Influence of Antioxidant Vitamin (L –ascorbic Acid) on Hypoxia Induced Oxidative and Nitrosative Stress in Physiological System of Male Albino Rats Exposed to Sodium Fluoride (NaF)

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Doctor of Philosophy in **Medical Physiology**

By

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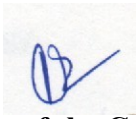
DECLARATION BY THE CANDIDATE

I hereby declare that this thesis entitled "***Influence of Antioxidant Vitamin (L–ascorbic Acid) on Hypoxia Induced Oxidative and Nitrosative Stress in Physiological System of Male Albino Rats Exposed to Sodium Fluoride (NaF)"*** is bonafide and genuine research work carried out by me under the supervision of Professor Kusal K. Das (Guide) Department of Physiology, Shri B. M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura, Karnataka, India and Dr. Raju H Taklikar (Co-Guide), Professor and HOD, Department of Physiology, Navodaya Medical College, Hospital and Research Centre, Raichur, Karnataka, India. No part of this thesis has been formed the basis for the award of any degree or fellowship previously.



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Dedication

This thesis is dedicated to our late dadaji

'In the memory of
Dr. A. Krishna Rao*'*
Stalwart giant in physiology,
KMC manipal

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LIST OF ABBREVIATIONS

µM/L	Micro moles per litre
µg/ml	Microgram per ml
µ L	Microliter
µ /dl	Microgram/dl
%	Percent
2,4, DNPH	2,4, dinitrophenylhydrazine
ABP	Avidin-Biotin-Peroxidase Complex
ADP	Adenosine diphosphate
Al2(SiO4)F2	Topaz
ANOVA	Analysis of varience
AP-1	Activator protein -1
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AT2	Angiotensin II receptor
BH4	Tetrahydrobiopterin
Bax	BCL2 associated X
Bcl -2	B-cell lymphoma- 2
BP	Blood pressure
b. wt	Body weight
CaF2	Fluorspar
Ca5(PO4)3F	Fluorapatite
CAT	Catalase
CBP	CREB-binding protein
C	Celsius
cDNA	Complementary DNA

CH	Chronic hypoxia
cNOS	Constitutive nitric oxide synthase
Co_2	Corban dioxide
CPCSEA	Committee for the purpose of control and supervision of experiments on animals
$CuSO_4$	Copper Sulphate
DBP	Diastolic blood pressure
DNA	Deoxy ribonucleic acid
dl	Decilitre
ECG	Electrocardiogram
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme-linked immune sorbent assay
eNOS	Endothelial nitric oxide synthase
Epo	Erythropoietin
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
F^-	Fluoride ion
F_2	Fluorine
Fe^{2+}	Ferrous
Fe^{3+}	Ferric
g/dl	Gram per decilitre
GLUT	Glucose transporter
GLP	Glucagon like peptide
GSH-Px	Glutathione peroxidase
GSSG	Glutathione disulfide
GST	Glutathione-s-transferase
GTPase	Guanosine triphosphatase

HB	Hemoglobin
HCl	Hydrochloric acid
HF	High frequency
HF	Hydrogen fluoride
HIF	Hypoxia Inducible Factor
H_2O_2	Hydrogen peroxide
HR	Heart Rate
HREs	Hypoxia response elements
HRP	Horseradish peroxidise
HRV	Heart Rate Variabilty
H_2SO_4	Sulphuric acid
IFN-γ	Interferon gamma
i.p	Intra peritoneal
iNOS	Inducible nitric oxide synthase
IL	Interleukin
JNK	c-Jun N-terminal kinase
Kg	Kilogram
LPO	Lipid peroxidation
LF	Low frequency
LF/HF ratio	Low frequency/High frequency ratio
MAP	Mean arterial pressure
MAPK	Mitogen- activated protein kinase
MDA	Malondialdehyde
ml	Milliliter
MIN	Minute
mg	Milligram
mg/dl	Milligram per decilitre

mg/kg	Milligram per kilogram
mIU/L	Milli International Units per Litre
mm Hg	Millimetre of mercury
mm3	Cubic millimetre
mmol/L	Millimoles per liter
Mros	Mitochondrial reactive oxygen species
Na3ALF6	Cryolite
NaF	Sodium Fluoride
NaNO2	Sodium Nitrite
NaoH	Sodium Hydroxide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIBP	Noinvasive blood pressure
nm	Nanometer
nNOS	Neuronal nitric oxide synthase
NOS3	Nitric oxide synthase3
NOX2	NADPH oxidase 2
ns	not significant
nu	Normalized units
O2	Oxygen
O2-	Superoxide anion
OD	Optical density
ODD	Oxygen-dependent domain
OH	Hydroxyl radical
OSI	Organo somatic index
p38 MAPK	p38 mitogen-activated protein kinases
p53	Phospo protein p53

Pg/ml	Picogram/millilitre
PAS Domain	Per-ARNT-sim domain
PCR	Polymerase chain reaction
PHD	Prolyl hydroxylase domain
PUFA	Polyunsaturated fatty acid
RBC	Red Blood cell
RPM	Revolutions per minute
RNA	Ribose nucleic acid
RNS	Reactive nitrogen species
ROS	Reactive Oxygen species
RR	Respiratory rate
SBP	Systolic blood pressure
SD	Standard deviation
Sec	Seconds
SIRT-1	Sirtuin- 1
SOD	Super oxide dismutase
SPSS	Statistical Package for the Social Sciences
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TMB	3,3',5,5'-Tetramethylbenzidine
TNF alpha	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor receptor 1
UV-B	Ultraviolet B
VEGF	Vascular endothelial growth factor
VHL	Von Hippel–Lindau
WBC	White Blood cell
$ZnSo_4$	Zinc Sulphate

ABSTRACT

Objective :

To evaluate the supplementation of L-ascorbic acid as an antioxidant on chronic hypoxia induced alterations of cardiac autonomic functions, biochemical, histopathological and oxygen sensing transcriptional pathways in male albino rats exposed to sodium fluoride toxicity.

Methods :

Male albino rats were randomly divided into 8 groups (n= 6/group), and groups were named accordingly .Group I(control) , group II (L-ascorbic acid ,50 mg / 100g. b.wt, orally), group III (Chronic hypoxia, 10%O_2), group IV (NaF ;20 mg/kg b.wt /day ; ip), group V (NaF + Chronic hypoxia, 10% O_2), group VI (L–ascorbic acid + Chronic hypoxia, 10% O_2), group VII (L-ascorbic acid + NaF) and group VIII (L-ascorbic acid + NaF + Chronic hypoxia, 10% O_2). The treatments were carried for 21 days. Animals of all the groups were weighed on the starting day of protocol and immediately after the end of 21st day. Percentage change of body weight gain and OSI was determined. Electrophysiological parameters like pneumogram, noninvasive blood pressure (NIBP) and ECG were recorded. HRV analysis was done to assess cardiac autonomic functions. Oxidant status were assessed by evaluating serum MDA and NO levels . Antioxidant status were assessed by evaluating serum SOD, vitamin E, vitamin C and hepatic Vitamin C. Oxygen sensing molecular markers like vascular endothelial growth factor (VEGF) and nitric oxide synthase 3 (NOS3) were also assessed. Histopathological evaluations were done to identify changes in myocardial tissue (ventricle), lungs, hepatic tissue and kidney **.**

Results :

Our results on gravimetry are indicative of decrease in % of body weight gain and OSI of heart, lungs, liver and kidneys in CH, NaF and CH + NaF treated groups. Electrophysiological studies in CH group are indicative of altered sympathovagal balance and sympathetic dominance. Increase in heart rate and MAP in CH group are also indicative of sympathetic stimulation. Whereas NaF treated rats showed parasympathetic dominance along with the decrease in heart rate and MAP, which also

indicates altered autonomic functions. Imbalance in oxidants and antioxidant status in CH, NaF and CH + NaF groups are indicative of oxidative and nitrosative stress with upregulation of oxygen sensing molecular markers like NOS3 and VEGF. Histopathological evaluation was also done to identify changes in myocardial tissue (ventricle), lungs, hepatic tissue and kidney. It is evident from the above results that fluoride toxicity and chronic hypoxia induce oxidative and nitrosative stress leading to cardiac autonomic dysfunctions by the molecular mechanisms mediated by HIF through VEGF and NOS3 pathways and also impacting the development of cardiopulmonary and hepatorenal pathophysiology.

In the groups treated with L-ascorbic acid showed improvements in gravimetry , sympathovagal balance, heart rate, MAP, it has also led to decrease in oxidative stress and nitrosative stress in chronic hypoxia and NaF treated groups. This could be due to potential antioxidant property of L- ascorbic acid.

CONCLUSION :

Results from our study shows that chronic hypoxia (CH) exposure and sodium fluoride(NaF) toxicity leads to cardiac autonomic dysfunctions, causes oxidative and nitrosative stress in cardiopulmonary and hepatorenal systems, enhances nitric oxide production by up regulation of VEGF & NOS3 genes leading to activation of apoptotic pathways. The supplementation of L-ascorbic acid has ameliorating effects on cardiac autonomic functions in chronic hypoxia (CH) induced male albino rats exposed to sodium fluoride (NaF). L-ascorbic acid supplementation is also salubrious to combat both chronic hypoxia (CH) and sodium fluoride (NaF) induced apoptotic cell signalling pathways leading to cellular adaptability.

Key words: L –ascorbic acid, sodium fluoride, chronic hypoxia, oxidative stress, Nitrosative stress.

INTRODUCTION

Existence of life is a complex process and is dependent on the utilization of oxygen but not on fluorine. Fluorine being strongly electronegative than oxygen ,could have been the alternative for oxygen, However oxygen being the most abundant element on earth and very less availability of fluorine during early conditions on earth has lead to the utilization of oxygen (Budisa *et al*., 2014) . Oxygen is essential for metabolism and ATP production, required for survival of multicellular organism. Fluorine (F_2) is an unstable atom and is quickly converted to fluoride ion (F^-). In the earth's crust, it is the 13th most abundant element, occurs as calcium fluoride. Topaz, fluoroapatite and cryolyte are the minerals in earth's crust which are mainly composed of calcium fluoride .Other elements to which fluoride has affinity is magnesium, aluminium, silicon and sodium. It belongs to Group VIIa of the periodic table and belongs to family of halogen elements . Availability of fluoride in ecosystem is dependent on factors like eruptions of volcanoes, dissolution from the rock, production of chemical fertilizers and many other industrial processes like thermal power plants, steel and iron plants leading to occupational hazards (Kanduti *et al*., 2016; Choubisa and Choubisa, 2016). Fluoride is present in the sources like water (mainly in ground water), plants like tea plants, can accumulate fluoride from soil and others sources are some pharmaceutical drugs contain fluoride. Fluoride is an nonessential element and hence there is no minimum required daily allowance for fluoride. Sodium fluoride is an inorganic salt of fluoride and it is soluble in water. Major amounts of fluoride is absorbed through gastrointestinal route ,rate of absorption depends on the solubility of fluoride compounds, highly soluble compounds like NaF and hydrogen fluoride gets absorbed at faster rate than magnesium fluoride and calcium fluoride (Martinez-Mier, 2012). Fluoride is excreted mainly by kidneys in the urine. Most of the injected fluoride is retained and it is an important route to induce toxicity. Fluoride due to its electro negativity can induce toxicity and inhibit enzymatic activity like ATPase and phosphatases (Miranda *et al*., 2018). Fluoride also inhibits the synthesis of proteins involved in cell signalling pathways (p53,AP-1 and NF-κB) (Lu *et al*., 2017).The effect of chronic fluoride toxicity on cardiovascular autonomic functions and the underlying mechanisms have not been extensively studied (Varol and Varol, 2012).

Hypoxia is a condition in which oxygen is not available in sufficient amounts at cellular level to maintain adequate homeostasis. Oxygen requirement in normoxic conditions is at 17% to 3% in various tissues. Oxygen requirement varies from tissues to tissues in

normoxic conditions. If oxygen content decreases at cellular level (0.5 -2%) then it leads to hypoxia (Lee *et al.*, 2020) . Fluoride toxicity and hypoxia exposure alter oxygen sensing cell signaling transduction pathways. Sodium fluoride and hypoxia can switch on several hypoxia inducible genes .Hypoxia-inducible factor-1 (HIF-1) is a transcription factor gene that regulates the adaptive response during its adaptation. It consists of a regulatory subunit HIF-1α which accumulates under hypoxic conditions. During hypoxia degradation of HIF-1α is suppressed, thus HIF-1α protein accumulates. During normoxic conditions also the HIF-1α gene is expressed but the HIF-1α protein undergoes degradation. For degradation of HIF- 1α requires hydroxylation of two proline residues. Hydroxylation is done by PHD and requires oxygen ,iron and α – ketoglutarate. HIF-1α induces expression of genes like erythropoietin, vascular endothelial growth factor and many others (Das *et al*., 2015). Oxidative and nitrosative stress is characterised by increase in reactive oxygen species (ROS) formation . ROS include free radicals like super oxide, peroxy nitrite and hydroxyl radicals. Hypoxia induces set of genes controlled by HIFs which are involved in cell survival, angiogenesis, glycolysis and metastasis. Studies have shown that like hypoxia , fluoride can also induce HIFs and other genes required for cellular adaptation (Lu *et al*., 2017) .

Vitamin C (L-ascorbic acid) is an important water soluble vitamin having antioxidant properties and is transported freely in the plasma. Vitamin C protects cells and tissues from oxidative stress due to the presence of 2, 3 -enediol in its structure. Vitamin C combats oxidative stress by acting against generated free radicals and down regulates the enzymes associated with free radical generation (Das, S *et al*.,2020 ; Reddy, R.C *et al*., 2020 ; Flora, 2009 ; Kaida *et al*., 2010). Vitamin C prevents lipid peroxidation and oxidative stress induced protein modification. Role of Vitamin C in preventing DNA damage have also been extensively studied. Invitro studies in human cells suggest that Vitamin C is important as antioxidant in maintaining genomic integrity by preventing oxidation induced mutations (Lutsenko *et al*., 2002).Studies also show neuroprotective effect of ascorbic acid is due to its antioxidant property in fluoride induced oxidative stress in hippocampus and motor cortex (Han *et al*., 2007). Many in vivo and in vitro experiments showed antiapoptotic effect of ascorbic acid by decreasing expression of Bax protein and by enhancing expression of Bcl-2 protein thereby preventing the apoptosis(Han *et al.,* 2007).Similarly in rats treated with vitamin E and C reduced endometrial apoptosis induced by fluoride toxicity (Guney *et al*.,

2007). Studies of Raina *et al*., have proved that Vitamin C supplementation is effective in preventing damage against fluoride and chlorpyrifos induced hepato toxicity . Vitamin C was also able to restore hepatic specific marker enzyme levels back to normal. Further few studies also evaluated the prophylactic role of Vitamin C. Vitamin C when given prophylactically has led to prevention of adverse effects like cardiomyopathy induced by chemotherapeutic drugs like doxorubicin used in the treatment of cancers. Vitamin C has also improved cardiac functions by reducing inflammation and also lead to up regulation of transporter proteins in cardiac muscle (Akolokar *et al*., 2017).Vitamin C also protects from hypoxic injury to cardiac myocyte structure and enhances myocardial metabolism for energy (Luo *et al*., 1998). Vitamin C protects transplanted kidney failure and its rejection caused by Ischemia reperfusion injury (Mohamed and Lasheen, 2014).Many lung diseases and chronic toxicity of lungs has been associated with decreased plasma levels of vitamin C. Administration of vitamin C in animal studies have led to resistance against such lung diseases and with improvements in plasma ascorbic acid levels (Hemila and Louhiala, 2007). Many researchers in their studies have enlightened the role of Vitamin C to modulate elements impacting genetic expressions and other functions related to cellular adaptation . Anti-apoptotic activity of Vitamin C against cell death induced by various environmental pollutants and protective role against metals can be attributed to its strong antioxidant property (Das *et al*., 2007;Flora, 2009).Vitamin C regulates the signaling pathways TNF alpha, NF-κB (nuclear factor kappa B), MAPK (mitogen- activated protein kinase), AP-1 (activator protein-1) during fluoride toxicity and oxidative stress (Bowie and O'Neill, 2000;Carcamo *et al*., 2002;Wholrab *et al*., 2019). Vitamin C prevents the activation of NF-kB in the endothelial cell line . NF-kB is stimulated by TNF and IL-1.. NF-kB is important in regulating gene expressions during cancers , inflammatory responses and in the development of atherosclerosis and altered cardiovascular functions (Bowie and O'Neill, 2000). Vitamin C regulates AP-1 activity by enhancing activity of Fos family transcription factors, prevents phosphorylation of JNK in UV –B induced oxidative cell injury and cell death (Catani *et al*., 2001) . Vitamin C modulates hypoxic pathway by enhancing HIF hydroxylase activity based on the intracellular concentration of vitamin C. PHDs are also dependent on increased intracellular Vitamin C levels to cause HIF-1α hydroxylation in clear cell renal carcinoma cell lines (Wholrab *et al*., 2019).So, with this framework ,it is our interest to find out the role of L- ascorbic acid in chronic hypoxia and sodium fluoride induced oxidative damage in cardiopulmonary and hepatorenal systems.

REVIEW OF LITERATURE

2.1.1 Physicochemical properties of Fluoride:

Fluorine is a nonmetal that belongs to the halogen family of the periodic table. Under standard conditions, fluorine is a univalent toxic pale, yellow-green acrid gas, has an atomic number of 9 and a molecular weight of 18.998gm/mol (Garcia and Borgnine, 2015)**.** Chemically, it is the most reactive electronegative element that readily forms fluoride compounds with other ions. Thus, never exist in the elementary state (US EPA, 1980).

Fluorine in an aqueous environment, predominantly occurs as fluoride ions (F^-). Fluoride compounds have unique chemical properties that make them highly diverse and thermodynamically stable. They are generally odorless, colorless, or white. Sodium fluoride (NaF) is a white powder or colorless crystalline solid with a moderate water solubility of 4%. (Sodium fluoride, PubChem; J. Fawell *et al*., 2006). Hydrogen fluoride (HF) is a highly corrosive colorless liquid or gas that has an exasperating odor (Hydrogen Fluoride, PubChem**).** When dissolved in water, it produces hydrofluoric acid, which is mainly used in industry. HF is highly toxic. Therefore, people who live in industrial areas or nearby high volcanic activity regions frequently exhibit fluoride-poisoning symptoms.

2.1.2 Distribution of Fluoride:

Fluorine is recognized as the 17th abundant element in the earth's crust, representing about 0.06%-0.09% (O' Mullane *et al*., 2016). It is widely distributed as inorganic fluoride in the lithosphere. Most fluoride sources are geological in origin, with few derived from anthropogenic contributions. Minerals such as hydrogen fluoride (HF), fluorspar (CaF_2), *villiuamite* , cryolite (Na_3ALF_6), fluorapatite ($Ca_5(PO_4)3F$), topaz ($Al_2(SiO_4)F_2$), and mica are noticeable forms of fluoride. They are abundant in areas of high volcanic activities, larger sedimentary basins in the semiarid condition, and regions that are underlain by igneous and metamorphic rocks, with concentrations as high as 2500 mg/kg (Singh *et al*., 2018; Garcia and Borgnino, 2015, O' Mullane *et al.*, 2016).

The fluoride dynamics in the natural ecosystem are based on two major geochemical processes: "1) dissolution and precipitation of fluoride-bearing minerals present in the dominant rocks and 2) fluoride adsorption/desorption from metal hydroxides and clay minerals" (Garcia and Borgnino, 2015).

Fluorides are naturally found in negligible amounts in the air, originating primarily from industrial pollution. Volcanic activities and other geochemical processes account for a fraction of the global fluoride atmospheric burden. In soil, fluoride usually is static and typically bound to other mineral compounds. Hence, present at a low concentration of 330 ppm. Soil acidity, particle size, and solubility of fluoride-bearing clay minerals are the few factors that govern the availability of free fluoride in the soil (O' Mullane *et al*., 2016; Liu and Zhang, 2014).

All water sources have some amount of fluoride in them, owing to the natural abundance. Nevertheless, the concentration varies according to geographic location and neighborhood to natural or human emission sources. Surface water such as lakes, streams, and wells have lower fluoride accumulation, generally below 0.3 mg/ L. Still, certain zones in the world, such as Lake Nakuru in the Rift Valley in Kenya, have recorded the highest fluoride concentration of 2800 mg/L(O' Mullane *et al*., 2016). In seawater, fluoride levels typically present within a narrow range of 1.2 to 1.4 mg/L . Whereas in the groundwater, which has fluoride levels below 1 mg/L , the concentration can reach up to a dangerously high level of 67 mg/L , attributed to pH and water-rock interactions by fluoride-bearing minerals (IPSC,2002, Narsimha and Sudarshan, 2016)**.** Geothermal waters, hot springs near volcanic areas, and alkaline and calcium deficient aquifers can also exhibit higher fluoride content (up to several hundred mg / L).

Human activities usually escalate undesirable levels of fluoride (particularly as HF) in the local environment. Among them, coal-burning factories, aluminium smelters, phosphate fertilizer manufacturers, fluoridation of drinking water supplies, and "other vast industries related to the ricks, glass, ceramic, iron, and steel production are the most important ones. (Garcia and Borgnino, 2015). Apart from this, using large quantities of chlorofluorocarbons in propellants and refrigerants also raises the global atmospheric fluoride burden, although the concentrations are relatively lower to natural environmental levels. (Garcia and Borgnino, 2015).

Fluoridated water and food are the two principal sources of fluoride exposure in humans, with levels varying depending on the region and individual. In fact, Asia is an epicentre for groundwater fluoride contamination. Many states in India, some parts of Pakistan, and southeastern China exceed the WHO standard of fluoride limits (Ali, 2006). Recently, some studies have highlighted the fluoride contribution of pharmaceutics and agrochemicals, ranging from 20%-40**%**. (Bharti, Giri and Kumar,

2017).Other sources that might cause increased fluoride exposure include inadvertent industrial or occupational exposure to sodium fluoride/ hydrofluoric acid, oral and dental care products like toothpaste and mouth rinse, beverages and dry foods contaminated with fluorinated water and salt. In addition, Teflon-coated cookware, fish, tea, and even cereal crops and vegetables grown in pesticide-laden fields and gardens are responsible for fluoride contamination (IPSC, 2002; Full and Parkin, 1975).

2.1.3 Pharmacokinetics of Sodium Fluoride

2.1.3a Absorption:

Fluoride absorption is a pH-dependent process, passive probably involving simple diffusion. Some fluoride compounds are absorbed better (e.g., sodium fluoride) than others. Typically, about 80%-90% of ingested fluoride is absorbed through the alimentary tract, with 40% taking place in the stomach as nonionic hydrogen fluoride**.** This process is inversely related to stomach acidity. Absorption across the oral mucosa is limited and accounts for <1% of the daily intake (O' Mullane *et al*., 2016).The absorption of fluoride in the small intestine is insensitive to a pH gradient. Fluoride absorption has a half-life of approximately 30 min after ingestion, with plasma fluoride levels rapidly increasing and peaking within 20–60 min. The base level is generally reached in 11–15 h due to the rapid uptake of fluoride ions by the calcified and soft tissues. Fluoride that has not been deposited in the body is excreted in the urine (Buzalaf and Whitford, 2011; Martínez-Mier, 2011).

The bioavailability of sodium fluoride is close to 100% on an empty stomach. However, some reports suggest gastric toxicity at higher fluoride levels, resulting in delayed absorption (Ekstrand and Spark, 1990). Also, foods rich in calcium, magnesium, and aluminium can form insoluble complexes with fluoride ions, lowering its systemic absorption and bio-availability to 70%-60%(IPSC, 2002; Ekstrand, Spak, and Vogel, 1990).

2.1.3b Distribution :

Fluoride distributes rapidly in body tissues after absorption, with 99 %of ions accumulating in ardent tissues such as teeth and bones (WHO, 2006). Plasma has similar or twice as high fluoride levels as the blood cells (IPSC, 2002).In plasma, fluoride exists in two forms - ionic and bound. Although the bound fluoride usually predominates and comprises lipid-soluble fluoride compounds, its biological role is not well understood

(Martínez-Mier, 2011). Alternatively, ionic fluoride directly reflects the fluoride content of water. It occurs in a dynamic equilibrium between plasma and other hard or soft tissues until a steady-state distribution is established (Figure-1).

