rep. 9(1): 42-46

Chattopadhyay, A., Podder, S., Agarwal, S and Bhattacharya, S. (2011): Fluoride-

induced histopathology and synthesis of stress protein in liver and kidney of mice, Arch. Toxicol. 85(4): 327-335.

Concepcion Navarro, M., Pilar Montilla, M., Martin, A., Jimenez, J and Pilar U. M. (1993): Free radical scavenger and antihepatotoxic activity of Rosmarinus tomentosus, Planta. Med. 59(4): 312–314.

Edmunds, W. M and Smedley, P. L. (1996): Groundwater geochemistry and health: An overview, Geol. Soc. Spec. Publ. 113: 91-105.

Ersoy, I. H., Alanoglu, E. G., Koroglu, B. K., Varol, S., Akcay, S., Ugan, Y., Ersoy, S and Tamer, M. N. (2010): Effect of endemic fluorosis on hematological parameters, Biol. Trace. Elem. Res. 138(1-3): 22-27.

Girotti, A. W. (1998): Lipid hydroperoxide generation, turnover, and effector action in biological systems, J. Lipid. Res. 39(8):1529-1542.

Guney, M., Oral, B., Demirin, H., Karahan, N., Mungan, T and Delibas, N. (2007): Protective effects of vitamins C and E against endometrial damage and oxidative stress in fluoride intoxication, Clin. Exp. Pharmacol. Physiol. 34: 467-474.

Guo, X. Y., Sun, G. F and Sun, Y. C. (2003): Oxidative stress from fluoride-induced hepatotoxicity in rats, Fluoride. 36(1): 25-29.

Hassan, H. A and Yousef, M. I. (2009): Mitigating effects of antioxidant properties of black berry juice on sodium fluoride induced hepatotoxicity and oxidative stress in rats, Food. Chem. Toxicol. 47(9): 2332-2337.

Jadhav, S. V., Bringas, E., Yadav, G. D., Rathod, V. K., Ortiz, I and Marathe, K. V.

Volume 27 June 2021
Indian Journal of Biological Sciences
(A Peer Reviewed Journal)
ISSN 0972-8503
http://dx.doi.org/10.62424/ IJBS.2021.27.01.01

(2015): Arsenic and fluoride contaminated groundwaters: A review of current technologies for contaminants removal, J. Environ. Manage. 162: 306-325.

Khan, A. M., Dubey, N., Raina, R., Singh, G and Beigh, S. A. (2013): Toxic effects of deltamethrin and fluoride on hematological parameters in rats, Fluoride. 46(1): 34-38.

Krechniak, J and Inkielewicz, I. (2005): Correlations between fluoride concentrations and free radical parameters in soft tissues of rats, Fluoride. 38(4): 293-296.

Levine, R. L., Williams, J. A., Stadtman, E. P and Shacter, E. (1994): Carbonyl assays for determination of oxidatively modified proteins, Methods. Enzymol. 233: 346–357.

Li, Y., Dunipace, A. J and Stookey, G. K. (1988): Genotoxic effects of fluoride: A controversial issue, Mutat. Res. Genet. Toxicol. Environ. Mutagen. 195: 127-136.

Lobo, V., Patil, A., Phatak, A and Chandra, N. (2010): Free radicals, antioxidants and functional foods: Impact on human health, Pharmacogn. Rev. 4(8):118-126.

Mondal, R., Biswas, S., Chatterjee, A., Mishra, R., Mukhopadhyay, A., Bhadra, R. K and Mukhopadhyay, P. K. (2016): Protection against arsenic-induced hematological and hepatic anomalies by supplementation of Vitamin C and vitamin e in adult male rats, J. Basic. Clin. Physiol. Pharmacol. 27(6)*:* 643–652.

Nabavi, S. M., Nabavi, S. F., Eslami, S and Moghaddam, A. H. (2012): In vivo protective effects of quercetin against sodium fluoride-induced oxidative stress in the hepatic tissue, Food. Chem. 132(2): 931-935.

Volume 27 June 2021
Indian Journal of Biological Sciences
(A Peer Reviewed Journal)
ISSN 0972-8503
http://dx.doi.org/10.62424/ IJBS.2021.27.01.01

Ohkawa, H., Ohishi, N and Yagi, K. (1979): Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95(2): 351–358.

Pak, C. Y. C., Zerwekh, J. E and Antich, P. (1995): Anabolic effects of fluoride on bone, Trends. Endocrinol. Metab. 6(7): 229-234.

Pal, P., Jha, N. K., Pal, D., Jha, S. K., Anand, U., Gopalakrishnan, A. V., Dey, A and Mukhopadhyay, P. K. (2022): Molecular basis of fluoride toxicities: Beyond benefits and implications in human disorders, Genes. Dis. 10(4): 1470-1493.

Pal, P and Mukhopadhyay, P. K. (2021): Fluoride induced testicular toxicities in adult Wistar rats, Toxicol. Mech. Methods. 31(5): 383–392.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M and Rice-Evans, C. (1999): Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free. Radic. Biol. Med. 26(9–10): 1231–1237.

Shanthakumari, D., Srinivasalu, S and Subramanian, S. (2004): Effect of fluoride intoxication on lipidperoxidation and antioxidant status in experimental rats, Toxicology. 204: 219-228.

Sharma, D., Singh, A., Verma, K., Paliwal, S., Sharma, S and Dwivedi, J. (2017): Fluoride: A review of pre-clinical and clinical studies, Environ. Toxicol. Pharmacol. 56: 297-313.

Sharma, P., Bhardwaj, A. K., Singh, M., Kumar, D., Sharma, A and Grover, A. (2018): Does fluorosis affect the intelligence profile of children? A cross sectional analysis of school children of district Una, Himachal Pradesh, India. International. J. Comm.

Med. Pub. Health. 5(3): 1047–1053.

Shashi, A., Singh, J. P and Thapar, S. P. (2002): Toxic effects of fluoride on rabbit kidney, Fluoride. 35(1): 35-50.

Shashi, A and Thapar, S. P. (2001): Histopathology of fluoride-induced hepatotoxicity in rabbits, Fluoride, 34(1): 34-42.

Shulman, J. D and Wells, L. M. (1997): Acute fluoride toxicity from ingesting home-use dental products in children, birth to 6 years of age, J. Public. Health. Dent. 57(3): 150-158.

Srivastava, R. N., Gill, D. S., Moudgil, A., Menon, R. K., Thomas, M and Dandona, P. (1989): Normal ionized calcium, parathyroid hypersecretion, and elevated osteocalcin in a family with fluorosis, Metabolism. 38(2): 120-124.

Thangapandiyan S, M. S. (2013): Molecular mechanism of fluoride induced oxidative stress and its possible reversal by chelation therapy, Res. Rev. J. Toxicol. 3(2): 1–11.

Vani, M. L and Reddy, K. P. (2000): Effects of fluoride accumulation on some enzymes of brain and gastrocnemius muscle of mice, Fluoride. 33(1):17-26.