Age has an inverse relationship with fluoride retention in the body. In adults, approximately 50% of fluoride is retained after absorption, while in infants, the corresponding Figure is about 75%. Other factors that can influence fluoride uptake include gender, modeling, and remodeling of bone. (Martínez-Mier, 2011; IPSC, 2002).

2.1.3c Elimination :

The kidney is the main route of fluoride elimination from the body, although a small quantity is also excreted through sweat (0.019-0.057 mg/L) and feces (10%). Saliva is also an important route of fluoride secretion, containing around 0.01 to 0.05 ppm. Salivary fluoride levels depend on plasma fluoride levels. Therefore, it is an important indicator in determining the role of fluoride in preventing dental caries (Dhar and Bhatnagar, 2009; O' Mullane *et al*., 2016).

In general, sodium fluoride renal clearance is higher (about 35 ml/min for a healthy adult) than other halides (less than 1.0 ml/min) and is regulated via urinary pH and flow rate variations. Low urine pH facilitates increased fluoride clearance and is therefore dependent on several factors such as age, diet, respiratory diseases, medications, and glomerular filtration rate (IPSC, 2002**).**

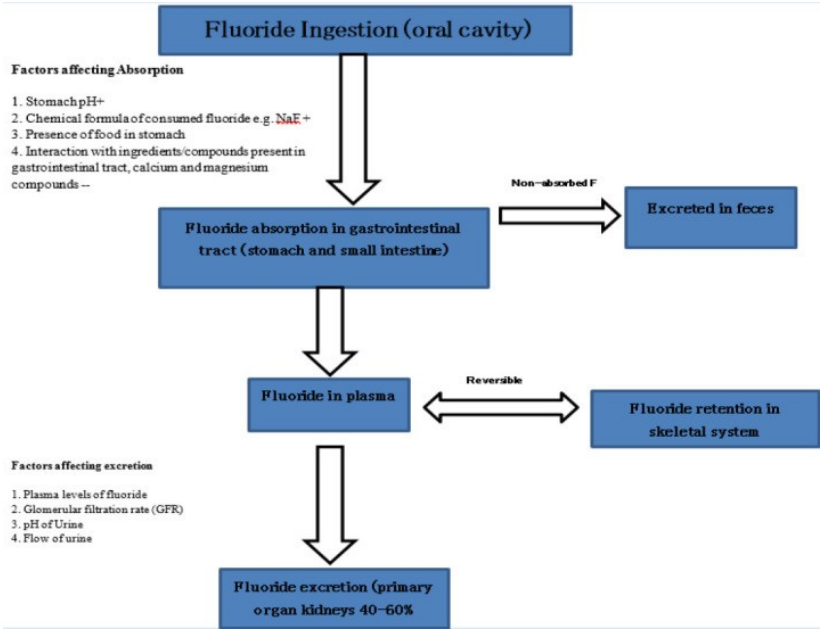


Figure-2.1 Schema of fluoride metabolism **(Source : Ullah, Zafar, and Shahani, 2017)**

2.1.4 Influence of Fluorosis on human health :

Fluoride toxicity can be severe or long-lasting in humans based on the dose and length of fluoride exposure. Acute toxicity mainly occurs due to accidental fluoridation overdosing. For a 70 kg adult, the lethal fluoride dose is 35–70 mg/kg body weight and for a 15 kg child, it is 1–2 grams (Dhar and Bhatnagar, 2009). Symptoms of acute fluoride exposure include nausea, abdominal pain, vomiting, cyanosis, hyperkalemia, hypocalcemia and even death within 2 to 4 hours. An expeditious administration of alkalizing agents in acute fluoride poisoning generally favors a good prognosis (Kanduti, Sterbenk, and Artnik, 2016).

Compelling data on chronic fluorosis have indicated serious toxic effects of fluoride on various body tissues, including the ones described below

2.1.4a : Dental and Skeletal effects :

Enamel/dental fluorosis refers to enamel hypomineralization resulting from avoidable fluoride intake during childhood. Clinical manifestations of dental fluorosis can vary from mild to severe. During the maturation of enamel, fluoride directly "affects the ameloblasts, the developing matrix, and processing of the matrix to cause dental fluorosis" (White and Meyer, 2019**).** Fluorotic teeth are resistant to tooth decay, but they afflict cosmetic appearance.

Skeletal fluorosis is known as endemic in several parts of the world, most notably in India, where fluoride consumption at a concentration up to 10 times lower than the WHO permissible limit of 1.5 mg L^{-1} has contributed to higher incidence (Srivastava and Flora, 2020**).** At the pre-clinical stage, the symptoms of skeletal fluorosis are not obvious and frequently misdiagnosed as rheumatoid arthritis or ankylosing spondylitis (San, Dey and Giri, 2016). The length and concentration of fluoride exposure determine the severity of symptoms. In some patients, bone changes are associated with osteomalacia, osteosclerosis, and secondary hyperparathyroidism.

2.1.4b : Genotoxic effects:

Fluoride's genotoxic effects have been debated for decades. Several in vivo animal studies have concluded the fluoride's role in inducing chromosome damage, with scanty human studies to back up this claim. Nevertheless, Manivannan *et al* (2013) have reported increased chromosomal aberration and DNA fragmentation in the bone marrow cells of

Swiss albino mice after 30 days of fluoride treatment. Similar findings were established in rat osteoblasts by Zhang *et al* (2006).Furthermore, positive results were reported from various genotoxic test systems, such as human cultured lymphocyte cells, foreskin fibroblast cells(JHU-1), promyelocytic HL-60, and zebrafish erythrocyte cells (Dey and Chattopadhyay , 2019).

2.1.4c : Reproductive effects:

Recently, fluoride effects on male and female reproductivity have gained enormous attention in the scientific community. Many identification studies have uncovered the impact of high fluoride doses on the reproductive health of the animal, for which currently the molecular mechanisms are not well understood. Commonly observed effects include increased luteinizing hormone, reduced testosterone and estrogen levels, oligospermia or azoospermia, decreased sperm motility, abnormal spermatozoa, decreased protein in the ovary and uterus, altered androgen to estrogen ratio, increased ROS generation, reduced SOD activity, and so on (Spittle, 2008 ; Dey and Chattopadhyay, 2019).

2.1.4d : Fluoride and Cancer :

Incidence of osteosarcoma was reported in experimental rat models when fed with fluoridated water (100 mg/L and 175 mg/L) for two years (O' Mullane et al., 2016). Similar descriptive studies on occupational fluoride exposure, particularly among aluminum smelters workers, have shown increased incidences of lung and bladder cancer and cancers of other sites (IPSC, 2002). Yet, none of the several population-based studies have given any conclusive evidence of fluoride causing cancer. Therefore, it is advised that available results must be interpreted with caution (Bajpai, 2013; Blakey *et al*., 2014; Crnosija, Choi, and Melikar, 2019). Higher fluoride levels have also been linked to thyroid dysfunction, type 2 diabetes, cognition, autoimmunity, and end-stage renal failure, with additional effects on the cell system being increasingly explored. (Kheradpishah et al., 2018; Krishna *et al*., 2020; Lantz *et al*., 1987; Grandjean, 2019).

2.1.5 Role of Fluoride on Oxidative and Nitrosative stress

Fluoride toxic effects on the function and physiology of the cell vary with time, dose, and cell type manner (Miranda et al., 2021). The main action of fluoride is to inhibit enzyme activity (such as for Na+/K +-ATPases, GTPases, and acid phosphatase).

However, it does stimulate enzymes at micromolar concentrations on occasions, thus exhibiting the hormesis effect (Strunecka and Strunecky, 2020).

According to growing literature reports, oxidative stress is the major malady of fluoride-mediated organotoxic effects (Barbier *et al*., 2010)**.** Oxidative stress refers to the oxidation-reduction imbalance characterized by excessive reactive oxygen species (ROS) relative to antioxidant production. Under normal conditions, the continuous production of ROS (namely, superoxide (O_2), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2)) during metabolic processes activates the antioxidant defense system, primarily the enzyme component for neutralizing ROS-induced cell injury. In chronic fluorosis, fluoride is thought to inhibit this enzyme component, including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and glutathione-s-transferase (GST), thereby generating increased oxidative stress. Despite this current knowledge, discordant results have been reported. Contrastingly, some studies have found that higher doses of fluoride administration significantly affect only CAT and GST levels but not SOD activity in human and rat liver cells (Miranda *et al*., 2018). Interestingly, a similar protective action of fluoride was noted by Reddy *et al*., (2003), where he spotted no significant difference in GSH, SOD, GSH-Px, and CAT activities in erythrocytes of fluorotic humans and fluoride-intoxicated rabbits.

Mitochondria is the primary site for ROS production; thus, the apparent target for fluoride toxicity. At mitochondria, fluoride alters the glutathione levels and results in excessive ROS generation (Liu, Chai, and Cui,2003;Miranda *et al*., 2021). In addition, it promotes ROS-induced lipid peroxidation of the mitochondrial membrane, releasing cytochrome C into the cytosol and attenuating ATP synthesis, eventually leading to apoptotic cell death.Accordingly, fluoride activates nitric oxide (NO) to hamper mitochondrial respiration and cause oxidation of mitochondrial DNA and proteins. (Miranda *et al*., 2021, Miranda *et al*., 2018)

ROS has various pathophysiological implications, owing to its ability to numerous cellular components, such as membrane lipids, DNA and protein, to trigger structural, biological and metabolic changes altering the intracellular redox homeostasis (Sharma and Chinoy, 1998).

Additionally, fluoride promotes the formation of malondialdehyde (MDA), an important biomarker of oxidative stress. Previously, Shivarajashankara *et al* (2001)

reported higher lipid peroxide levels in children with skeletal fluorosis in their erythrocytes. Shanthakumari *et al* (2004) reported similar findings in an experimental rat model. Even so, some investigators reported contradictory results. For example, Soni *et al* (1984) noted the increased generation of lipid peroxides in multiple rat tissues at lower fluoride doses. However, no such dose-effect relationship was established in the liver, testis, or lungs at higher fluoride concentrations. Similarly, Chlubek *et al* (1999) reported that higher fluoride concentrations had a less potent effect on increasing MDA levels on the studied mitochondrial fraction of the placenta. Conversely, the low fluoride concentrations had the most potent effect.

Furthermore, it is noticed that fluoride-mediated oxidative stress directly impacts protein regulatory activity, either by changing the structural conformation or chelating the cofactors (e.g., glutamine synthase) involved in amino acid biosynthesis. (Shashi, Singh, and Thapar, 1992; Trivedi, Verma, and Chinoy, 2006).Fluoride also interferes with the disulfide bond formation in the endoplasmic reticulum (ER), resulting in protein misfolding, ER stress, and ROS generation. (Barbier *et al*, 2010). Currently, studies on the genotoxic capabilities of fluoride are inconclusive. Although, a possible link between DNA damage and oxidative stress has been investigated. Recent work by Deng *et al* (2017) has reported ROS-dependent NF-Kappaβ signaling pathway in mediating the fluoride-induced DNA fragmentation and apoptosis in mouse splenocytes. Nevertheless, with this finding alone, no conclusion can be ascertained whether the increase or decrease of NF- Kappaβ is a defensive or adverse health response to fluoride intoxication. Some other investigators have highlighted the generation of ROS by SIRT1/autophagy through c-Jun N-terminal kinase (JNK) signaling in ameloblasts (Ribeiro *et al*., 2017).

Notably, the underlying fluoride cytotoxicity mechanisms rely upon the nature of the cell. However, these mechanisms and their regulations are poorly understood. Work on different mammalian cells and in vitro test systems has provided an insight into the fluoride's role in inducing oxidative stress and corresponding cell death (the extrinsic and intrinsic pathways) through MAP kinase signal transduction pathways. For instance, it has been observed that in pulmonary artery endothelial cells, fluoride intoxication activates the Ras/Raf/MEKK/MEK pathway to stimulate extracellular signal-regulated kinase (ERK). Likewise, a positive correlation with p38 MAPK activation was determined in the fluorotic individual's peripheral blood cells. (Agalakova and Gusev,

2012; Barbier *et al.,* 2010)**.** Fluoride is also known to cause excessive production of nitric oxide. However, it depends on the tissue source of fluoride action. In testis and gastric mucosa, fluoride induces nitrosative stress by triggering inducible nitric oxide synthase (iNOS) to produce excessive nitric oxide (NO) (Akimov and Kostenko, 2020). In blood, the fluoride effect could come from constitutive NOS (c NOS) (Sireli and Bulbul, 2004).

In fact, the role of oxidative and nitrosative stress in the molecular pathogenesis of fluoride is complex. Although different probable hypotheses have been proposed to explain the molecular pathogenesis of fluoride toxicity, none thus far has been conclusively established. Further research is thus necessary to fully comprehend the molecular mechanisms and markers of oxidative stress associated with fluoride toxicity.

2.2 Hypoxia :

Molecular oxygen (O_2) is necessary for all living organisms as it aids in energy production and acts as a crucial substrate in several biochemical reactions. In normoxia, the cell uses oxygen to maintain a high cellular ATP/ADP ratio, necessary for cell survival. Hypoxia can cause unbalanced cellular functions and even cell death if left untreated. The term hypoxia refers to the oxygen-deprived state of the body at the tissue and cellular level. Research on animals suggests that lacking oxygen can restrain numerous cellular and biological processes, compromising cell viability.Consequently, hypoxia plays an important role in many pathophysiological conditions, including chronic heart and kidney disease, ischemic diseases, cancer, inflammation, bacterial infections, metabolic disorders, and numerous reproductive diseases like preeclampsia and endometriosis (Chen *et al*., 2020).Variations in arterial blood oxygen may also occur as part of normal physiology during embryonic development, bone marrow microenvironment, or vigorous physical exercise.

A hypoxic cellular response can be transient, intermittent, or permanent, depending on the time scale. Chronic hypoxia is the long-term inadequacy of oxygen supply in the vasculature, as seen in the high-altitude residents, or during long-term inflammatory disease. The phase is characterized by altered gene transcription and protein synthesis to maintain oxygen homeostasis (Tomanek, 2013).

Unlike the upper respiratory tract (21% of oxygen), in many human cells and tissues, the normoxic oxygen tension does not reflect the ambient atmospheric oxygen

levels and typically ranges between 2 and 9%. Normoxia is only 10%–13% in arteries, lungs, and liver. Whereas for other tissues, the specific oxygen levels include "5% in venous blood, 1%–7% in the bone marrow, 0.5%–7% in the brain, and 1% in the cartilage" (Brennan et al., 2014).

2.3 Hypoxia- HIF cell signaling mechanism :

For all aerobic species, maintaining normoxic conditions is critical. Therefore, any change in the oxygen tension during hypoxia, anorexia, or hyperoxia could trigger an adaptive biological response. Despite this, the scientific minds are baffled how the cells detect acute and chronic oxygen tension changes and prepare the body for anti-hypoxia strategies. The answer lies in the underpinning mechanism of the transcriptional response pathway that is mediated through hypoxia-inducible factors (HIFs), which is regulated by the von Hippel–Lindau (VHL) and prolyl hydroxylases (PHD).

HIF is a heterodimeric helix-loop-helix PAS domain transcription factor that masterminds cellular and systemic adaptive responses to adapt to low oxygen levels and preserve oxygen homeostasis (Lee, Chandel, and Simon, 2020)**.** HIF activates a cascade of genes and transcriptional factors, including NF-kappaβ. The transcription factor has three members, HIF-1, HIF-2, and HIF-3, of which HIF-1 is best characterized (Brennan *et al*., 2014). The dimer has two subunits: 1) HIF-1α, which is the primary protein subunit that facilitates hypoxia-driven transcriptional response, and 2) HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator (ARNT), expressed constitutively in the cell (Berchner-Pfannschmidt *et al*., 2008).

During normoxia, HIF-1α undergoes several post-translational modifications to oppose hypoxia adaptive genetic programs. The transcriptional activity of HIF-1 α is delicately balanced, involving constant synthesis and proteasomal elimination degraded. The oxygen-activated prolyl hydroxylases (PHD) hydroxylate two proline residues (402 and 564) within the oxygen-dependent domain (ODD) of the HIF-1α protein **(**Fabian, 2018; Lee, Chandel, and Simon, 2020)**.** This modification "increases the affinity of pVHL-E3 ubiquitin ligase complex, which then mediates the rapid ubiquitination and degradation of HIF-1α by the 26S proteasome" (Yang *et al*., 2014**).** The second level of HIF-1 α hydroxylation occurs by asparaginyl hydroxylase, a Fe2+ and ketoglutarate-dependent dioxygenase family member. This "enzyme hydroxylates an asparagine residue in the C-terminal transactivation domain of the HIF-1α protein, preventing the attachment

of coactivators CBP(CREB binding protein) and p300 and curbing transcriptional activation of HIF-1α" (Lee, Chandel, and Simon, 2020).

Hypoxia prevents the hydroxylation and degradation of HIF-1 α, attributed to the oxygen dependence of all PHDs (PHD1-3). Once stabilized, HIF-1 α translocates into the nucleus and forms a dimer with HIF-1β. Subsequently, the complex recognizes hypoxia response elements (HREs) in the promoter regions of genes that regulate the switching of metabolism between oxygen-dependent to low oxygen adaptive state.

Fig. 2 summarises the cell signaling pathways that can be activated in response to hypoxic conditions. For example, angiogenesis, erythropoiesis, tumorigenesis, iron metabolism, intracellular pH regulation, multi-drug resistance, stem cell regeneration/ proliferation, and cell survival and proliferation are physiological responses in a cell experiencing hypoxia.

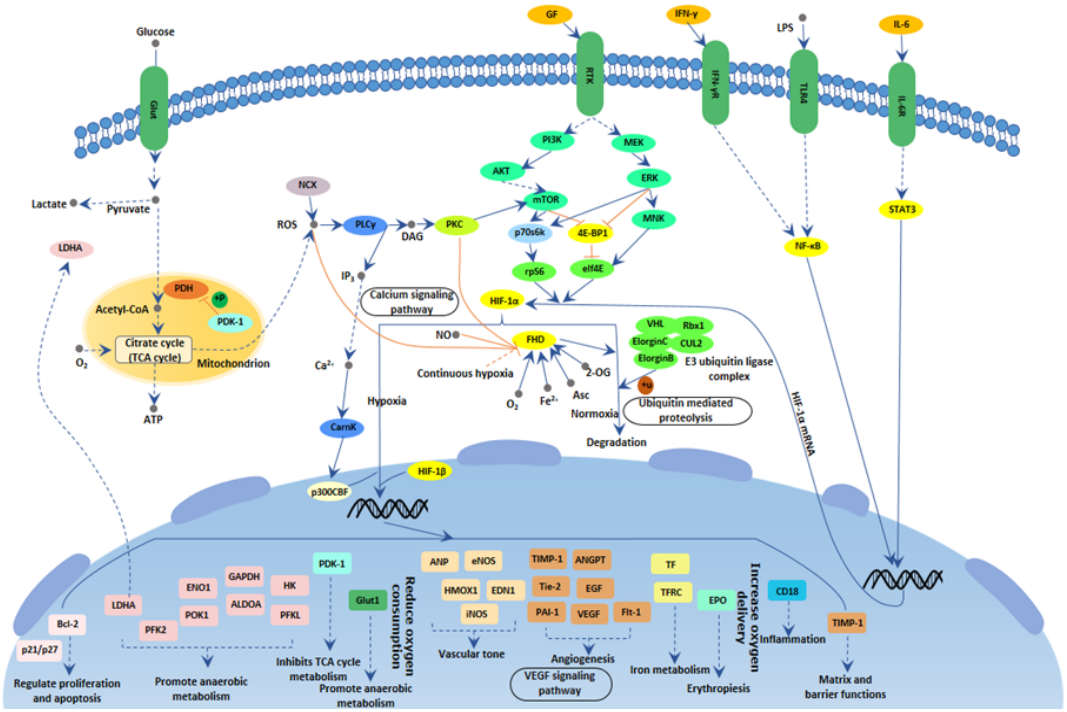


Figure 2.2 : Cell signaling pathways triggered during activation of HIF-1 transcription factors (Source: www.cusabio.com) accessed on 2/5/2021

2.4 L- Ascorbic Acid ($AscH_2$)

Ascorbic acid is a reduced and stable form of vitamin C found in the plasma in most plants and animals. It is a necessary micronutrient in humans due to the lack of l-gulonolactone oxidase enzyme for its biosynthesis. Ascorbic acid in most tissues is stored

in its oxidized state (dehydroascorbic acid), with the peak concentrations in the pituitary gland, adrenal glands, and neutrophils. However, the plasma level of ascorbate varies depending on the external dose supplementation**.** (Luck *et al*., 1995)**.** According to the current US daily allowance (RDA), an adult requirement for ascorbic acid falls between 100 and 120 mg/per day (Naidu, 2003).

Ascorbic acid is a highly effective redox buffer that quenches ROS and RNS reactivity that resulted from the mechanism of free radical generation, as shown in the reaction below.

$$AscH2 \text{ (or } AscH^{-}) + R^{\bullet} \rightarrow AscH^{\bullet} \text{ (or } Asc^{\bullet -}) + RH$$

Legend: $AscH^{-}$: Ascorbate ion; $R^{\bullet}$: free radical ion

Furthermore, it functions as a cofactor for several hydroxylases and oxygenases (such as dopamine β-hydroxylase, cytochrome b561 (Cytb561), and ascorbate peroxidase), keeping them in the reduced state (Akhbari *et al*., 2016)**.** As a cytosolic antioxidant, ascorbate efficiently regenerates the antioxidant abilities of oxidized alpha-tocopherol (via vitamin E redox cycle) and glutathione. Both antioxidant and cofactor properties of ascorbic acid are dose-dependent and differ depending on ascorbate concentration and the redox potential of the local cellular environment (Akhbari *et al*., 2016)**.**

The presence of redox iron (Fe2+) makes ascorbic acid pro-oxidant, leading to free radicals formation via Fenton-like reaction, preceding the oxidative modification of biomolecules. Nevertheless, in vivo, this mechanism is insignificant, considering the availability of iron (Fe 3+) ions in the system usually occupied by metal-binding proteins. Additionally, oxidized ascorbate is relatively unreactive and thus may not cause cellular damage (Pehlivan, 2017).

Various physiological processes in the human body are ascorbate-dependent, including immune stimulation, iron absorption, collagen synthesis, cholesterol to bile conversion, carnitine synthesis, cell division/proliferation, and hormones/ neurotransmitters synthesis (Rodríguez *et al*., 2017; Erkan *et al*. 2021). Its anti-inflammatory properties are attributed to the downgrading of the NF kappaβ signaling pathway, curbing the generation of proinflammatory cytokines (IL-6, TNF-α, and IFN-γ**)** (Jang *et al*., 2014; Ellulu *et al*., 2015**).**

Ascorbic acid is the first line of defense against oxidative stress generated by environmental toxicants. In a study by Ozkan *et al* (2011), ascorbic acid attenuated the sub-lethal doses of chlorpyrifos induced oxidative damage in the fish (*Oreochromis*

niloticus) liver and brain tissue. Another study by Fetoui *et al* (2008) reported that dietary supplementation of ascorbic acid had a protective role in reducing lambda-cyhalothrin's toxic effects. A previous study obtained similar results with the concurrent exposure of vitamin C and α-tocopherol against lead and nickel-treated rat brain tissue (Das *et al*., 2010).

More recently, Bhardwaj and colleagues (2018) have demonstrated the protective role of vitamin C against the oxidative stress parameters and apoptotic effect of glyco-phosphates in the goat antral follicles. They observed that supplementing vitamin C and E together can counteract the GLP effects, thus preventing granulosa cell apoptosis. Moreover, with the same dose and exposure length, vitamin C displayed more significant attenuation than vitamin E, probably because of the hydrophilic nature and redox-buffering capacity of vitamin C to restore oxidized tocopherol. Further, the combined exposure of both vitamins effectively boosted the antioxidant enzyme activity in granulosa cells, thus, successfully contravening the ROS chain reactions. Perez-Cruz, Carcamo, and Golde (2003) demonstrated that vitamin C inhibits Fas-mediated apoptosis in monocytes and U937 cells by decreasing the activities of caspase-3, 8, and 10, and lowering ROS levels, thereby protecting mitochondrial membrane integrity. A very recent study also examined the regulatory effects of ascorbic acid on the signaling pathways triggered during the toxic response to heavy metals (Das *et al*., 2020), supporting the hypothesis that ascorbic acid works in multiple ways on cells.

2.4.1 Role of L-ascorbic acid in mitigating sodium fluoride-induced toxicity :

Recently, several herbs and extracellular antioxidants have proven to help mitigate the toxic effects of sodium fluoride. Therefore, studies supporting ascorbic acid's protective role as a free radical scavenger suggest that it can counteract the toxic effect of fluoride on the body. Considerably, a study by Reddy, Sudhakar, and Nageshwar (2018) found that vitamin C enhanced the antioxidant system and reduced LPO and GSSG levels in the neural cells induced by fluoride. Furthermore, in a study by Yilmaz and Erkan (2015) Sertoli cells were exposed to 4 ppm and 20ppm concentrations of sodium fluoride to evaluate fluoride's role in reproductive toxicity. A significant decline in the biosynthesis of testosterone and viability of Sertoli cells at 20 ppm fluoride concentration was noted after 24h and 48 h of administration. Subsequently, vitamin C reversed the apoptosis/necrosis rate induced by fluoride.

In the same way, several other researchers have found the protective effects of vitamin C in various other tissues (the rat epididymis, the embryo, the liver, and the endometrium) against fluoride-intoxication. (Raina *et al*., 2015; Gunay *et al*., 2007; Gunay *et al*. 2007; Verma and Sherlin 2002; Serbecic and Beutelspacher 2005; Chinoy, Momin and Jhala, 2005). Of late, Peng and colleagues (2018) in F9 embryonic carcinoma cells have shown that vitamin C promotes the Sirt1-SOD2 signaling pathway to nullify sodium fluoride-induced mitochondrial oxidative distress. However, the precise underlying molecular mechanisms to explain how ascorbic acid negates oxidative stress and interacts with DNA and proteins is yet unidentified.

2.4.2 Role of L-ascorbic acid in mitigating hypoxia

Exposure to chronic hypoxia triggers several adaptive regulatory responses for cell survival, particularly at the transcriptional level. While on one side, short-term hypoxia supports the role of HIF-1, prolonged exposure has pathological consequences on organ structure and function. Hydrophilic antioxidant vitamin C has been used successfully against various tissue hypoxic effects. In the myocardium, vitamin C treatment improved mitochondrial function and prevented hypoxia-induced structural injury (Luo *et al*., 1998). According to Richter *et al* (2012), maternal supplementation with ascorbic acid may reduce maternal and placental indices of oxidative stress, thereby protecting the growing fetus from hypoxic injury and increasing the birth weight of the rat. Subsequently, in vivo maternal administration of vitamin C has been reported preventive against prenatal fetal hypoxia associated with adult cardiac dysfunction (Kane *et al*., 2013). Bautista-Oretega and Ruiz-Feria (2010) noted that the simultaneous supplementation of vitamins C, E, and arginine to broiler chickens helped reduce cardiovascular oxidative stress, thus improving pulmonary vascular performance. In another study, ROS scavenger in a concentration-dependent manner inhibited platelet aggregation by blocking platelet apoptosis during ischemia/perfusion (Liu *et al*., 2020).

AIM AND OBJECTIVES

3.1 AIM OF THE STUDY:

To evaluate the role of L-ascorbic acid on cardiopulmonary and hepatorenal system of normobaric-normoxic and normobaric hypoxic rats exposed to sodium fluoride.

3.2 OBJECTIVES OF THE STUDY:

1. To evaluate the supplementation of L-ascorbic acid as an antioxidant on chronic hypoxia induced alterations of cardiac autonomic functions with or without exposure to sodium fluoride.
2. To evaluate the supplementation of L-ascorbic acid as an antioxidant on chronic hypoxia induced alterations of hematological, biochemical parameters with or without exposure to sodium fluoride.
3. To evaluate the supplementation of L-ascorbic acid as an antioxidant on chronic hypoxia induced histopathological alterations of heart, lungs , kidneys and liver with or without exposure to sodium fluoride.
4. To investigate the exposure of sodium fluoride or hypoxia alone or in combination with or without administration of L- ascorbic acid on oxygen sensing molecules like VEGF and NOS3 (eNOS).

3.3 HYPOTHESIS

3.3.1 NULL HYPOTHESIS:

There will not be any significant effect of L-ascorbic acid supplementation on hypoxia-induced alterations of cardiopulmonary and hepatorenal pathophysiology in male albino rats with or without exposure to sodium fluoride.

3.3.2 ALTERNATE HYPOTHESIS :

L-ascorbic acid supplementation may improve hypoxia-induced alterations of cardiopulmonary and hepatorenal pathophysiology in male albino rats with or without exposure to sodium fluoride.

MATREIALS AND METHODS

4. MATERIALS AND METHODS

4.1 Study design: Animal study

4.2 Inclusion criteria: Healthy adult male albino wistar rats (Rattus Norvegicus*)*,8 -10 weeks old, weighing about 150g-180g were included in the study.

4.3 Exclusion criteria: Old rats , unhealthy and diseased rats were excluded from the study.

4.4 Ethical approval: The study was approved by the Institutional animal ethical committee(Ref: NMC/RCR/IAEC/2016-17/02) of Navodaya medical college , Raichur dated 18/01/2017.

4.5 Source of Data: Laboratory bred healthy adult male Wistar strain albino rats, aged 8-10 weeks weighing about 150-180 g were accommodated in the animal house of Navodaya medical college and hospital , Raichur, Karnataka, India. All the animals were allowed to acclimatize to the laboratory conditions for 7 days. The animals were maintained at 22-24^{0}C, exposed to 12 h light: dark cycle with water and food made available ad libitum. The rats were randomly assigned to one of the eight groups as follows (n=6).

4.5.1 Experimental groups

Table 4.1: Random allocation of experimental animals into groups

Sl.No	Group Name **(Intervention for 21 days)**
I	Control
II	L-ascorbic acid (50 mg / 100g. b.wt) (Hasanien P and Shaidi.S 2010)
III	Chronic Hypoxia (CH) (Das *et al*., 2015)
IV	NaF (20 mg / kg.b.wt., i.p) (Chen *j et al*., 2002)
V	Chronic Hypoxia (CH)+ NaF
VI	L-ascorbic acid + Chronic Hypoxia (CH)
VII	L-ascorbic acid + NaF
VIII	L-ascorbic acid + Chronic Hypoxia (CH)+NaF

NaF– Sodium fluoride; b.wt- body weight; i.p – intraperitonial

4.5.2 Experimental protocol:

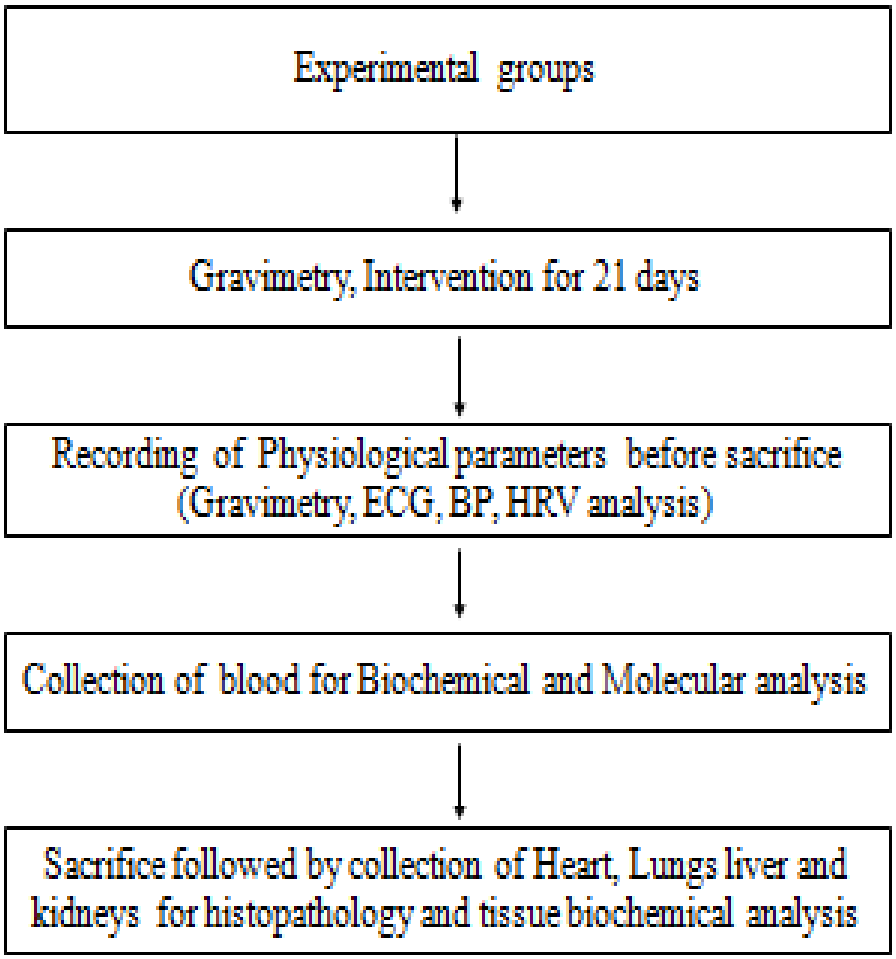


Figure 4.1 Summary of experimental protocol

4. 5.3 Exposure of animals to Chronic Hypoxia (CH):

For Chronic normobaric hypoxia treatment, caged rats (4 per cage) were kept in 300-liter acrylic chamber and were given mixture of 10% oxygen and 90% nitrogen to induce normobaric hypoxia. CO_2 was absorbed by soda lime 27 granules and excess humidity was removed by a desicator. Temperature was maintained at 24-26°C. The chamber was opened twice a week for 1 h to clean the cages and replenish food and water. Rats were exposed to hypoxiafor 21 days (Das *et al*., 2015)

4.6 Method of data collection:

4.6.1 Gravimerty :

Animals of all groups were weighed on the starting day of protocol (Initial body weight determination) and immediately after the end of 21st day (Final body weight determination) i.e. on the day of sacrifice using electronic weighing balance (Shimadzu BL3200HL Japan) . Percentage change of body weight gain was determined. Organs weight was determined and organosomatic index was also calculated.

$$\%\ change\ of\ \ body\ weight\ \ gain\ =\ \frac{Final\ body\ weight - Initial\ body\ weight}{Initial\ body\ weight}\ \times 100$$

$$Organosomatic\ \ index\ =\ \frac{Organ\ Weight}{Body\ Weight}\ \times 100$$

4.6.2 Evaluation of electrophysiological parameters:

4.6.2a Recording of Pneumogram:

The respiratory rate was recorded by using the respiratory pad transducer (BioPac student lab system) at the end of 21 days intervention period.

4.6.2b Recording of Blood Pressure:

Blood Pressure was recorded noninvasively (NIBP) using a tail-cuff sensor (BioPac 200A) after placing the animal in a restrainer. Systolic blood pressure (SBP) and Diastolic blood pressure (DBP) were obtained. Average of the three readings was considered from each animal. Mean arterial pressure (MAP) was calculated using the following formula.

$$Mean\ Arterial\ Pressure\ (MAP) =\ DBP + \frac{1}{3}\ (Pulse\ Pressure)$$

$$Pulse\ Pressure = SBP - DBP$$

4.6.2c Recording of ECG :

ECG was recorded after the intervention period using needle electrodes connected to Biopac Student Lab software 4.1 (BSL 4.1) .Animals were anaesthetized (Ketamine, 60mg/kg and Xylazine, 6mg/kg) and ECG was recorded in the morning hours following overnight fasting in animals. Heart rate was obtained from ECG.

4.6.2d Heart rate variability analysis for autonomic functions

HRV analysis was done by taking RR intervals from recorded ECG and RR intervals were exported to Kubios software. HRV analysis was performed by frequency domain method. Sympathetic activity, parasympathetic activity and sympathovagal balance were evaluated by the frequency domain method .

Table 4.2: Components of the frequency domain method of HRV analysis

Sl. No	Component	Indicator of
1.	Low frequency component (LF) (nu)	Sympathetic activity
2.	High frequency component (HF) (nu)	Parasympathetic activity
3.	LF/HF ratio	Sympathovagal balance

4.6.3. Collection of Blood

At the end of 21st day blood was collected by retro-orbital puncture from anaesthetized rats in Ethylene Diamine Tetra Acetic Acid (EDTA) coated tubes and plain tubes with clot activator for the purpose of haematological and biochemical analysis respectively.

4.6.4. The sacrifice of animals and tissue collection

The animals were sacrificed after 21 days of intervention by cervical dislocation . Rats were carefully dissected. Heart, lungs ,liver and kidneys were taken and each organ was weighed separately. The organosomatic index was calculated for each organ. Tissues were stored in 10% neutral buffered formalin for further histopathological examination and part of the liver was also stored at -20^{o}C for tissue homogenate preparation.

4.6.5 Haematological analysis: Analysis of blood was done by an automated hematology analyzer (Siemens ADIVA360) for determination of RBC count (million/cumm), WBC count(thousands/cumm), platelets(lakh/cumm), Hemoglobin (g%) and blood indicies.

4.6.6 Biochemical Analysis

4.6.6.1 Assessment of oxidant status:

4.6.6.1a Estimation of Serum Nitric oxide concentration by Greiss reaction (Moshage Han *et al*., 1995; Cortas and Wakid, 1990; Green *et al*., 1982)

- **Principle**

The stable product of nitric oxide is nitrate, nitrate was reduced to nitrite by cadmium reduction method after deproteination and coupling to N-naphthylethylene diamine. Spectrophotometer was used to measure the coloured complex product at a wavelength of 540 nm .

- **Reagents**

1. Glycine-NaOH buffer (pH-9.7): 7.5 grams of glycine was dissolved in 200 ml of distilled water. The pH was then adjusted to 9.7 by 2M NaOH and was diluted to 500 ml by distilled water.
2. Sulphanilamide: 2.5 grams of sulphanilamide was dissolved in 250ml of warm 3M/L HCl and allowed to cool.
3. N-Naphthylethylene diamine: 50mg N-Naphthylethylene diamine was dissolved in distilled water and the volume was adjusted to 250ml .
4. Cadmium granules: 2.5-3gm granules stored in 0.1M/L H_2SO_4.
5. Standard sodium nitrite solution.:

a. Stock standard(0.1mol/L)

690 mg of sodium nitrite was dissolved in 100ml of 10mmol/L sodium borate solution.

b. Working standard(10µmol/L)

10µl of stock sodium nitrite ($NaNO_2$) was diluted up to 100ml with 10mmol/L sodium borate solution.

6. ZnSO4 solution (75mmol/L)
7. NaOH solution (55mmol/L)
8. H_2SO_4 solution (0.1mol/L)
9. $CuSO_4$ solution (5mmol/L)

125mg of $CuSO_4$ was dissolved in 100ml of glycine-NaOH buffer.

- **Procedure**

a. Deproteinization: 0.5ml of serum was taken in a centrifuge tube. Clean and dry centrifuge tube was taken for this purpose. To this 2.0 ml of 75mmol/L $ZnSO_4$ solution was added and mixed. To this 2.5 ml of 55mmol/L of NaOH reagent was added. After this it was mixed well and centrifugation was done for 10 minutes. The supernatant was treated as a protein-free filtrate.

b. Activation of cadmium granules: Cadmium granules stored in 0.1 mol/L H_2SO_4 solution, were rinsed three times with distilled water during analysis. The granules were then swirled in 5mmol/L $CuSO_4$ solution for 1-2 minutes. The copper-coated granules were drained and washed by the glycine-NaOH buffer. These activated granules were used within 10min after activation. The granules after use were washed by distilled water and stored in 0.1mmol/L H_2SO_4 solution. Granules were activated every time by the same method .

c. Nitrite Assay:

1. Three Erlenmeyer flasks were taken and labelled as Blank (B), Test (T) and Standard (S).
2. 1ml of glycine-NaOH buffer was added to each Erlenmeyer flask. To the flasks labelled B (Blank), T (Test), S (Standard) 1ml of deionised water, 1ml of deproteinized sample and 1ml of the working standard were added respectively.
3. With a spatula, 2.5-3gm of freshly activated cadmium granules were added to each flask.
4. The contents were stirred to swirl the granules of all the flasks .
5. After 90 mins the mixture in all three flasks was diluted to 4ml with distilled water.
6. 2ml of this solution from respective flasks were pipetted in 3 clean dry test tubes labelled B, S, T respectively.

7. 1ml of sulfanilamide followed by 1ml of N-napthylethylene diamine solution were added to each tube.
8. All the three tubes were shaken well and after 20 mins, OD of S and T was read against blank at 540 nm on a spectrophotometer.

- **Calculations**

$$Nitric\ Oxide\ (\mu mol/L) = \frac{\text{OD of Sample}}{OD\ of\ Standard} \times concentration\ of\ standard \times Diluting\ factor$$

4.6.6.1b Estimation of Serum MDA by the method of Buege and Aust (Buege ,J.A. and Aust,S.D.1978)

- **Introduction**

  MDA (malondialdehyde) is produced by the degradation of polyunsaturated fatty acid (PUFA) .It is an indicator of lipid peroxidation. MDA is a reactive aldehyde and it is an reactive electrophile species causing oxidative stress.

- **Principle**

  MDA reacts with thiobarbituric acid (TBA) to form pink coloured compound. It is read at 535 nm by using spectrophotometer. MDA is the most reliable index to determine the extent of lipid peroxidation

- **Sample**: serum
- **Chemicals required**:

1. Malonaldehyde bis (dimethyl acetal)
2. Hydrochloric acid (HCl)
3. 2-Thiobarbituric acid (TBA)
4. Trichloroacetic acid (TCA)

- **Preparation:**

  1. MDA standard (stock-164 µg/ml)

     16.4µl of standard MDA solution was taken and made up to 100 ml using distilled water.

2. MDA working standard (working- 1.64 µg/ml)

   100 ml of the stock was made up to 10 ml using distilled water

3. TCA-TBA-HCl reagent

0.25 N HCl: 2.21 ml of concentrated HCl was made up to 100 ml with distilled water (DW). 15% TCA and 0.375% TBA was dissolved in 100ml of 0.25 N HCl. The reaction mixture was warmed to dissolve the contents and stored at 4^0C.

- **The procedure for standardization:**

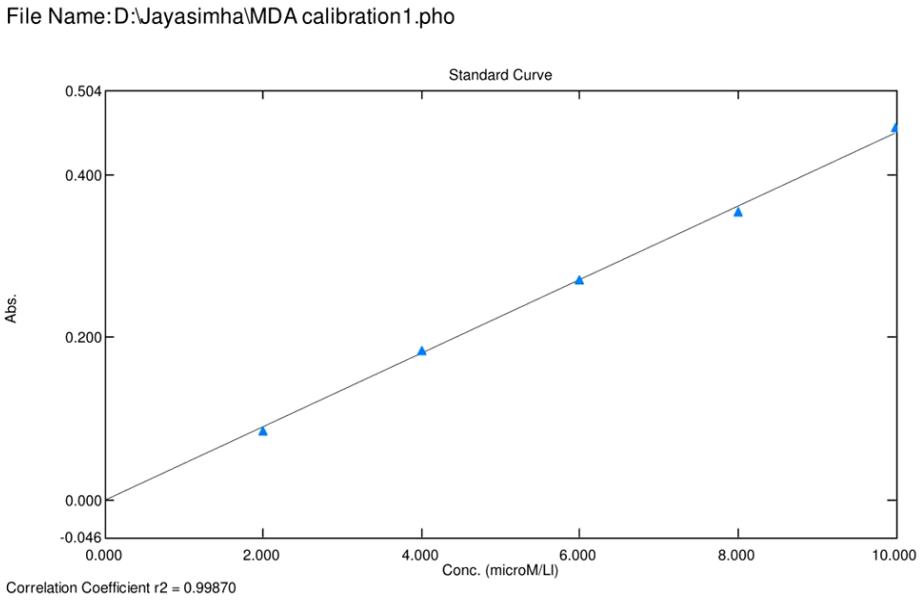
  Standardisation (Range 2-10 µM/L)

The standardization was done according to the table and all the reagents were added according to the values given in the table

Sl .No	Vol. of MDA (ml)	Vol. of DW (ml)	Conc. of MDA (µM/L)	TBA-TCA-HCl (ml)	Keep in boiling water bath for 15 min
B	0.0	1	0.0	1	
1	0.2	0.8	2.0	1	
2	0.4	0.6	4.0	1	
3	0.6	0.4	6.0	1	
4	0.8	0.2	8.0	1	
5	1	-	10.0	1	

Read O.D. absorbance at 535nm. The optical densities were plotted against the concentration on a graph.

Standard curve



- **Estimation of MDA in the sample:**

  Serum : 100µl serum was diluted to 500µl with distilled water

- **Procedure**

1. The diluted sample was taken to this 1ml of TCA-TBA-HCl reagent was added .
2. Samples were maintained for 15 minutes in a boiling water bath .
3. Then the reaction mixture was cooled and centrifuged.
4. The supernatant was pipetted and the optical densities of the pink colour formed were read at 535 nm by UV visible spectrophotometer (Shimadzu, Model: UV 1800).

- **Calculations**

The observed OD of the given test samples were directly proportional to the concentration of MDA and is calculated by plotting against the standard graph and multiplied by the respective dilution factors. The final concentration of MDA was expressed in µM/L.

4.6. 6.2 Assessment of Antioxidant status :

4.6. 6.2a Estimation of Serum Superoxide Dismutase (SOD) Activity

(Method – Marklund S & Marklund G.1998)

- **Principle :**

Auto-oxidation of pyrogallol involves Superoxide anion at pH - 8.5. The superoxide dismutase inhibits auto-oxidation of pyrogallol which can be determined as an increase in absorbance at 420 nm.

- **Reagents :**

1. Pyrogallol (20mM): 25 mg was dissolved in 10mL distilled water.
2. Tris buffer (0.05M) : 50mM of tris buffer and 1mM of EDTA was mixed with distilled water and HCL was added to adjust the pH at 8.5. A final volume of 100ml solution at pH 8.5 was prepared.

- **Procedure :**

1. Control : 2.9 ml of tris buffer was taken in a cuvette to which 0.1 ml of pyrogallol was added. Then absorbance (OD) was read at 420nm after 1min 30 sec and 3min 30 sec.
2. Test: 2.8 ml of tris buffer and 0.1 ml of serum was taken in a cuvette to which 0.1 ml pyrogallol was added. Then absorbance (OD) was read at 420nm after 1min 30 sec and 3min 30 sec.
3. Difference in absorbance (ΔA/min) was calculated as

$$\Delta A/min = \frac{OD\ at\ 3min\ 30\ sec - OD\ at\ 1min\ 30\ sec}{2}$$

Calculation

$$serum\ SOD\ activity = \frac{\Delta A/\min of\ control - \Delta A/\min of\ test}{\Delta A/\min of\ control \times 50} \times 100 \times \frac{1}{volume\ of\ sample}$$

$$= \frac{Control - Test}{Control \times 50} \times 100 \times \frac{1}{0.1}$$

$$= \frac{Control - Test}{Control \times 50} \times 1000 = \ldots u/ml$$

One unit of SOD is defined as the amount of enzyme required to cause 50 % inhibition of pyrogallol auto- oxidation .

4.6. 6.2b Estimation of Serum Vitamin E (α-tocopherol)

By the method of Jargar JG and Das KK (2012)

- **Reagents**

a. N-propanol
b. Absolute ethanol (aldehyde free)
c. Xylene (extra pure)
d. Ferric chloride (0.12%)
e. DL-α-tocopherol acetate
f. 2, 2'-Bipyridyl
g. Distilled water

- **Reagent preparation**

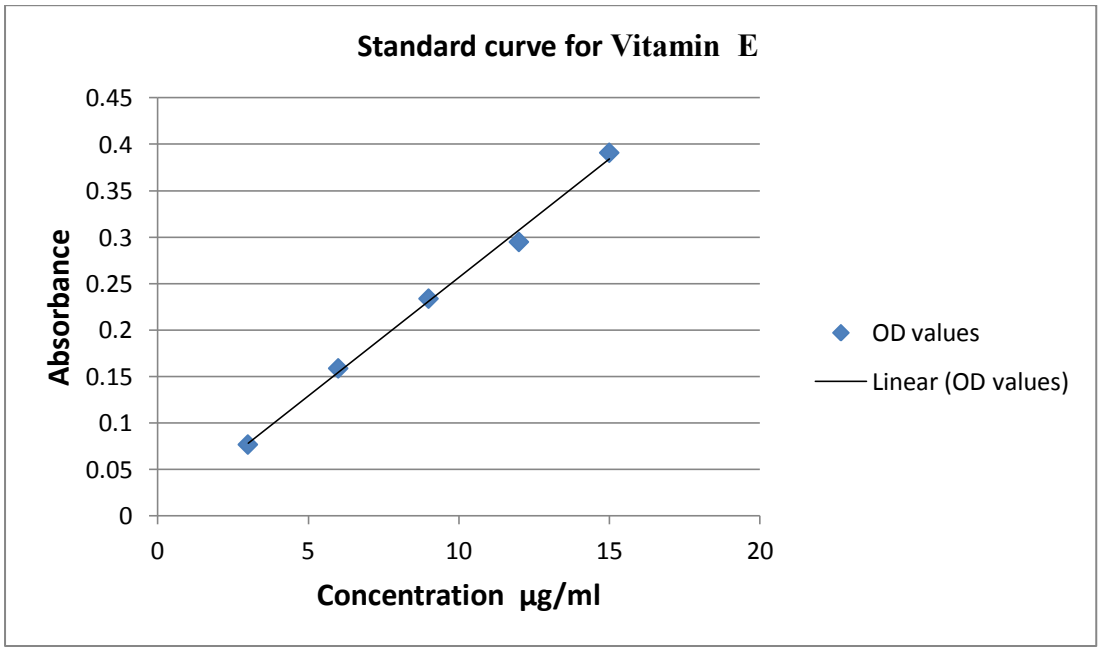
1. Ferric Chloride: (0.12% w/v): 120 mg of ferric chloride dissolved in 100 ml absolute ethanol and stored in a brown bottle
2. 2, 2' – Bipyridyl (0.12% w/v): 120 mg of 2, 2' bipyridyl dissolved and volume was made up to 100 ml in n-propanol and kept in a brown bottle.
3. Stock Standard (0.27% w/v): 270 mg of α-tocopherol acetate was diluted in 100ml of absolute ethanol and mixed properly.
4. Working Standard: (27µg/ml): 1ml of the stock standard was diluted to 100ml with absolute ethanol to obtain a concentration of 27µg/ml.

- **The procedure of standardisation**

6 centrifuge tubes were taken and labelled as B (Blank), S1, S2, S3 S4 and S5. 0, 150, 300, 450, 600 and 750µl of the working standard were added in respective tubes and the volume adjusted to 750µl using absolute ethanol. These solutions S1-S5 were equivalent to 4, 8, 12, 14, 16 and 20 µg/ml of α-tocopherol respectively using these solutions in the routine procedure as shown in the following table. The absorbance was read by using 200 µl of solutions prepared above including blank on plain ELISA microplate (non-antibody-coated) and read in ELISA reader at 492nm. A standard curve absorbance vs α-tocopherol (µg/ml) was plotted.

Sl. No.	Working Standard		Ethanol (µl)	Distilled Water	Xylene µl	Centrifuge for 10 min at 3000 rpm. Take 500µl of xylene layer	2,2'-Bipyridyl (µl)	$FeCl_3$ (µl)
	α-tocopherol µl	Conc. µg/ml						
B	0	0	750	750	750		500	100
S1	150	4	600	750	750		500	100
S2	300	8	450	750	750		500	100
S3	450	12	300	750	750		500	100
S4	600	14	150	750	750		500	100
S5	750	16	0	750	750		500	100

After 2 minutes, OD was read at 492 nm using UV visible spectrophotometer (Shimadzu, Model: UV 1800). The curve was drawn to determine the extent of adherence to the Beer-Lambert Law with various photoelectric instruments.



Analysis of serum Vitamin E:

- Sample preparation: 3ml of blood is allowed to clot in a centrifuge tube for 2 hours at room temperature and then centrifuged at 3000 rpm for 15 minutes. Serum sample is obtained. Serum for α- tocopherol estimation should be protected from sunlight and undue agitation. α- tocopherol darkens on exposure to light and slowly gets oxidised by atmospheric oxygen.

α-tocopherol was found to be stable in the collected serum at 25^0 C for 1 day, at 4^0C for 2 weeks and at -20^0 C for 2 months.

- **Procedure**

STEP 1*:* Two centrifuge tubes labelled as S and B (i.e sample and blank). To the sample tube, add 750µl absolute ethanol and 750 µl serum. Add the serum slowly with shaking to obtain a finely divided protein precipitate. To the blank add 750 µl of DW and 750 µl absolute ethanol. Stopper the tubes tightly by wrap paper and shake vigorously for at least 30 sec. To all the tubes add 750 µl of n-heptane. Again stopper the tubes tightly by wrap paper and shake vigorously for at least 30 sec and centrifuge all the tubes for 10 min at 3000rpm.

STEP 2*:* Transfer 500µl of xylene layer (supernatant) into properly labelled clean small size test tubes. To each tube add 500µl of 2, 2'-bipyridyl and 100µl of $Fecl_3$ solution and wait for 2 min.

STEP 3*:* Transfer 200µl solution from these tubes to plain microplate respectively. Readings are taken in ELISA reader at 492nm within 4 min.

- **Calculations**

$$Concentration\ of\ Vitamin\ E\ (\mu g/ml) = \frac{OD\ of\ Test - OD\ of\ Blank}{Slope} \times Dilution\ Factor$$

$$Slope = \frac{Y2 - Y1}{X2 - X1}$$

X and Y are concentration and absorbance of standard respectively

4.6.6.2c Estimation of Vitamin C (Ascorbic acid)

By Roe & kuether method

Vitamin C assay comprises standardization of chemicals and estimation of vitamin C in the sample.

- **Standardization of Vitamin C :**

Principle: The ascorbic acid is oxidized to diketogluconic acid in presence of strong acid solution which reacts with 2, 4, dinitrophenylhydrazine to form diphenylhydrazone which dissolves in strong H_2SO_4 solution to produce red colour which can be measured at 505nm (range of vitamin C 500-520 nm) spectrophotometrically.

- **Reagents**

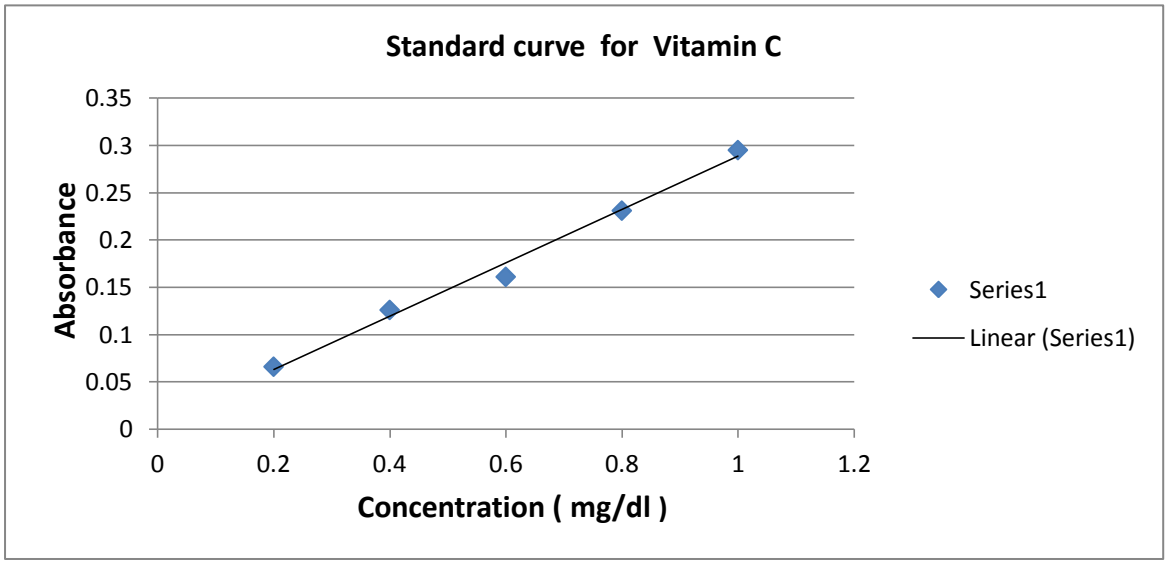
1. 2,4, dinitrophenylhydrazine (2,4, DNPH) : 2gm of 2,4, dinitrophenylhydrazine was dissolved in 9N H_2SO_4 and volume was made up to 100ml.
2. TCA (10%): 10gm of trichloroacetic acid (TCA) was dissolved in distilled water and the volume was made up to 100ml.
3. $CuSO_4$: 1.5 gm of $CuSO_4$ was dissolved in distilled water up to 100ml.
4. Thiourea: 10gm of Thiourea was dissolved in 100ml of 50% ethanol.
5. Combined color reagent (prepared fresh at the time of assay): 5ml 2,4 DNPH + 0.1 ml of $CuSO_4$ + 1ml Thiourea.
6. 85% H_2SO_4: 85 ml of H_2SO_4 was added in distilled water to make up to 100ml.
7. Stock Solution: 1gm of vitamin C dissolved in distilled water and made up to 100ml.
8. Working Standard: 1ml of stock solution was made up to 100ml with distilled water.

- The procedure of standardization:

Sl.No.	Conc. of Vit C (mg/dl)	Vol. of working standard (ml)	DW (ml)	TCA (ml)	Colour reagent (ml)	Mix well and keep in a warm water bath at 56^{0}C for 1 hour	Cooled in an ice bath for 5 min	85% H_2SO_4 (ml)
B	0	0	0.5	0.5	0.4			**2**
S1	0.2	0.1	0.4	0.5	0.4			**2**
S2	0.4	0.2	0.3	0.5	0.4			2
S3	0.6	0.3	0.2	0.5	0.4			2
S4	0.8	0.4	0.1	0.5	0.4			2
S5	1.0	0.5	0	0.5	0.4			2

20 minutes after adding 2ml of chilled 85% H_2SO_4 the reading was taken at 505nm by UV visible spectrophotometer (Shimadzu, Model: UV 1800). The optical densities were plotted against the concentration on a graph.

Standard Curve :



Estimation of Vitamin C in the sample

- **Sample preparation:**

1. Serum/plasma : 500 µl of the sample was added to 500 µl of 10% TCA. Mixed well for 10-15 sec. Centrifuged for 10min at 3500 rpm and supernatant is taken.
2. Tissue: To 500 µl of supernatant, 500µl of 10% TCA was added and centrifuged for 10 min at 3500 rpm and the supernatant was taken.

(Tissue homogenate prepration) : 500mg of tissue in 5ml of 0.9% NaCl was taken and homogenized for a few minutes. Centrifuged and the supernatant was taken for estimation).

- **Procedure**

After sample preparation, 500µl of supernatant was taken, 0.4ml of a colour reagent was added and kept in water bath at 56^0C for 1 hour. Then cooled and 2ml of chilled 85% H_2SO_4 was added and after 20 minutes the readings were taken at 505nm spectrophotometrically (Shimadzu, Model: UV 1800).

- **Calculations**

$$Vitamin\ C\ concentration = \frac{OD\ of\ TEST}{OD\ of\ Standard} \times \frac{\text{Concentration of Standard}}{\text{Volume of Test}} \times 100 = \ldots \text{mg/dl}$$

4.6.7 Molecular Markers:

4.6.7a. Estimation of Serum NOS3 by ELISA method

It was estimated with the help of Rat NOS3 ELISA Kit (Catalog No.: BYEK2703 Chongqing Biospes Co., Ltd, Chongqing, China). Instructors manual was followed during entire procedure

- **Principle**

Principle is based on sandwich enzyme-linked immune-sorbent assay. 96 well plates coated with the purified anti-NOS3 antibody are used. The anti-NOS3 antibody conjugated with HRP was used as detection antibodies. The standards, test samples and HRP conjugated detection antibody were added to the wells, mixed and incubated, and then unbound conjugates are washed away with wash buffer. TMB substrates (A&B) are used to visualize HRP enzymatic reaction. HRP catalyzes TMB to produce a blue colour product that changes into yellow after adding acidic stop solution. The density of yellow is proportional to the amount of NOS3 in the sample captured in plate. O.D. absorbance was read at 450 nm in a microplate reader, and the NOS3 concentration was calculated.

- **Range of detection** : 3 - 120 pg/ml
- **Kit components:**
    a. HRP conjugated anti-rat NOS3 antibody
    b. Standard (180 pg/ml)
    c. Standard diluent buffer
    d. Sample diluent buffer
    e. Wash buffer (30X): Dilution (1:30)
    f. Stop solution
    g. TMB substrate A
    h. TMB substrate B
    i. Plate sealer
    j. Hermetic bag
    k. One 96-well plate pre-coated with anti-rat NOS3 antibody

- **Preparation of sample and reagents**

Sample : Blood sample is allowed to clot for 10-20 min at 26^0C, and then centrifuged at 2000-3000 rpm for 20 min. Supernatant is collected. The supernatant is aliquoted and stored at -20^0C. Frequent freezing and thawing were avoided .

Wash buffer : It was diluted 30 fold (1:30) with distilled water.

Standard: 10 standard wells were set on the pre-coated plates and in the 1st and 2nd well, 100µl of the standard was added. Standard diluent buffer was taken and then 50µl of it was added to the above two wells and mixed properly. 100µl from the 1st and 2nd were transferred to the 3rd and 4th well respectively. 50µl of the standard diluent buffer was added to the 3rd and 4th well and mixed properly. 50µl was taken from the 3rd and 4th well and discarded and 50µl was transferred to 5th and 6th well. 50µl of the standard diluent buffer was added to 5th and 6th well and mixed . 50µl was transferred from 5th and 6th well to 7th and the 8th well. 50µl of standard diluents buffer was added to 7th and 8th well and mixed properly. 50µl from 7th and 8th well was transferred to 9th and 10th well. 50 µl of standard diluents buffer was added to 9th and 10th well and mixed properly. 50 µl was taken out from 9th and 10th well and discarded. After diluting, the loading volume for each well was 50 µl and the concentrations were 120 pg/ml, 80 pg/ml, 40 pg/ml, 20 pg/ml, 10 pg/ml.

- **Procedure to perform assay :**
    1. Kit components were taken and kept out for 15-30 minutes at room temperature before the assay procedure.
    2. Standard, test sample and control (zero) were set in the wells on pre-coated plate respectively. Their positions were noted. 50 µl of diluted standards (120 pg/ml, 80 pg/ml, 40 pg/ml, 20 pg/ml, 10 pg/ml) were added into standard wells. 50 µl of the standard diluent buffer was added into control well.
    3. In test sample wells, 40 µl of sample diluents buffer was added and then 10µl of the sample was added. The solution was added at the bottom of each well without touching the side well. The plate was mildly shaken to mix properly.
    4. Plate sealer was used to cover the plate and then incubated at 37^0 C for 30 min.
    5. Plate was washed manually after removing sealer. For this, the solution in the plate was discarded without touching the sidewalls. The plate was clapped on

absorbent filter paper. Each well was filled completely with wash buffer (1x) and vortexed mildly on ELISA shaker for 2 minutes, the contents of the plate were then taken. The plate was clapped on absorbent filter paper. The same procedure was repeated 4 more times for a total of 5 washes.

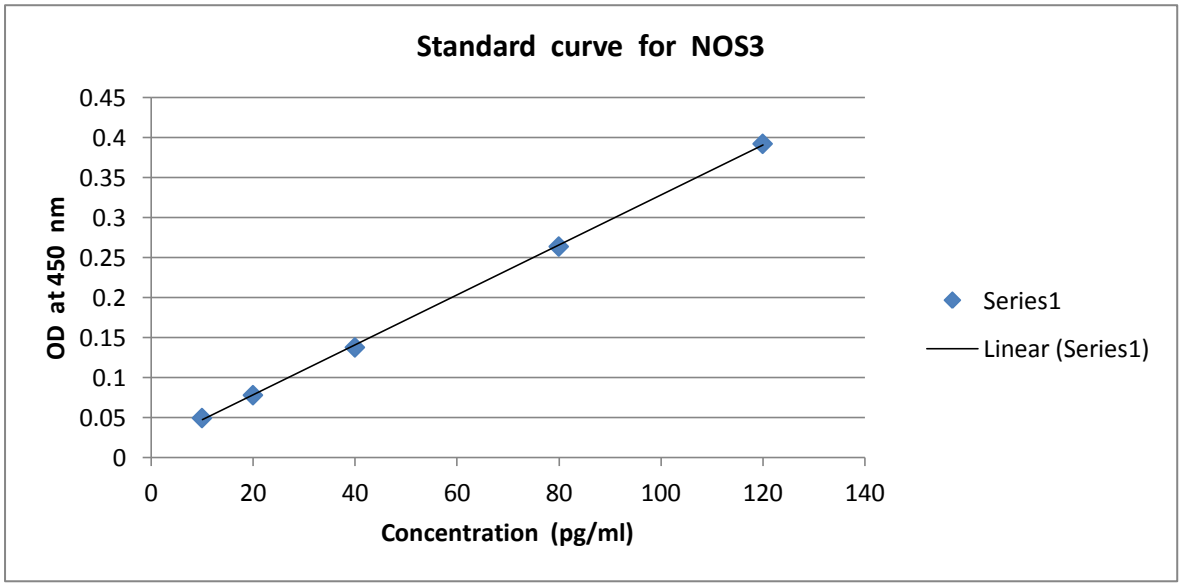
6. HRP conjugated anti-NOS3 antibody was added into each well (except control well). 50 µl of it was added.
7. Plates were sealed with plate cover and incubated at 37^0 C for 30 minutes and sealer was removed after 30 minutes and the plate was washed.
8. 50 µl of TMB substrate A was added into each well, followed by 50 µl of TMB substrate B. The plate was gently shaken by hand for 30 sec and incubated in dark at 37^0C for 15 min. The shades of blue were seen in the wells.
9. 50 µl of Stop solution was added into each well and mixed thoroughly. The colour changed into yellow immediately.
10. In a microplate reader, OD absorbance was read at 450nm .It was done within 15 min after adding the stop solution.

- **Calculations :**

Relative $O.D._{450}$= (O.D. $_{450}$ of each well) – (O.D. $_{450}$ of Zero well). The standard curve was plotted as the relative O.D. $_{450}$ of each standard solution (Y) vs. the respective concentration of the standard solutions (X). The NOS3 concentration of the samples was interpolated from the standard curve.

Standard Curve :

X	pg/ml	0	10	20	40	80	120
Y	OD_{450}	0	0.0498	0.0784	0.1381	0.2642	0.3926



4.6.7b Estimation of Serum VEGF by ELISA method

It was estimated by using Rat VEGF ELISA Kit (Catalog No.: BEK1228 Chongqing Biospes Co., Ltd, Chongqing, China) .Instructors manual was followed during entire procedure.

Principle :

Principle was based on sandwich enzyme-linked immune-sorbent assay technique. 96 well plates pre-coated with Anti-VEGF antibody were used. The biotin-conjugated anti-VEGF antibody was used as detection antibodies. The standards, test samples and biotin conjugated detection antibody were added to the wells and washed with wash buffer. The avidin-biotin-peroxidase complex was added and unbound conjugates were washed away with wash buffer. TMB substrates were used to visualize Avidin-Biotin-Peroxidase complex enzymatic reaction. TMB was catalyzed by Avidin-Biotin-Peroxidase to produce a blue colour product that changed into yellow after adding acidic stop solution. The density of yellow colour is proportional to the VEGF amount of sample captured in plate. OD absorbance was read at 450 nm in a microplate reader, and accordingly VEGF concentration can be calculated.

• **Range:** 15.6 pg/ml-1000 pg/ml

• **Sensitivity** < 1 pg/ml

• **Kit components:**

1. Lyophilized rat VEGF standards (10 ng/tube)
2. Sample/Standard diluents buffer
3. Biotin conjugated anti-rat VEGF antibody (1:100)
4. Antibody diluent buffer
5. Avidin-Biotin-Peroxidase Complex (ABC) (1:100)
6. ABC diluents buffer
7. TMB substrate
8. Stop solution
9. Wash buffer (25X)
10. One 96-well plate precoated with an anti-Rat VEGF antibody

- **Protocol :**

Preparation of sample and reagents

1. **Sample:** Blood was allowed to clot at room temperature and kept for 4 hours. Centrifuged at approximately 1500 rpm for 15 min. The serum was taken and stored at -20^0C.
2. **Wash buffer :** Wash buffer was diluted 25-fold (1:25) with distilled water.
3. **Standard*:*** Reconstitution of the lyophilized rat VEGF: the standard solution has to be prepared 2 hours prior to the experiment.
    a. 10,000 pg/ml of a standard solution : 1ml of sample/standard diluents buffer was added into one standard tube and kept at room temperature for 10 min and mixed.
    b. 1000 pg/ml of standard solution: 0.1ml of the above 10ng/ml standard solution was added into 0.9 ml of sample diluents buffer and mixed properly.
    c. 500 pg/ml of standard solution to 15.6 pg/ml of standard solutions : 6

Eppendorf tubes were labelled with 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml, 15.6 pg/ml, respectively 0.3 ml of Sample/Standard diluents buffer was aliquoted into each tube. To this 0.3 ml of the above 1000 pg/ml of standard solution was added into the 1st tube and mixed thoroughly. 0.3 ml from the 1st tube was transferred into 2nd tube and mixed properly. 0.3 ml form the 2nd was transferred to 3rd tube and mixed properly and so on.

4. **Preparation of biotin-conjugated anti-rat VEGF antibody working solution:** It should just be prepared 2 hours before the experiment.

    a. To Calculate the total volume of working solution: 0.1 ml/well X quantity of wells.
    b. Biotin conjugated anti-rat VEGF antibody was diluted with antibody diluent buffer at 1:100 and mixed . i.e. 1 µl of Biotin conjugated anti-rat VEGF antibody was added into 99 µl of antibody diluent buffer.

5. **Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution:** Prepared just 1 hour before the experiment.
    a. Calculation of the total volume of working solution : 0.1 ml/well X quantity of wells.
    b. Avidin-Biotin-Peroxidase Complex (ABC) is diluted with ABC diluents buffer at 1:100 and mixed thoroughly i.e 1 µl of Avidin-Biotin-Peroxidase Complex (ABC) is added into 99 µl of ABC diluent buffer.

- **Assay Procedure**

1. ABC working solution and TMB substrate were brought to room temperature,30 minutes before start of procedure.
2. Standard, test sample and control wells are set on pre-coated plate respectively and their positions are noted.
3. 0.1ml of 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml, 15.6pg/ml standard solutions are aliquoted into standard wells.

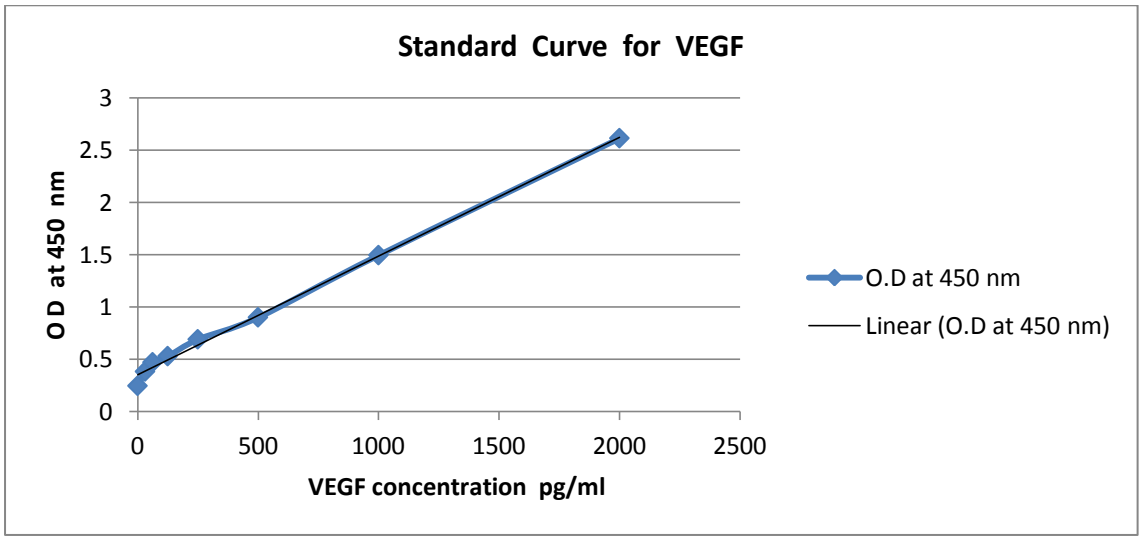
4. 0.1ml of sample/standard diluents buffer was added into control (zero) well.
5. 0.1ml of properly diluted sample was added into test sample wells.
6. The plate is sealed with a cover and incubated at 37^0C for 90 min.
7. The cover is removed and the plate contents are discarded and the plate is claped on the absorbent filter paper. Care should be taken not to let wells to dry at any time. The plate must not be washed.
8. 0.1ml of biotin-conjugated anti-rat VEGF antibody working solution was added into above wells (standard, test samples and zero wells). The solution was added at the bottom of each well without touching the sidewall.
9. The plate was sealed with a cover and incubated at 37^0C for 60 min.
10. The cover is removed and the plate was washed with wash buffer by manual washing. For this, the solution in the plate was discarded without touching the sidewalls. The plate was clapped on absorbent filter paper. Each well was filled completely with wash buffer and vortexed mildly on ELISA shaker for 2 min, the contents of the plate were aspirated. The plate was clapped on absorbent filter paper. The same procedure was repeated two more times for a total of three washes.
11. 0.1ml of ABC working solution was added into each well. The plate was covered and incubated at 37^0 C for 30 min.
12. The cover was removed and the plate was washed 5 times with wash buffer and each time the wash buffer was allowed to stay in wells for 1-2 min.
13. 90 µl of TMB substrate was added into each well. The plate was covered and incubated in dark for 25-30 min at 37^0 C. The shades of blue could be seen in the first 3-4 wells (with most concentrated rat VEGF standard solutions), the other wells showed no obvious colour.
14. 0.1ml of stop solution was added into each well and mixed thoroughly. The colour changed to yellow immediately.
15. In a microplate reader, OD absorbance was read at 450nm .It was done within 30 minutes after adding the stop solution.

- **Calculations**

  Relative O.D.$_{450}$ = (O.D. $_{450}$ of each well) – (O.D. $_{450}$ of Zero well). The standard curve was plotted as the relative O.D. $_{450}$ of each standard solution (Y) *vs*. the respective concentration of the standard solutions (X). The Rat VEGF concentration of the samples was interpolated from the standard curve.

- **Standard Curve :**

X	Pg/ml	0	15.6	31.2	62.5	125	250	500	1000	2000
Y	OD_{450}	0	0.247	0.384	0.472	0.531	0.692	0.902	1.497	2.616



4.7.. Histopathological examination :

The left ventricular myocardium, lungs, liver and kidneys were carefully collected, isolated immediately and fixed in freshly prepared 10 % formalin for 24 hours. All the fixed tissues were embedded in paraffin and thin sections were taken. Staining was done with hematoxylin and eosin and were subjected to Histopathological evaluations

4.8. Statistical Analysis :

- SPSS software version 16.0 (SPSS Inc., Chicago, USA) was used to analyse data.
- All the data obtained from control and experimental groups were analysed for the level of significance by using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test were done to find out intergroup significant differences.
- All values were represented as mean ± SD
- $p \leq 0.05$ was considered as statistically significant.
- Pearson's correlation was done between HRV analysis and oxidative parameters.

4.9. Ethical statement :

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) (vide letter: NMC/RCR/IAEC/2016-17/02 dated 18/01/2017). Guidelines of CPCSEA(Committee for the Purpose of Control and Supervision of Experiments on Animals) , Government of India were strictly followed during experimental procedures.

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RESULTS

5.1 GRAVIMETRY

Table 5.1.1: Comparison of body weight gain between experimental groups

Parameter	Group I	Group II	Group III	Group IV	Group V	GroupVI	Group VII	Group VIII	ANOVA	
									F Value	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]	0.24	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[b]	35.71	<0.0001*
% 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[b]	45.71	<0.0001*

Values expressed as Mean ± SD , one way ANOVA followed by Post Hoc Tukey's multiple comparison test, $p \leq 0.05$ was considered statistically significant, values with different superscripts a, b, c, d, e are significantly different from each other .

Our results from gravimetry **Table 5.1.1** shows initial body weight, final body weight and % of body weight gain values in experimental groups. Initial body weight was nearly same in all the groups and we did not observe any statistical significant difference in groups . Final body weight of all the groups was measured at the end of 21 days intervention. Results were analysed and compared by one way ANOVA followed by Post Hoc Tukey's test.We observed significant changes in final body weight in between groups. Rats of all groups showed an increase in body weight at the end of 21 days as indicated by % body weight gain. % body weight gain was determined and interpreted in

Figure 5.1 ,which illustrates comparison of % body weight gain in experimental groups of rats. The % body weight gain was significantly lower in CH, NaF and CH + NaF groups when compared to control and L-ascorbic acid supplemented groups. But supplementation of L –ascorbic acid on L-ascorbic acid + CH, L-ascorbic acid + NaF, L-ascorbic acid + CH+ NaF groups showed significant increase in % body weight gain when compared to CH, NaF and CH+NaF groups respectively.

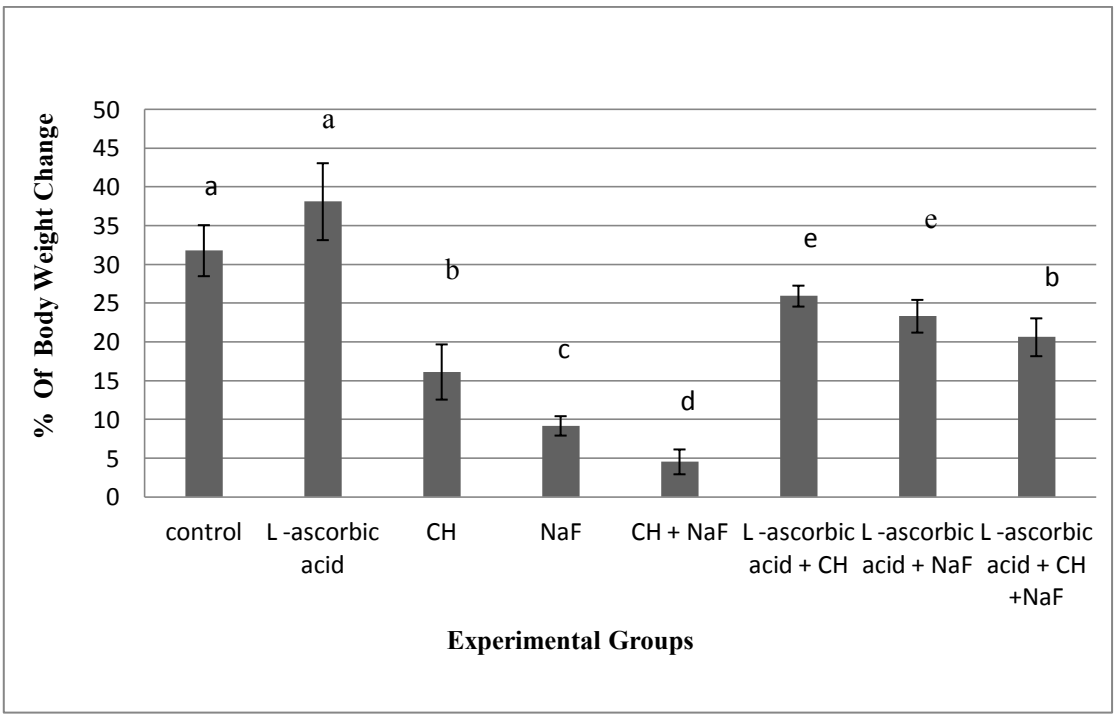


Figure -5.1.1 Comparison of % body weight change between groups

Values are expressed as Mean ± SD. Values with different superscripts (a, b c, d and e) are significantly different from each other ($p<0.05$), n=6 rats in each group.CH - chronic hypoxia.

5. 1.2 Organ weight determination and organosomatic index

Table 5.1.2 Determination of organ weight

Parameter	Group I	Group II	Group III	Group IV	Group V	GroupVI	Group VII	Group VIII	ANOVA	
									F Value	P value
Heart weight (gm)	0.78± 0.03[a]	0.83± 0.03[a]	0.52± 0.02[b]	0.49± 0.03[b]	0.38± 0.02[b]	0.65± 0.01[c]	0.61± 0.01[c]	0.61± 0.01[c]	147.10	<0.0001*
Lungs weight (gm)	1.04± 0.09[a]	1.23± 0.17[a]	0.70± 0.01[b]	0.67± 0.02[b]	0.87± 0.05[c]	0.8± 0.01[c]	0.6± 0.01[b]	0.76± 0.03[c]	25.87	<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]	111.20	<0.0001*
Kidneys weight (gm)	0.85± 0.02[a]	0.89± 0.04[a]	0.6± 02[b]	0.56± 0.04[c]	0.69± 0.02[b]	0.72± 0.02[b]	0.43± 0.02[c]	0.66± 0.03[b]	100.50	<0.0001*

values expressed as Mean ± SD , One way ANOVA followed by Post Hoc Tukey's multiple comparison test, $p \leq 0.05$ was considered statistically significant, values with different superscripts a, b, c, d are significantly different from each other.

Table 5.1.2 shows cardiopulmonary and hepatorenal organs weight. Statistically significant decrease in organ weight of heart, lungs, liver and kidneys were observed in CH, NaF and CH + NaF groups when compared to control and L-ascorbic acid supplemented groups. But L-ascorbic acid + CH, L-ascorbic acid + NaF, L-ascorbic acid + CH + NaF groups showed significant improvements in organs weight of heart, lungs, liver and kidneys when compared to CH, NaF and CH + NaF groups respectively.

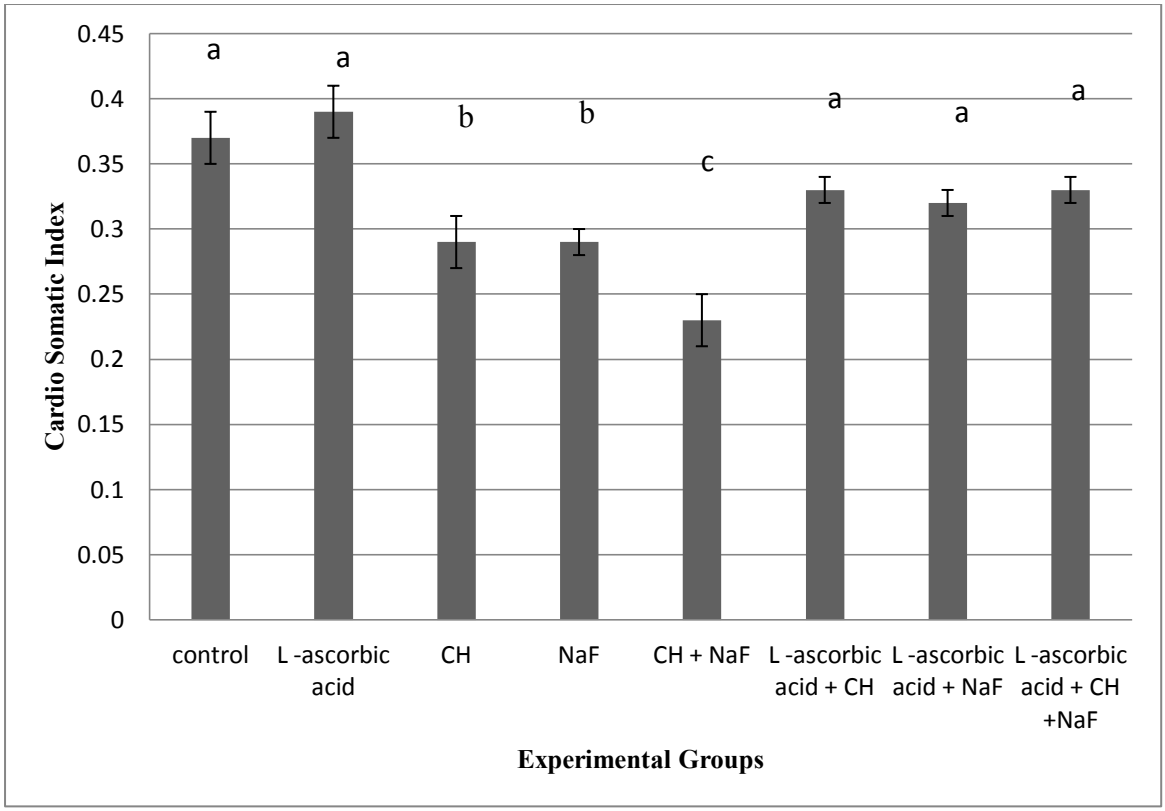


Figure -5.1.2a Comparison of cardio somatic index in experimental groups

Values are expressed as Mean ± SD. Values with different superscripts (a, b and c) are significantly different from each other ($p<0.05$), n=6 rats in each group. CH – chronic hypoxia.

Figure 5.1.2a shows comparison of cardio somatic index in experimental groups. Cardio somatic index was significantly decreased in CH, NaF and CH + NaF groups when compared to control and L-ascorbic acid supplemented groups. But L-ascorbic acid + CH, L-ascorbic acid + NaF, L-ascorbic acid + CH + NaF groups showed significant improvements in cardio somatic index when compared to CH, NaF and CH + NaF groups respectively.

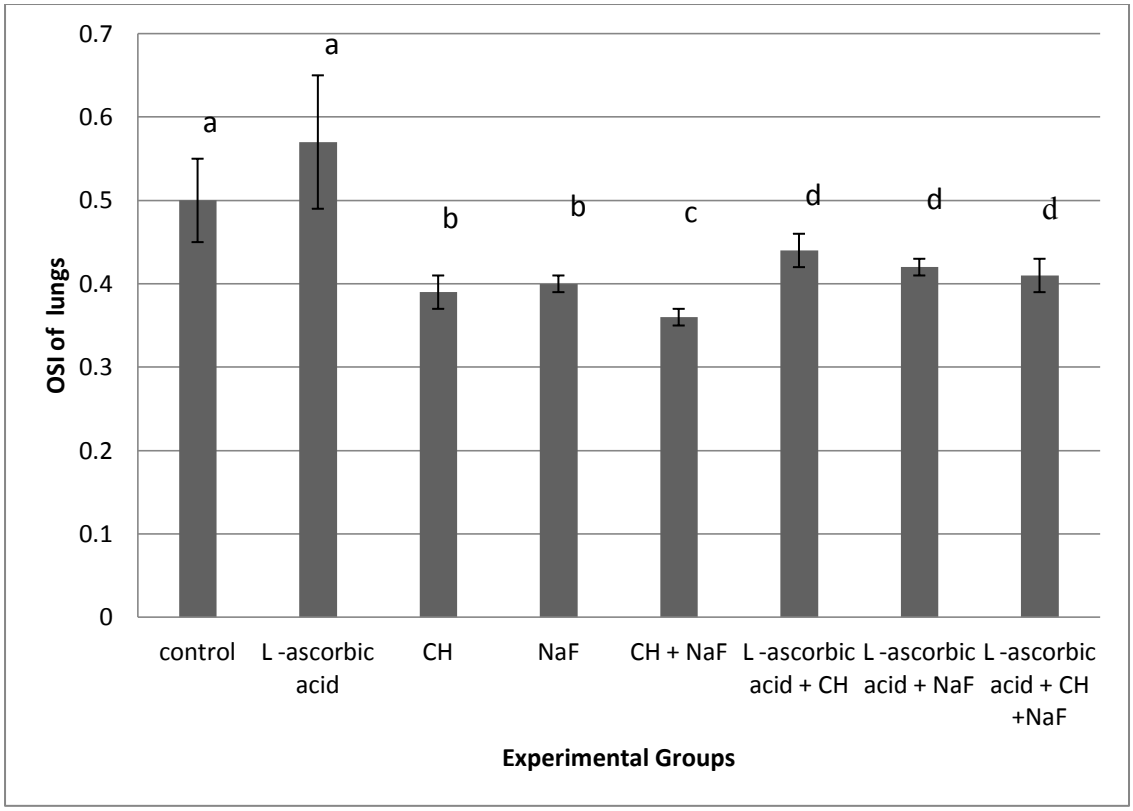


Figure -5.1.2b Comparison of organo somatic index of lungs in experimental groups

Values are expressed as Mean + SD. Values with different superscripts (a, b,c and d) are significantly different from each other ($p<0.05$), n=6 rats in each group.CH – chronic hypoxia .

Figure 5.1.2b shows comparison of pulmonosomatic index in experimental groups. Pulmonosomatic index was significantly decreased in CH, NaF and CH + NaF groups when compared to control and L-ascorbic acid supplemented groups. But L-ascorbic acid + CH, L-ascorbic acid + NaF, L-ascorbic acid + CH + NaF groups showed significant improvements in pulmonosomatic index when compared to CH, NaF and CH + NaF groups respectively.

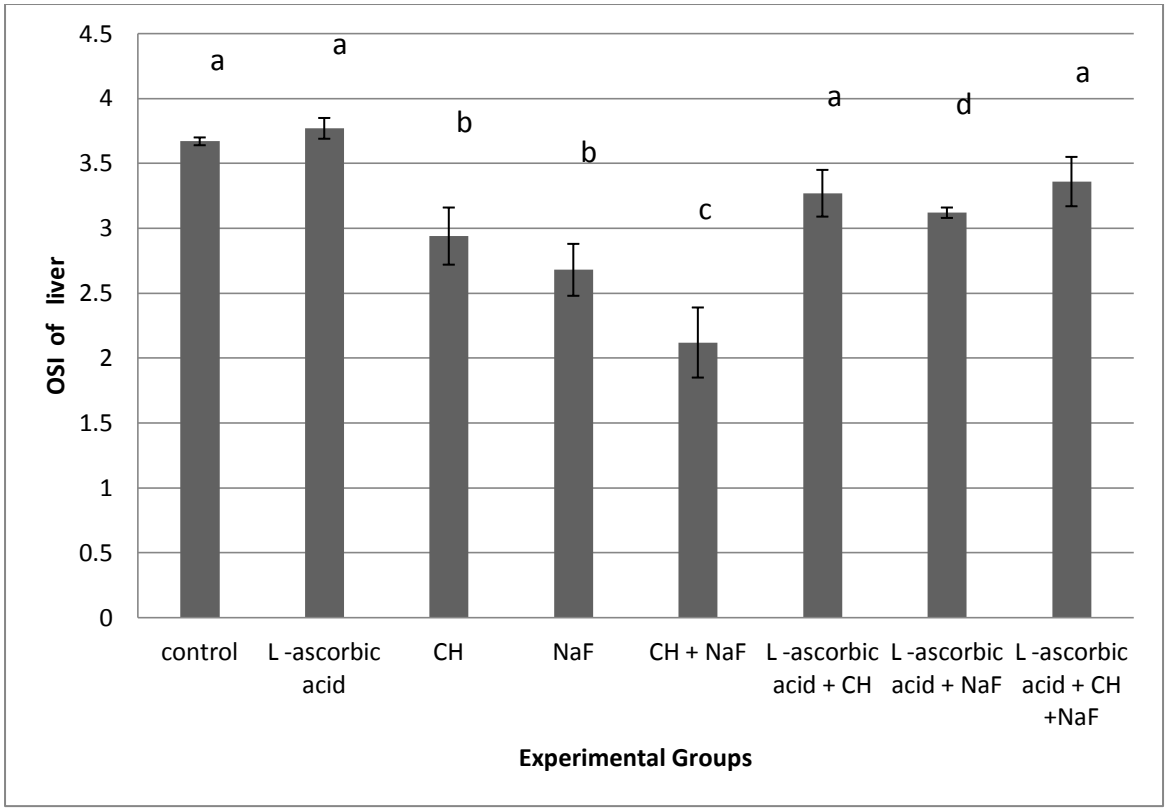


Figure5.1.2.c Comparison of organo somatic index of liver in experimental groups

Values are expressed as Mean + SD. Values with different superscripts (a, b, c and d) are significantly different from each other ($p<0.05$), n=6 rats in each group. CH – chronic hypoxia.

Figure5.1.2.c shows comparison of hepatosomatic index in experimental groups. Hepatosomatic index was significantly decreased in CH, NaF and CH + NaF groups when compared to control and L-ascorbic acid supplemented groups. But L-ascorbic acid + CH, L-ascorbic acid + NaF, L-ascorbic acid + CH + NaF groups showed significant improvements in hepatosomatic index when compared to CH, NaF and CH+ NaF groups respectively.

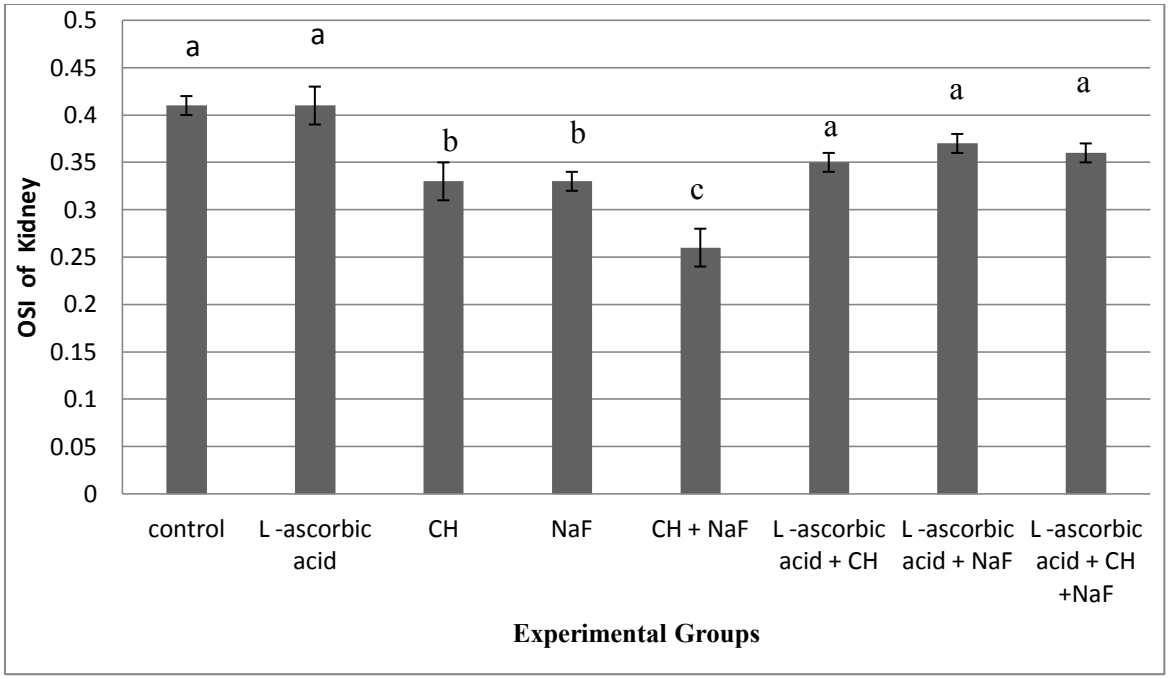


Figure 5.1.2.d Comparison of organo somatic index of kidney in experimental groups

Values are expressed as Mean + SD. Values with different superscripts (a, b and c) are significantly different from each other ($p<0.05$), n=6 rats in each group. CH – chronic hypoxia

Figure 5.1.2.d Shows Reno-somatic index among experimental groups. Reno-somatic index was significantly decreased in CH, NaF and CH + NaF groups compared to control and L-ascorbic acid supplemented groups. But L-ascorbic acid + CH, L-ascorbic acid + NaF, L-ascorbic acid + CH + NaF groups showed significant improvements in reno-somatic index when compared to CH, NaF and CH + NaF groups respectively.

5.2 ELECTROPHYSIOLOGY

5.2.1 Respiratory Rate and Heart Rate

Table 5.2.1: Comparison of respiratory rate and heart rate in experimental groups

Parameter	Group I	Group II	Group III	Group IV	Group V	GroupVI	Group VII	Group VIII	ANOVA	
									F Value	P value
Respiratory rate (cycles/ min)	18.33± 0.58[a]	16.67± 1.53[a]	26.67±2.52[b]	13.67 ± 1.53[c]	15.33±1.53[a]	18.33±1.53[a]	16± 1[a]	15.67± 0.58[a]	21.97	<0.0001*
Heart Rate (bpm)	313± 8[a]	308± 9[a]	360± 16[b]	156± 10[c]	211± 4[d]	311± 1[a]	256 ± 1[e]	227± 5[d]	146	<0.0001*

Values are expressed as Mean + SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison test. Values with different superscripts (a, b, c, d and e) are significantly different from each other ($p<0.05$), n=6 rats in each group.

Table 5.2.1 shows comparison of respiratory rate and heart rate in experimental groups. Statistically significant increase in respiratory rate was observed only in CH group when compared to control , L-ascorbic acid supplemented and other groups. Statistically significant increase of heart rate was observed in CH group. Whereas in case of NaF and CH+ NaF groups showed decrease of heart rate as compared to their respective controls. However L-ascorbic acid + CH and L-ascorbic acid + NaF, L-ascorbic acid + CH + NaF groups showed significant improvements in heart rate .

5.2.2 Mean arterial pressure (MAP):

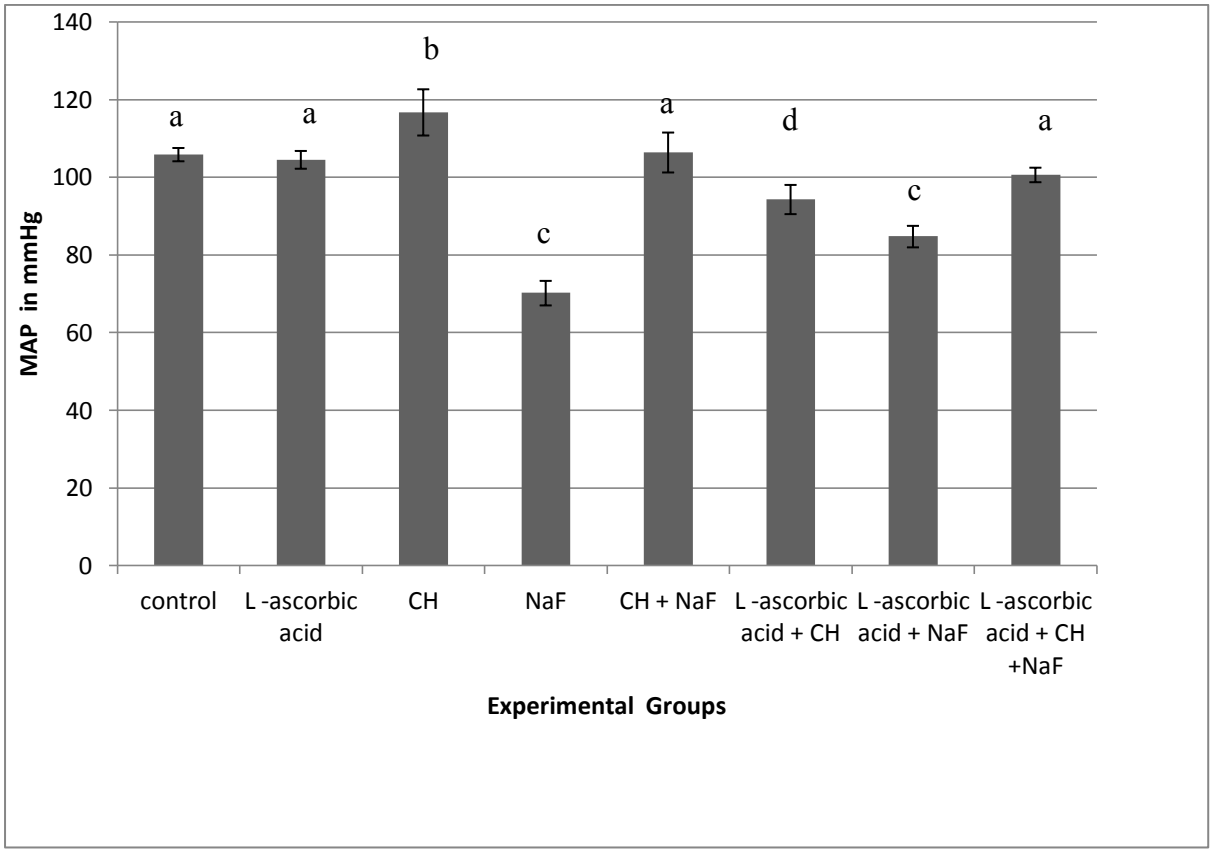


Figure 5.2.2: Comparison of MAP in experimental groups

Values are expressed as Mean ± SD. Values with different superscripts (a, b,c and d) are significantly different from each other ($p<0.05$), n=6 rats in each group. CH- chronic hypoxia,MAP- mean arterial pressure.

Figure 5.2.2 shows comparison of MAP in experimental groups. Statistically significant increase of MAP was observed in CH group. Whereas in case of NaF and CH + NaF groups showed decrease of MAP as compared to their respective controls. However L-ascorbic acid + CH, L-ascorbic acid + NaF and L-ascorbic acid + CH+ NaF rats showed significant improvements of MAP.

5.2.3 Heart rate variability(HRV) for cardiac autonomic functions : Frequency domain analysis

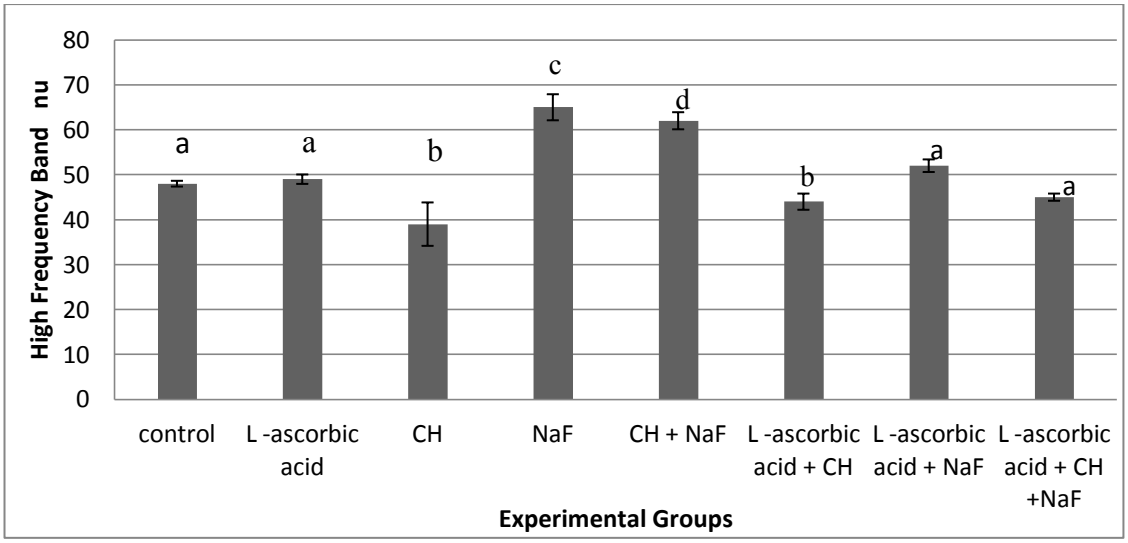


Figure 5.2.3a: Comparison of high frequency band in experimental groups

Values are expressed as Mean ± SD. Values with different superscripts (a, b ,c and d) are significantly different from each other ($p<0.05$), n=6 rats in each group. CH – chronic hypoxia.

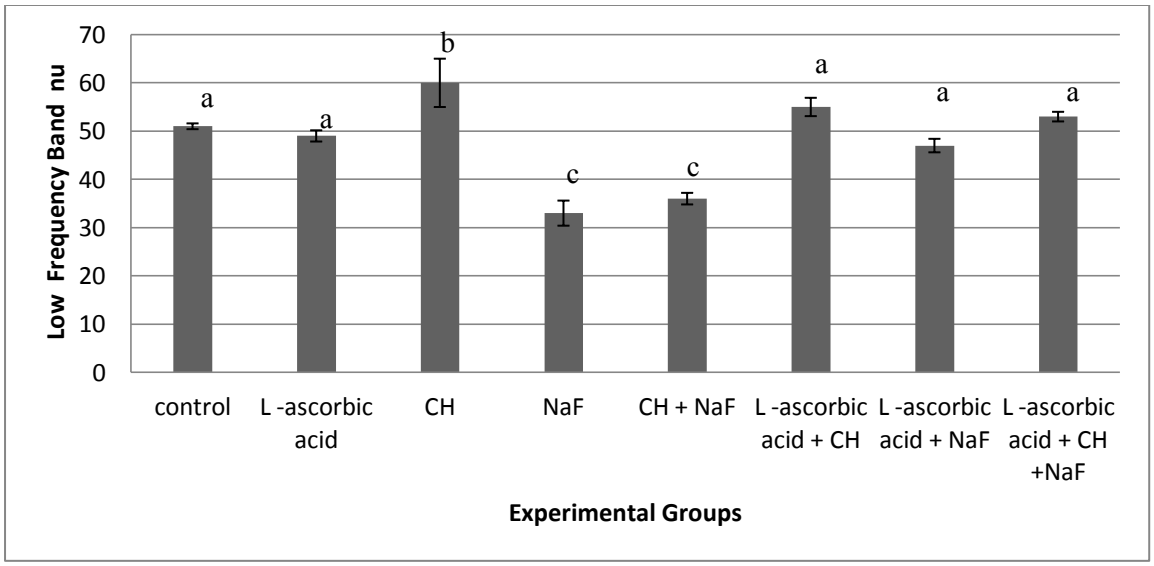


Figure 5.2.3b: Comparison of low frequency band in experimental groups

Values are expressed as Mean ± SD. Values with different superscripts (a, b and c) are significantly different from each other ($p<0.05$), n=6 rats in each group. CH – chronic hypoxia.

HRV analysis was done to assess the level of sympathetic activity(LF band), parasympathetic activity(HF band) and sympathovagal balance(LF/HF ratio).

Figure 5.2.3a and 5.2.3b shows comparison of HF and LF power band among experimental groups. HF power band of HRV analysis showed a significant increase in NaF and CH+ NaF groups as compared to control group. In case of LF power band, NaF and CH+ NaF groups showed significant decrease as compared to control group. In case of CH group increase in LF power band and decrease in HF power band were noticed. However L-ascorbic acid + CH, L-ascorbic acid + NaF and L-ascorbic acid + CH+ NaF groups showed significant improvements in LF and HF power band.

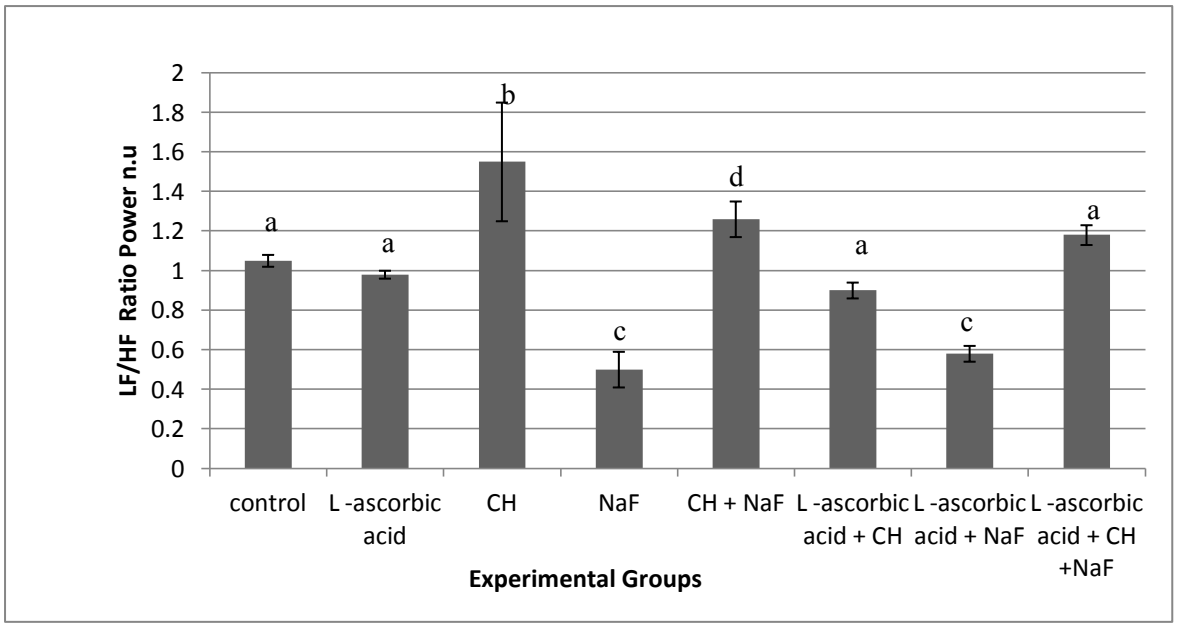


Figure 5.2.3c: Comparison of LF/HF ratio in experimental groups

Values are expressed as Mean ± SD. Values with different superscripts (a, b,c and d) are significantly different from each other ($p<0.05$), n=6 rats in each group. CH – chronic hypoxia.

Figure 5.2.3c shows comparison of LF/HF ratio among experimental groups increased sympathetic activity was observed due to increase in LF/HF ratio in CH group whereas increased parasympathetic activity was observed due to decrease of LF/HF ratio in NaF and CH+ NaF groups as compared to their respective controls. However L- ascorbic acid+ CH, L -ascorbic acid + NaF and L-ascorbic acid +CH + NaF groups showed significant improvements in LF/HF ratio.

Analysis Of Frequency Domain Results Of Rat In Each Group

Control (Rat-2)

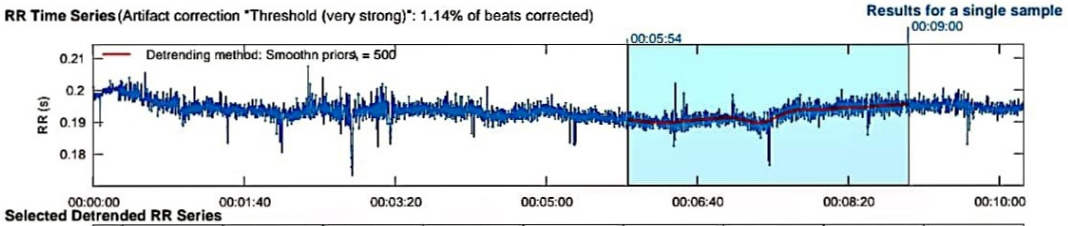
gp2b hrv.txt - xx/xx/xx - xx:xx:xx

HRV Analysis Results

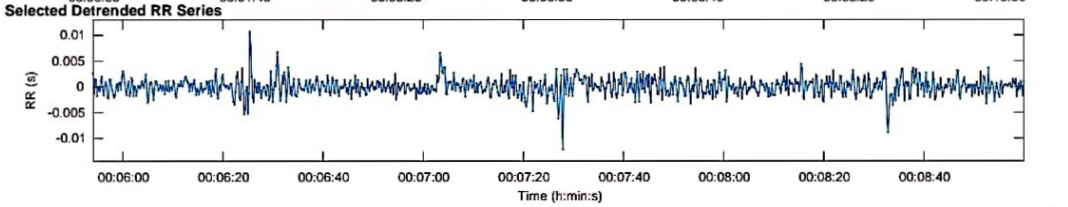
Page 1/1

RR Time Series (Artifact correction *Threshold (very strong)*: 1.14% of beats corrected)

Results for a single sample



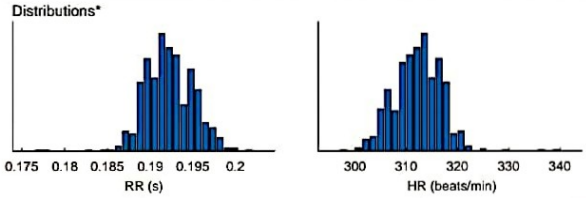
Selected Detrended RR Series



Time-Domain Results

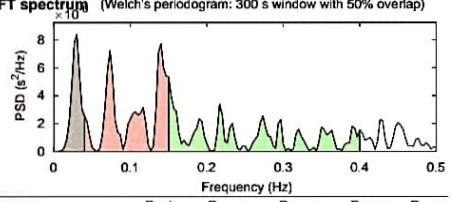
Variable	Units	Value
Mean RR*	(ms)	192.2
STD RR (SDNN)	(ms)	1.7
Mean HR*	(beats/min)	312.11
Min/Max HR	(beats/min)	303.64/321.54
RMSSD	(ms)	2.0
NNxx	(beats)	0
pNNxx	(%)	0.0
RR triangular index		1.042
TINN	(ms)	15.0

Distributions*



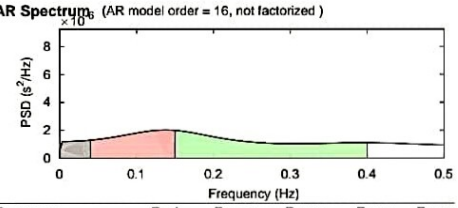
Frequency-Domain Results

FFT spectrum (Welch's periodogram: 300 s window with 50% overlap)



Frequency Band	Peak (Hz)	Power (ms²)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0300	0	-2.167	17.0	
LF (0.04-0.15 Hz)	0.1400	0	-1.243	42.9	51.8
HF (0.15-0.4 Hz)	0.1500	0	-1.324	39.6	47.7
Total		1	-0.397		
LF/HF		1.085			

AR Spectrum (AR model order = 16, not factorized)

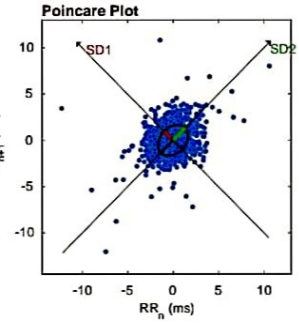


Frequency Band	Peak (Hz)	Power (ms²)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0400	0	-3.031	8.7	
LF (0.04-0.15 Hz)	0.1367	0	-1.661	34.2	37.5
HF (0.15-0.4 Hz)	0.1500	0	-1.157	56.7	62.1
Total		1	-0.589		
LF/HF		0.604			

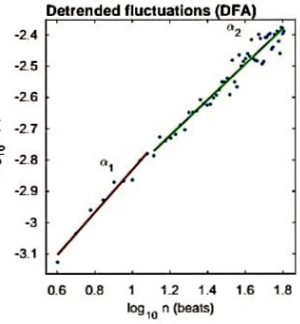
Nonlinear Results

Variable	Units	Value
Poincare Plot		
SD1	(ms)	1.4
SD2	(ms)	1.9
SD2/SD1		1.362
Approximate Entropy (ApEn)		1.529
Sample Entropy (SampEn)		1.837
Detrended Fluctutation Analysis (DFA)		
Short-term fluctuations $\alpha 1$		0.682
Long-term fluctuations $\alpha 2$		0.570

Poincare Plot



Detrended fluctuations (DFA)



*Results are calculated from the non-detrended selected RR series.

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Kubios HRV Standard (ver. 3.0.2)
Kubios Ltd. - www.kubios.com

Rat Treated With L-Ascorbic Acid (Rat-2)

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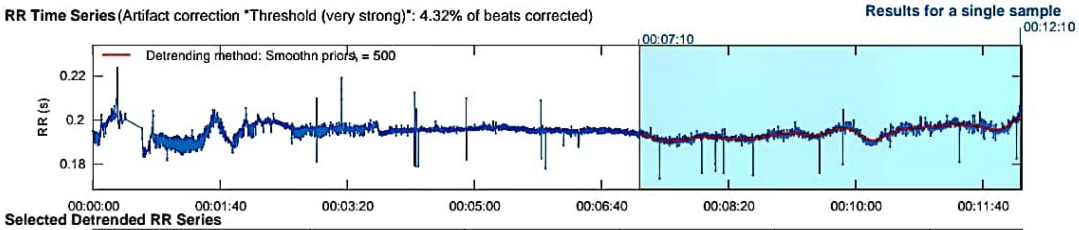
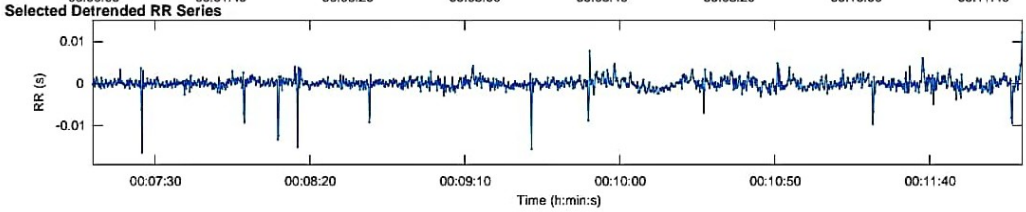
HRV Analysis Results

RR Time Series (Artifact correction "Threshold (very strong)": 4.32% of beats corrected)

Results for a single sample



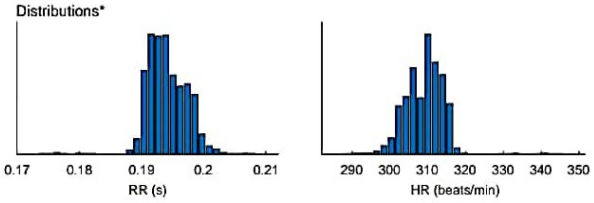
Selected Detrended RR Series



Time-Domain Results

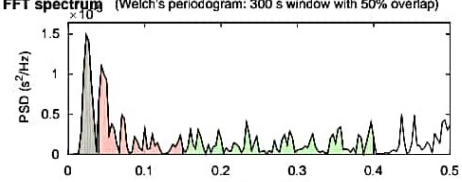
Variable	Units	Value
Mean RR*	(ms)	194.1
STD RR (SDNN)	(ms)	1.6
Mean HR*	(beats/min)	309.09
Min/Max HR	(beats/min)	291.40/320.62
RMSSD	(ms)	1.7
NNxx	(beats)	0
pNNxx	(%)	0.0
RR triangular index		1.032
TINN	(ms)	19.0

Distributions*



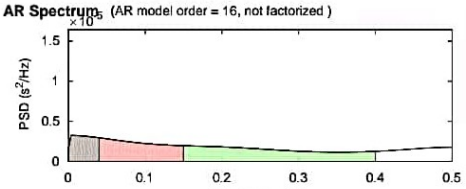
Frequency-Domain Results

FFT spectrum (Welch's periodogram: 300 s window with 50% overlap)



Frequency Band	Peak (Hz)	Power (ms^2)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0233	0	-1.609	25.9	
LF (0.04-0.15 Hz)	0.0433	0	-1.344	33.7	45.5
HF (0.15-0.4 Hz)	0.3967	0	-1.179	39.8	53.8
Total		1	-0.258		
LF/HF		0.847			

AR Spectrum (AR model order = 16, not factorized)

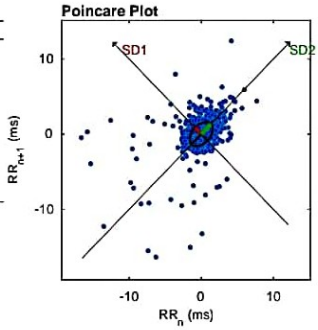


Frequency Band	Peak (Hz)	Power (ms^2)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0033	0	-2.094	16.4	
LF (0.04-0.15 Hz)	0.0400	0	-1.354	34.4	41.1
HF (0.15-0.4 Hz)	0.1500	0	-1.003	48.9	58.5
Total		1	-0.287		
LF/HF		0.704			

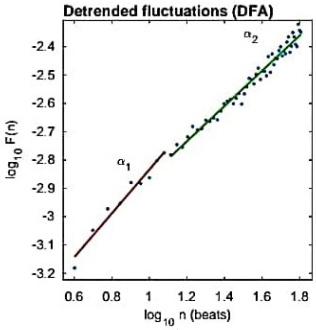
Nonlinear Results

Variable	Units	Value
Poincare Plot		
SD1	(ms)	1.2
SD2	(ms)	1.9
SD2/SD1		1.575
Approximate Entropy (ApEn)		1.356
Sample Entropy (SampEn)		1.338
Detrended Fluctuation Analysis (DFA)		
Short-term fluctuations $\alpha 1$		0.774
Long-term fluctuations $\alpha 2$		0.622

Poincare Plot



Detrended fluctuations (DFA)



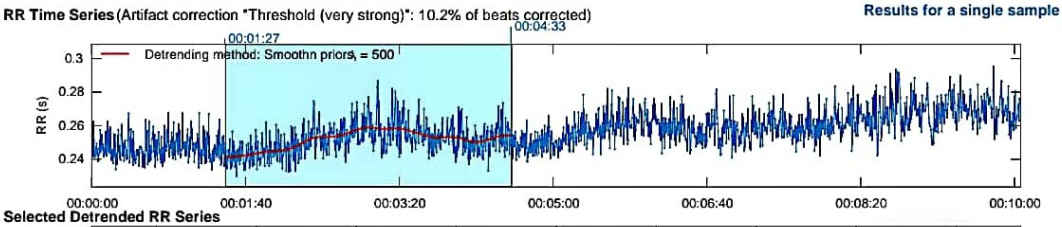
*Results are calculated from the non-detrended selected RR series.

Rat Exposed To Chronic Hypoxia (Rat-2)

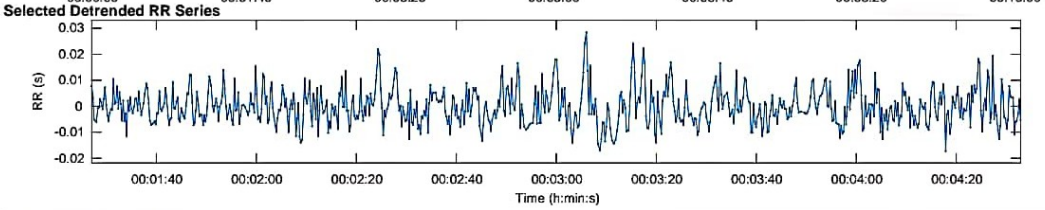
gp3a hrv.txt - xx/xx/xx - xx:xx:xx

HRV Analysis Results

RR Time Series (Artifact correction "Threshold (very strong)": 10.2% of beats corrected) — **Results for a single sample**



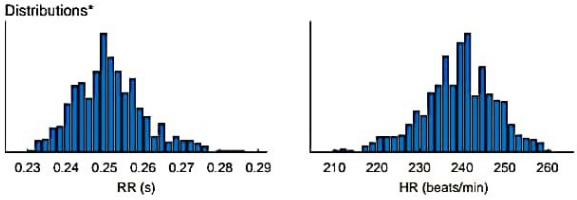
Selected Detrended RR Series



Time-Domain Results

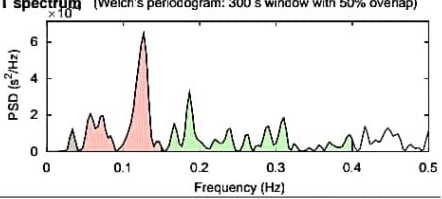
Variable	Units	Value
Mean RR*	(ms)	251.3
STD RR (SDNN)	(ms)	6.8
Mean HR*	(beats/min)	238.73
Min/Max HR	(beats/min)	218.10/254.99
RMSSD	(ms)	7.1
NNxx	(beats)	0
pNNxx	(%)	0.0
RR triangular index		2.368
TINN	(ms)	36.0

Distributions*



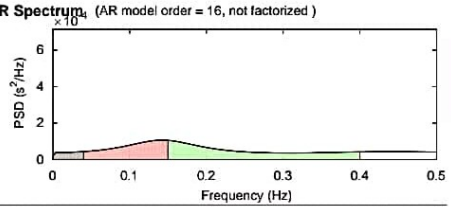
Frequency-Domain Results

FFT spectrum (Welch's periodogram: 300 s window with 50% overlap)



Frequency Band	Peak (Hz)	Power (ms^2)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0333	1	-0.017	2.9	
LF (0.04-0.15 Hz)	0.1267	18	2.894	53.1	54.7
HF (0.15-0.4 Hz)	0.1867	15	2.697	43.6	44.9
Total		34	3.527		
LF/HF		1.218			

AR Spectrum (AR model order = 16, not factorized)

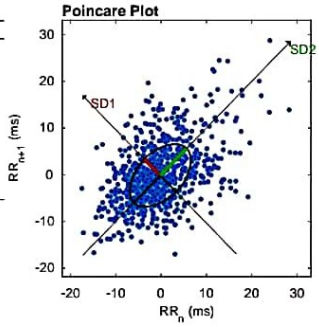


Frequency Band	Peak (Hz)	Power (ms^2)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0400	2	0.458	7.0	
LF (0.04-0.15 Hz)	0.1433	8	2.124	36.8	39.6
HF (0.15-0.4 Hz)	0.1500	13	2.539	55.8	60.0
Total		23	3.122		
LF/HF		0.660			

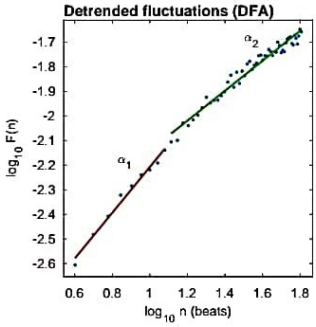
Nonlinear Results

Variable	Units	Value
Poincare Plot		
SD1	(ms)	5.0
SD2	(ms)	8.2
SD2/SD1		1.650
Approximate Entropy (ApEn)		1.489
Sample Entropy (SampEn)		2.011
Detrended Fluctuation Analysis (DFA)		
Short-term fluctuations $\alpha 1$		0.930
Long-term fluctuations $\alpha 2$		0.608

Poincare Plot



Detrended fluctuations (DFA)



*Results are calculated from the non-detrended selected RR series.

Rat Treated with Sodium Fluoride (Rat-2)

gp 4a hrv.txt - xx/xx/xx - xx:xx:xx

HRV Analysis Results

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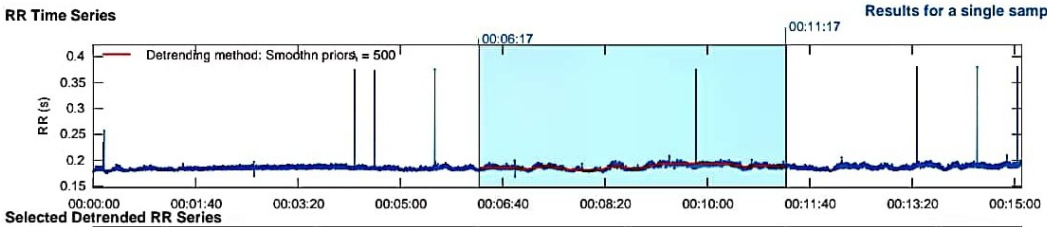
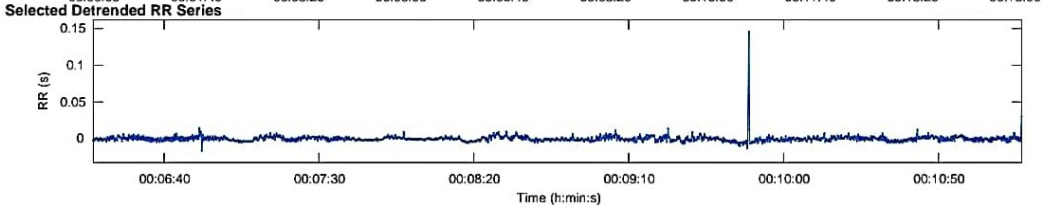
RR Time Series — Results for a single sample



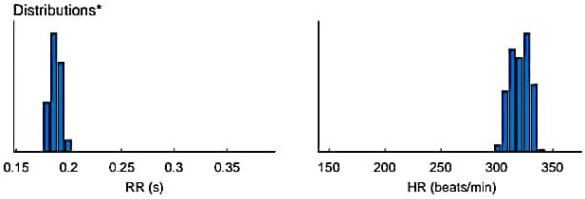
Selected Detrended RR Series



Time-Domain Results

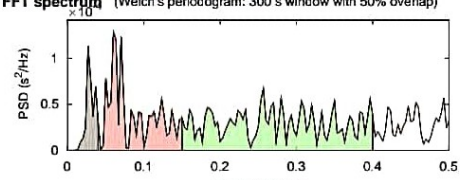
Variable	Units	Value
Mean RR*	(ms)	187.5
STD RR (SDNN)	(ms)	4.7
Mean HR*	(beats/min)	319.96
Min/Max HR	(beats/min)	264.32/337.08
RMSSD	(ms)	5.8
NNxx	(beats)	2
pNNxx	(%)	0.1
RR triangular index		1.799
TINN	(ms)	108.0

Distributions*



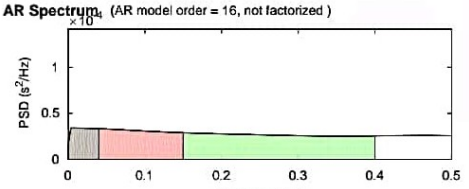
Frequency-Domain Results

FFT spectrum (Welch's periodogram: 300 s window with 50% overlap)



Frequency Band	Peak (Hz)	Power (ms^2)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0267	1	0.185	9.2	
LF (0.04-0.15 Hz)	0.0600	4	1.468	33.3	36.7
HF (0.15-0.4 Hz)	0.2567	7	2.001	56.8	62.6
Total		13	2.566		
LF/HF		0.587			

AR Spectrum (AR model order = 16, not factorized)

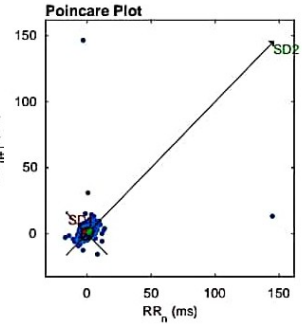


Frequency Band	Peak (Hz)	Power (ms^2)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0033	1	0.281	11.6	
LF (0.04-0.15 Hz)	0.0400	3	1.229	30.0	34.0
HF (0.15-0.4 Hz)	0.1500	7	1.886	57.9	65.5
Total		11	2.432		
LF/HF		0.519			

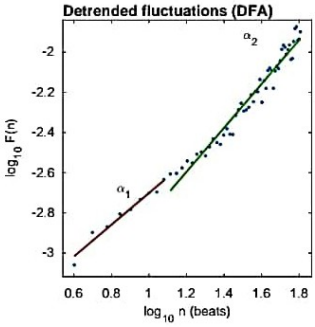
Nonlinear Results

Variable	Units	Value
Poincare Plot		
SD1	(ms)	4.1
SD2	(ms)	5.2
SD2/SD1		1.262
Approximate Entropy (ApEn)		1.504
Sample Entropy (SampEn)		1.644
Detrended Fluctutation Analysis (DFA)		
Short-term fluctuations $\alpha 1$		0.790
Long-term fluctuations $\alpha 2$		1.094

Poincare Plot



Detrended fluctuations (DFA)



*Results are calculated from the non-detrended selected RR series.

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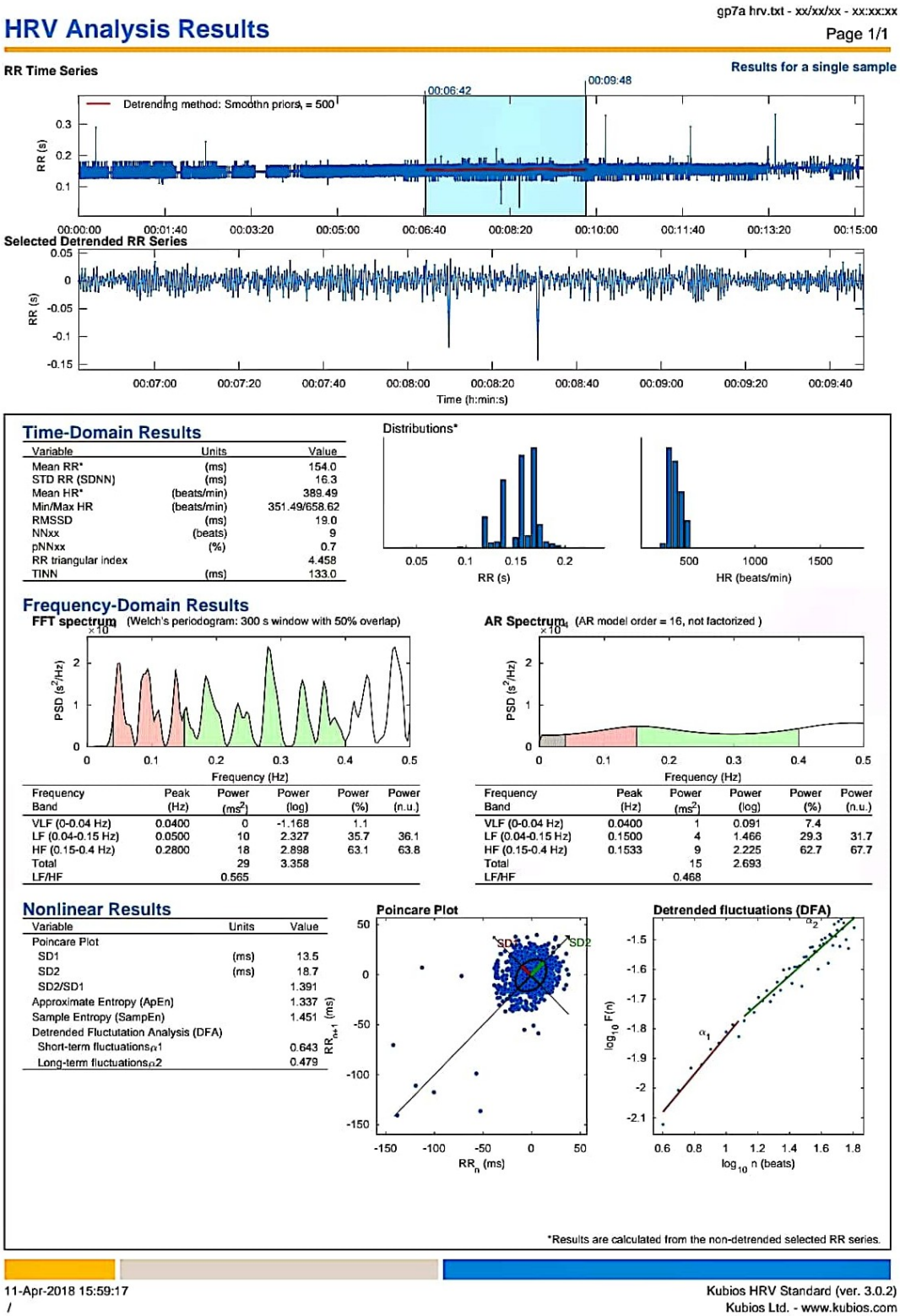
Rat Exposed To Chronic Hypoxia And Sodium Fluoride(CH + NaF) (Rat-2)

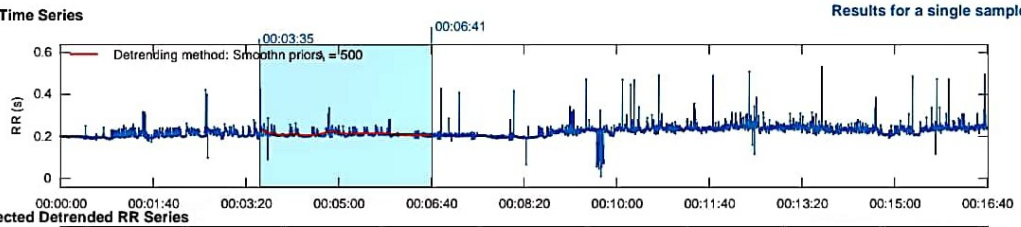


L-Ascorbic Acid + CH Exposed Rat (Rat-2)

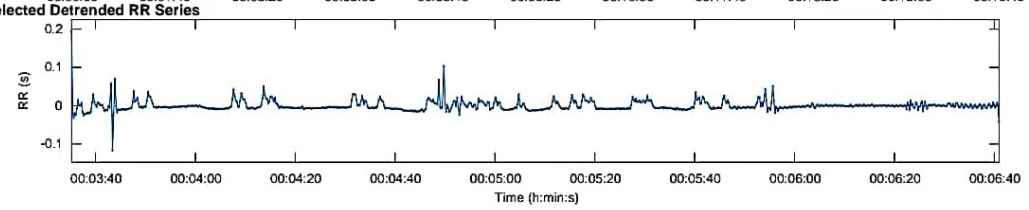
HRV Analysis Results

Results for a single sample

RR Time Series



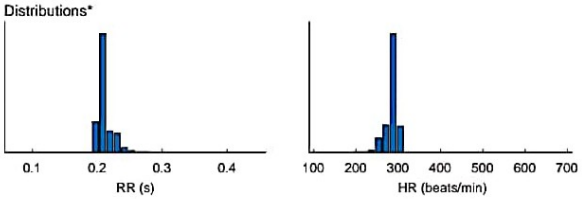
Selected Detrended RR Series



Time-Domain Results

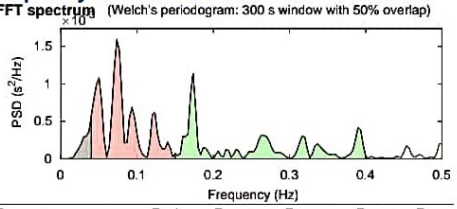
Variable	Units	Value
Mean RR*	(ms)	211.6
STD RR (SDNN)	(ms)	13.6
Mean HR*	(beats/min)	283.62
Min/Max HR	(beats/min)	233.46/301.51
RMSSD	(ms)	13.8
NNxx	(beats)	11
pNNxx	(%)	1.3
RR triangular index		2.085
TINN	(ms)	209.0

Distributions*



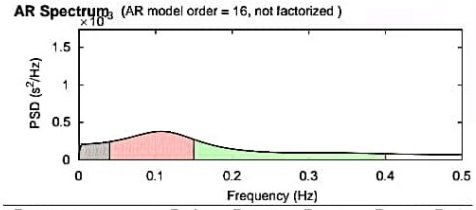
Frequency-Domain Results

FFT spectrum (Welch's periodogram: 300 s window with 50% overlap)



Frequency Band	Peak (Hz)	Power (ms²)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0400	6	1.749	6.0	
LF (0.04-0.15 Hz)	0.0733	52	3.943	54.2	57.7
HF (0.15-0.4 Hz)	0.1733	38	3.632	39.7	42.2
Total		95	4.556		
LF/HF		1.365			

AR Spectrum (AR model order = 16, not factorized)

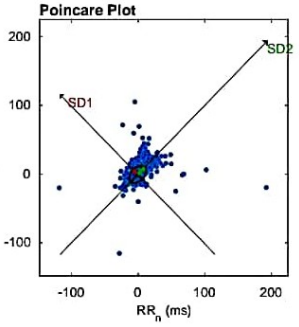


Frequency Band	Peak (Hz)	Power (ms²)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0400	9	2.158	11.7	
LF (0.04-0.15 Hz)	0.1067	36	3.578	48.3	54.6
HF (0.15-0.4 Hz)	0.1500	30	3.386	39.8	45.1
Total		74	4.306		
LF/HF		1.212			

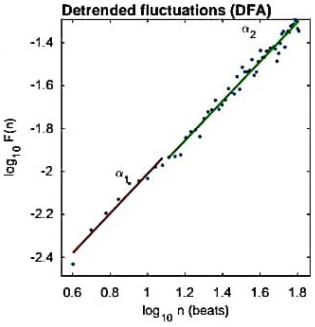
Nonlinear Results

Variable	Units	Value
Poincare Plot		
SD1	(ms)	9.8
SD2	(ms)	15.1
SD2/SD1		1.545
Approximate Entropy (ApEn)		0.804
Sample Entropy (SampEn)		0.522
Detrended Fluctutation Analysis (DFA)		
Short-term fluctuations $\alpha 1$		0.927
Long-term fluctuations $\alpha 2$		0.918

Poincare Plot



Detrended fluctuations (DFA)



*Results are calculated from the non-detrended selected RR series.

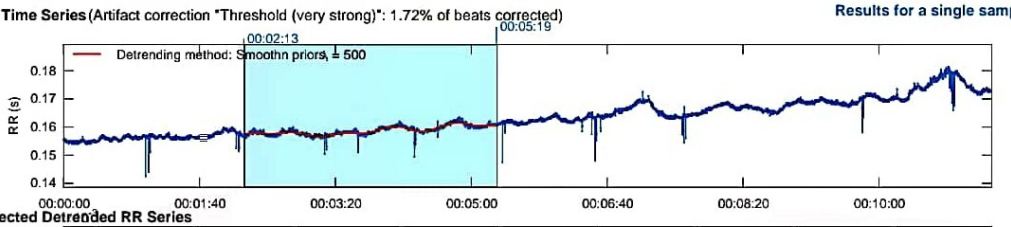
L-Ascorbic Acid + Sodium Fluoride Exposed Rat (Rat-2)

gp6A hrv.txt - xx/xx/xx - xx:xx:xx

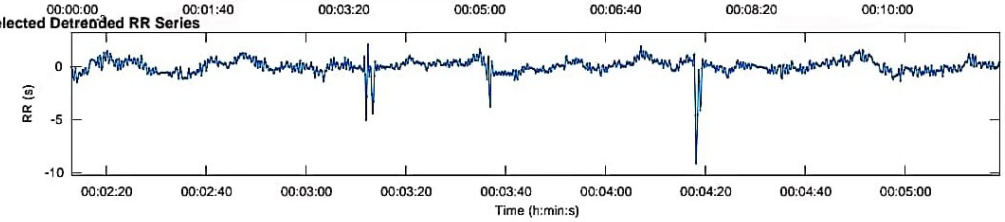
HRV Analysis Results

RR Time Series (Artifact correction "Threshold (very strong)": 1.72% of beats corrected)

Results for a single sample



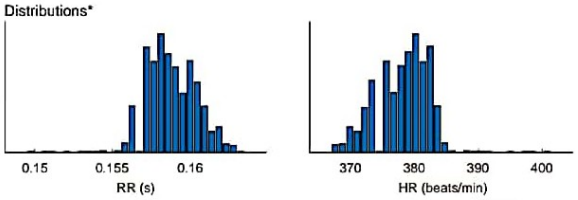
Selected Detrended RR Series



Time-Domain Results

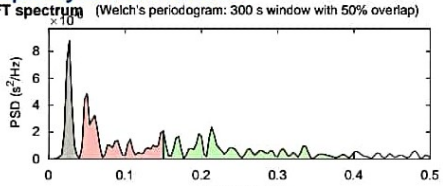
Variable	Units	Value
Mean RR*	(ms)	158.8
STD RR (SDNN)	(ms)	0.7
Mean HR*	(beats/min)	377.90
Min/Max HR	(beats/min)	368.32/388.40
RMSSD	(ms)	0.5
NNxx	(beats)	0
pNNxx	(%)	0.0
RR triangular index		NaN
TINN	(ms)	8.0

Distributions*



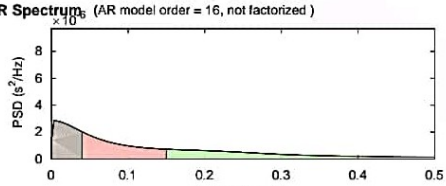
Frequency-Domain Results

FFT spectrum (Welch's periodogram: 300 s window with 50% overlap)



Frequency Band	Peak (Hz)	Power (ms²)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0267	0	-2.527	21.6	
LF (0.04-0.15 Hz)	0.0500	0	-1.952	38.4	49.0
HF (0.15-0.4 Hz)	0.2133	0	-1.918	39.7	50.7
Total		0	-0.995		
LF/HF		0.967			

AR Spectrum (AR model order = 16, not factorized)

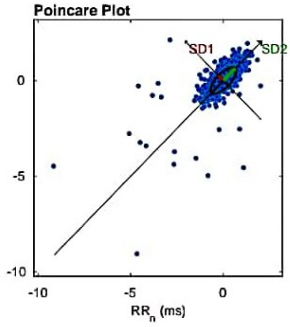


Frequency Band	Peak (Hz)	Power (ms²)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0033	0	-2.311	29.8	
LF (0.04-0.15 Hz)	0.0400	0	-2.076	37.7	53.6
HF (0.15-0.4 Hz)	0.1500	0	-2.225	32.4	46.2
Total		0	-1.100		
LF/HF		1.161			

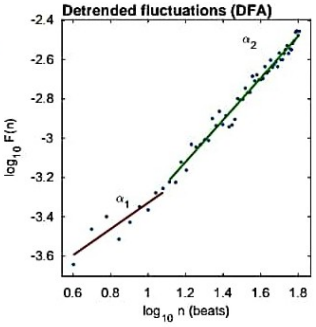
Nonlinear Results

Variable	Units	Value
Poincare Plot		
SD1	(ms)	0.4
SD2	(ms)	0.9
SD2/SD1		2.410
Approximate Entropy (ApEn)		1.253
Sample Entropy (SampEn)		1.197
Detrended Fluctutation Analysis (DFA)		
Short-term fluctuations $\alpha 1$		0.667
Long-term fluctuations $\alpha 2$		1.068

Poincare Plot



Detrended fluctuations (DFA)



*Results are calculated from the non-detrended selected RR series.

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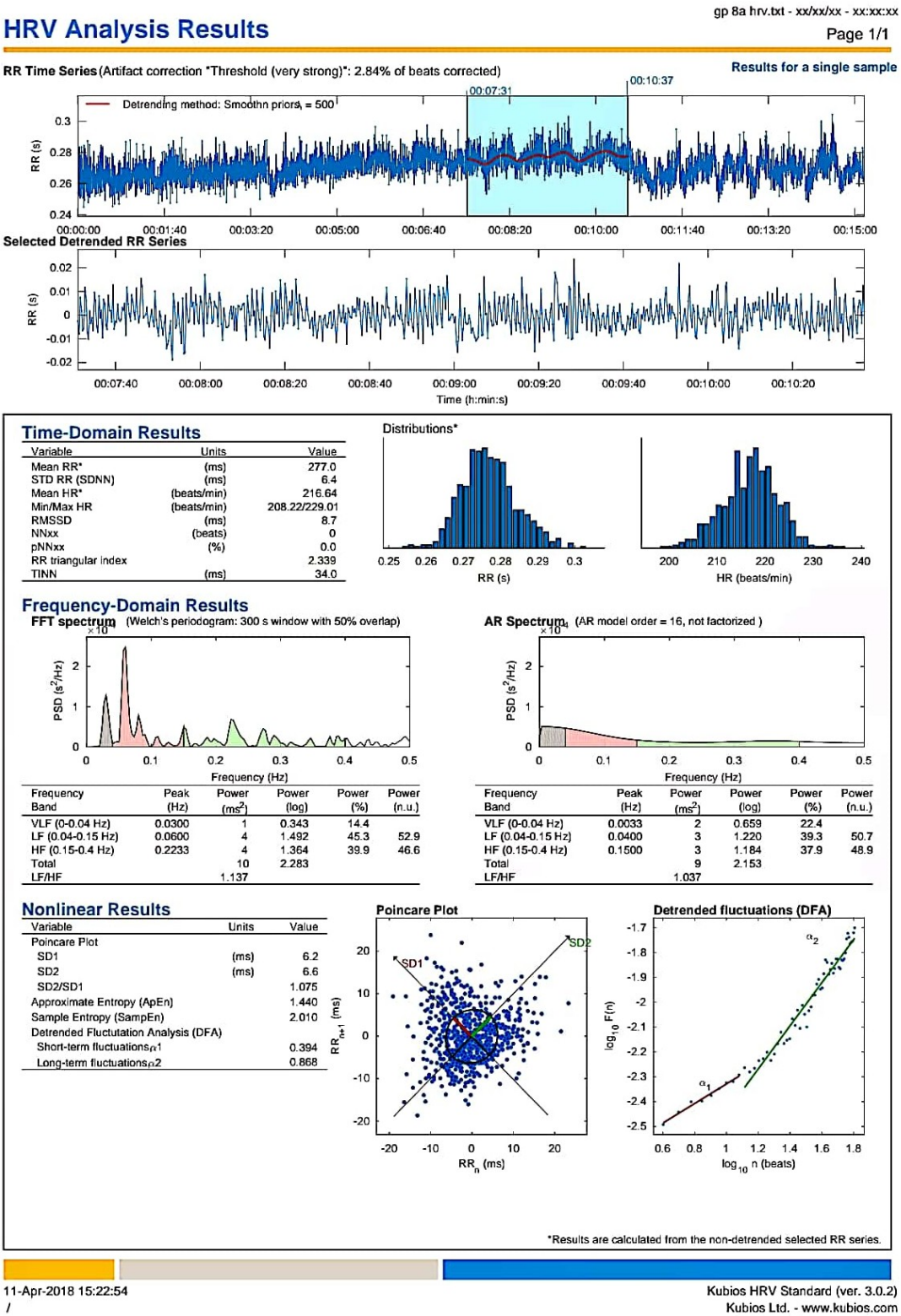
L- Ascorbic Acid + Chronic Hypoxia + Sodium Fluoride Treated Rat (Rat-2)



5.3 HEMOGRAM

Table -5.3.1: Comparison of haematological parameters in experimental groups

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Group VIII	ANOVA	
									F Value	P Value
RBC	7.67 ±0.12[a]	7.62 ±0.26[a]	9.46 ±049[b]	6.09 ±0.95[c]	9.18 ±0.13[b]	7.04 ±0.7[b]	5.95 ±0.8[c]	7.22 ±0.28[a]	16.01	<0.0001
WBC	6663.33 ±296	76450 ±476	9739 ±79.37	9076.67 ±467.58	5520 ±507.15	6366.67 ±485.63	10690 ±615	6 3164 ±406	1.171	0.375,NS
Hb	14.13 ±0.17[a]	14.36 ±0.62[a]	16.74 ±0.71[a]	12.9 ±0.78[b]	16.37 ±0.12 [a]	13.82 ±1.04[b]	12.3 ±1.07[b]	13.93 ±0.18[b]	15.02	<0.0001

Values are expressed as Mean ± SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison test. $p<0.05$ was considered statistically significant, values with different superscripts a, b and c are significantly different from each other .RBC-Red blood cell, WBC- White blood cell, Hb- Hemoglobin.

Table -5.3.1 illustrates comparison of haematological parameters in experimental groups. There was statistically significant increase of RBC count in CH group and CH +NaF group of rats. Statistically significant decrease of RBC count was seen in NaF groups when compared to control. L-ascorbic acid + CH group showed significant decrease in RBC count when compared to CH alone group. But L-ascorbic acid + NaF did not show any significant change when compared to NaF alone .We have not observed any significant differences in WBC count, hemoglobin concentrations among experimental groups.

5.4 Biochemical analysis

5.4.1 Oxidant status

Table 5.4.1 shows oxidant status in experimental groups. Statistically significant increase in serum MDA and nitrite concentration were observed in CH , NaF, CH+NaF groups as compared to respective controls. However simultaneous supplementation with L-ascorbic acid in L-ascorbic acid + CH, L-ascorbic acid + NaF and L-ascorbic acid +CH+ NaF groups showed statistically significant improvements of serum MDA and nitrite.

Table -5.4.1: Comparison of oxidant status parameters among groups

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Group VIII	ANOVA	
									F - value	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]	9.353	<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]	61.27	<0.0001*

Values are expressed as Mean ± SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison test. $p<0.05$ was considered statistically significant, values with different superscripts a, b, c, d ,e are significantly different from each other

5.4.2 Correlation between frequency domain indices (LF, HF and LF/HF ratio) of HRV analysis and serum MDA among experimental groups

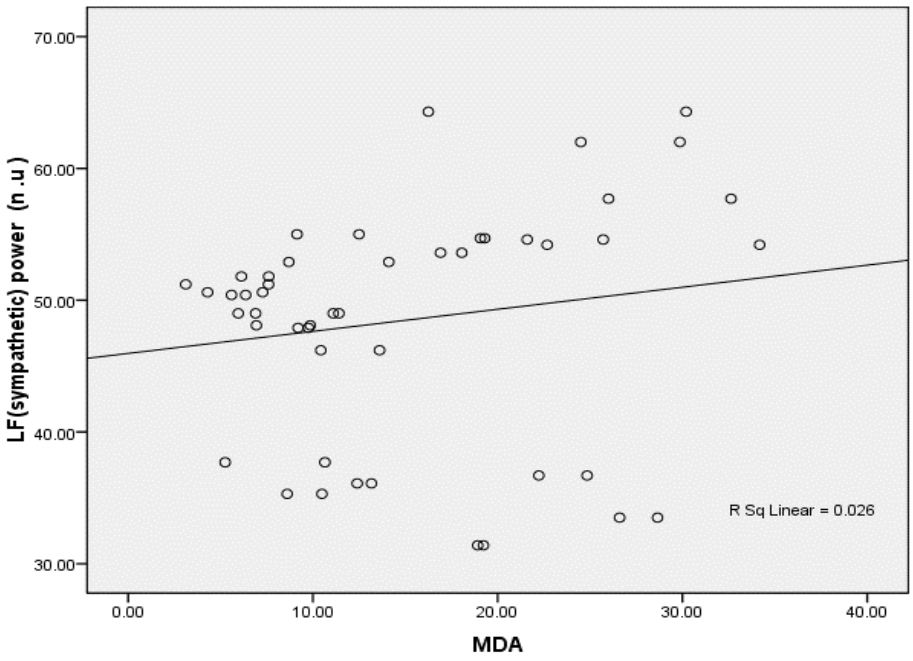


Figure 5.4.2a: Correlation between LF and serum MDA (µmol/L) ; r=0.1612 , p = ns

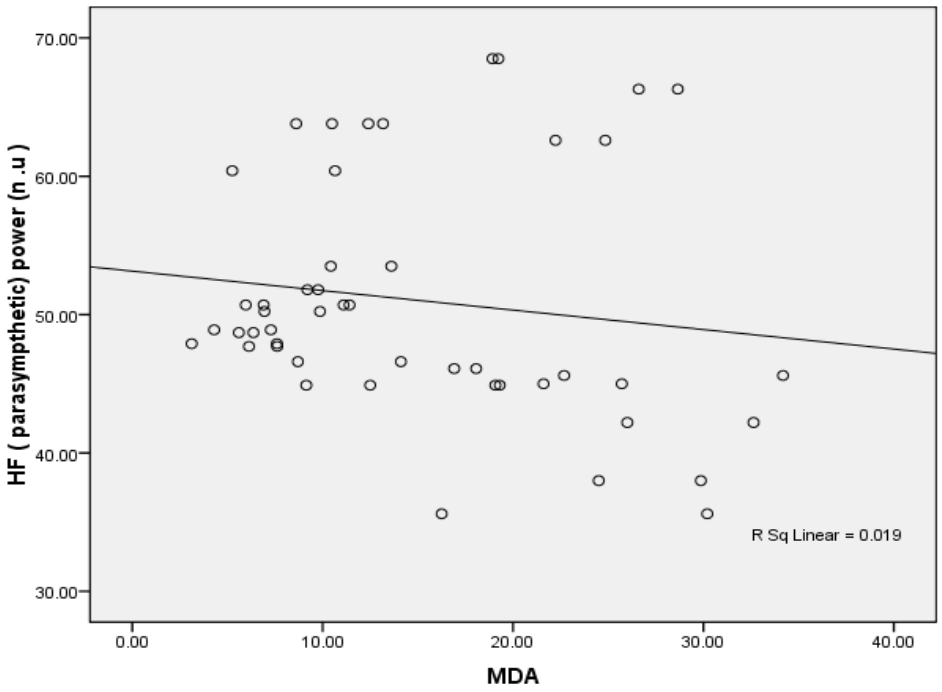


Figure 5.4.2b: Correlation between HF and serum MDA (µmol/L) ; r =0.137 ,p = ns

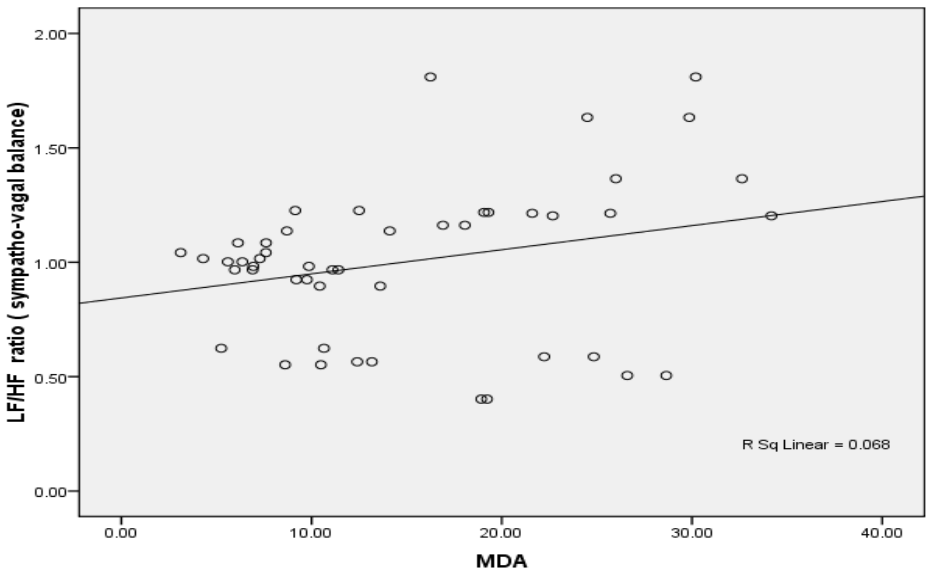


Figure 5.4.2c: Correlation between LF/HF ratio and serum MDA (µmol/L) ; r =0.260 , p= ns

The correlation between LF with serum MDA, HF with serum MDA and LF/HF ratio with serum MDA was assessed by Pearson's correlation. Serum MDA is an marker of oxidant status . Pearson's correlation does not show any statistical significance.

5.4.3 Correlation between frequency domain indices (LF, HF and LF/HF ratio) of HRV analysis and serum SOD among experimental groups

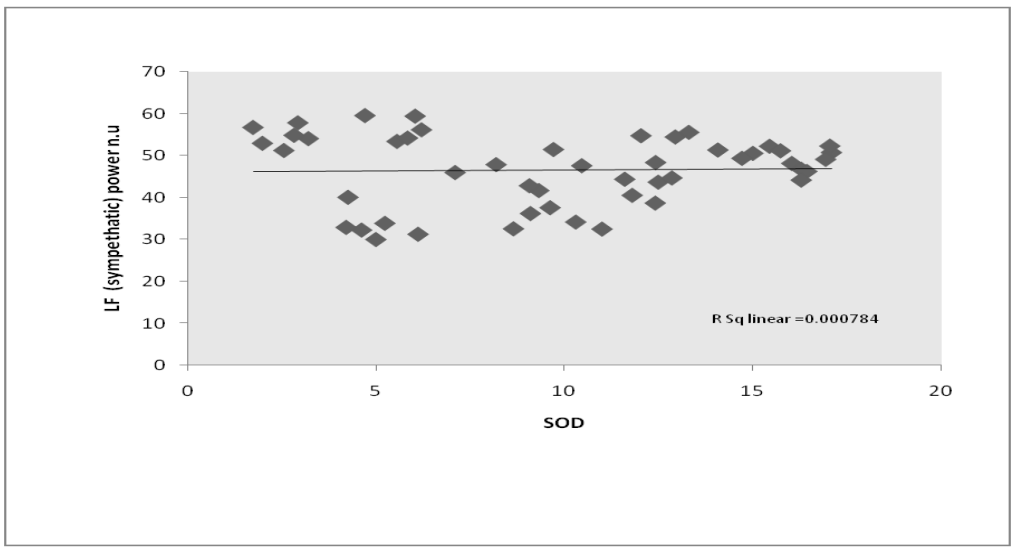


Figure 5.4.3a: Correlation between LF and serum SOD (units/ L) ; r = 0.028 , p= ns

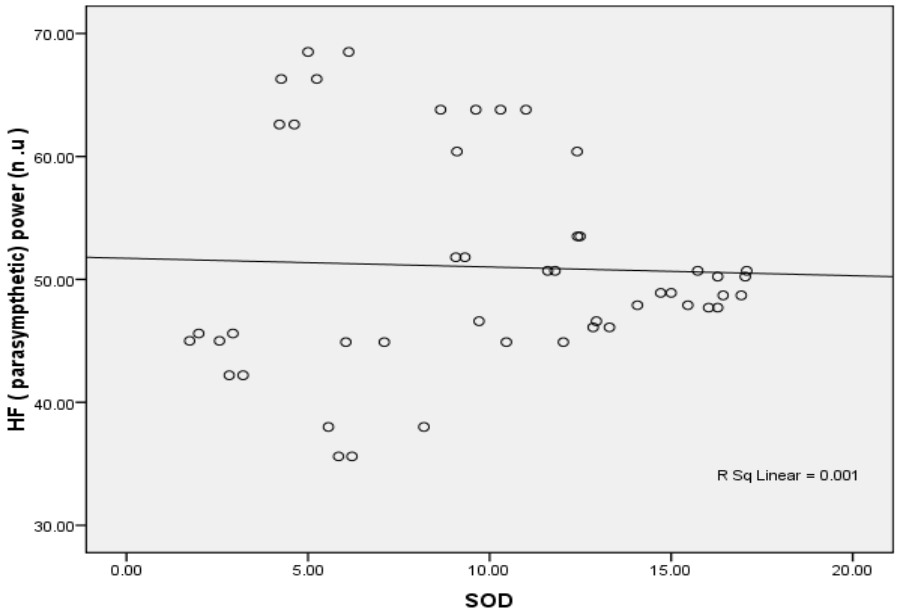


Figure 5.4.3b: Correlation between HF and serum SOD (units/ L) ; r =0.031 , p=ns

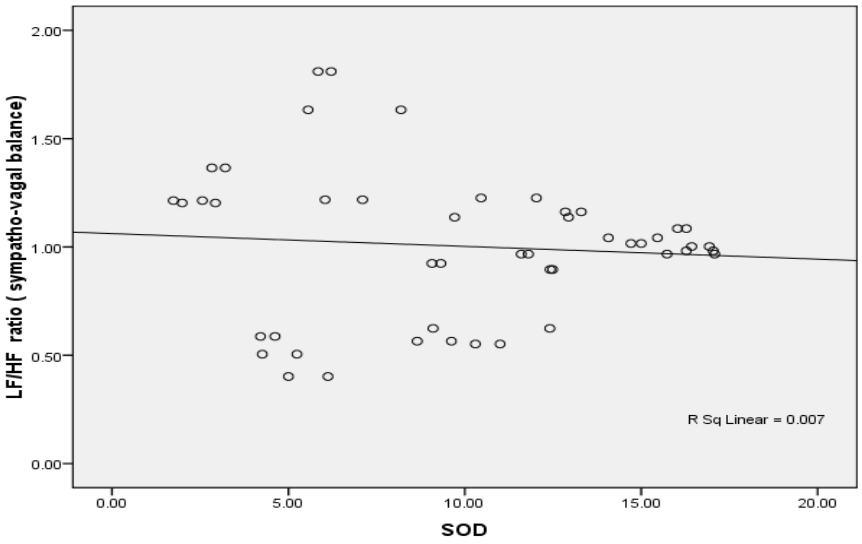


Figure 5.4.3c: Correlation between LF/HF ratio and serum SOD (units L) ; r = 0.083, p= ns

The correlation between LF with serum SOD, HF with serum SOD and LF/HF ratio with serum SOD was assessed by Pearson's correlation. Serum SOD is an biological indicator of antioxidant status in oxidative stress. Pearson's correlation does not show any statistical significance.

5.4.4 Antioxidant status:

Table 5.4.4.1 : Comparison of antioxidant status among experimental groups

Parameter	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII	Group VIII	ANOVA	
									F-value	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]	50. 68	<0.0 001*
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]	19. 86	<0.0 001*
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]	76. 05	<0.0 001*
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]	23. 9	<0.0 001*

Values are expressed as Mean ± SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison test. $p<0.05$ was considered statistically significant, values with different superscripts a, b, c, d, e, f and g are significantly different from each other

Table 5.4.2 shows antioxidant status in experimental groups. Statistically significant decrease in serum SOD, vitamin C, hepatic vitamin C and serum vitamin E concentration in CH , NaF, CH+ NaF groups as compared to respective controls. However simultaneous supplementation with L-ascorbic acid in L-ascorbic acid +CH, L-ascorbic acid + NaF and L-ascorbic acid +CH+ NaF groups showed significant improvements of serum SOD ,serum vitamin C ,hepatic vitamin C and serum vitamin E concentration.

5.5 MOLECULAR MARKERS

5.5 : Molecular Markers

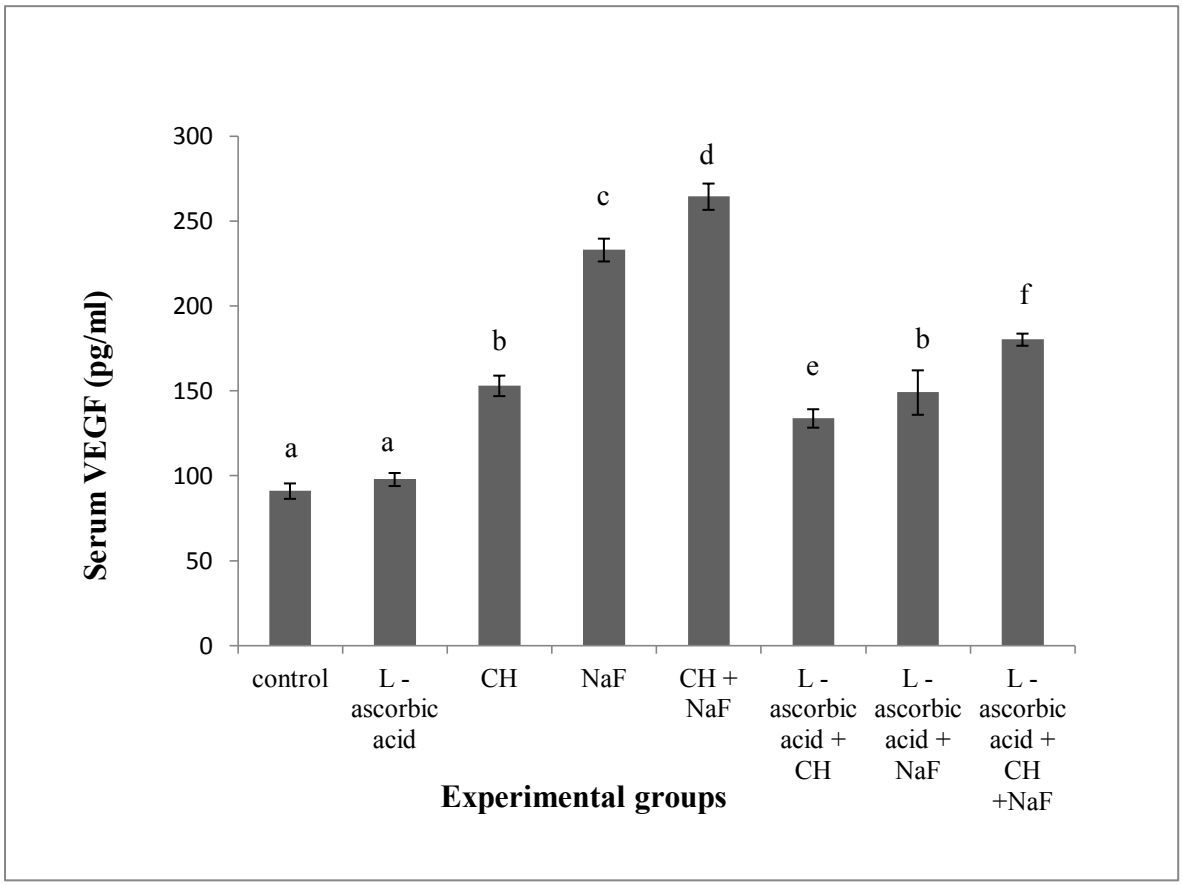


Figure 5.5.1: Comparison of serum VEGF levels among experimental groups

Values are expressed as Mean ± SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison test. Values with different superscripts (a, b, c, d, e and f) are significantly different from each other ($p<0.05$), n=6 rats in each group

Figure 5.5.1 shows statistically significant increase in serum VEGF concentration in CH, NaF, CH+ NaF groups as compared to respective controls. However simultaneous supplementation with L-ascorbic acid in L-ascorbic acid +CH, L-ascorbic acid + NaF and L-ascorbic acid +CH+ NaF groups showed significant improvements in serum VEGF concentration.

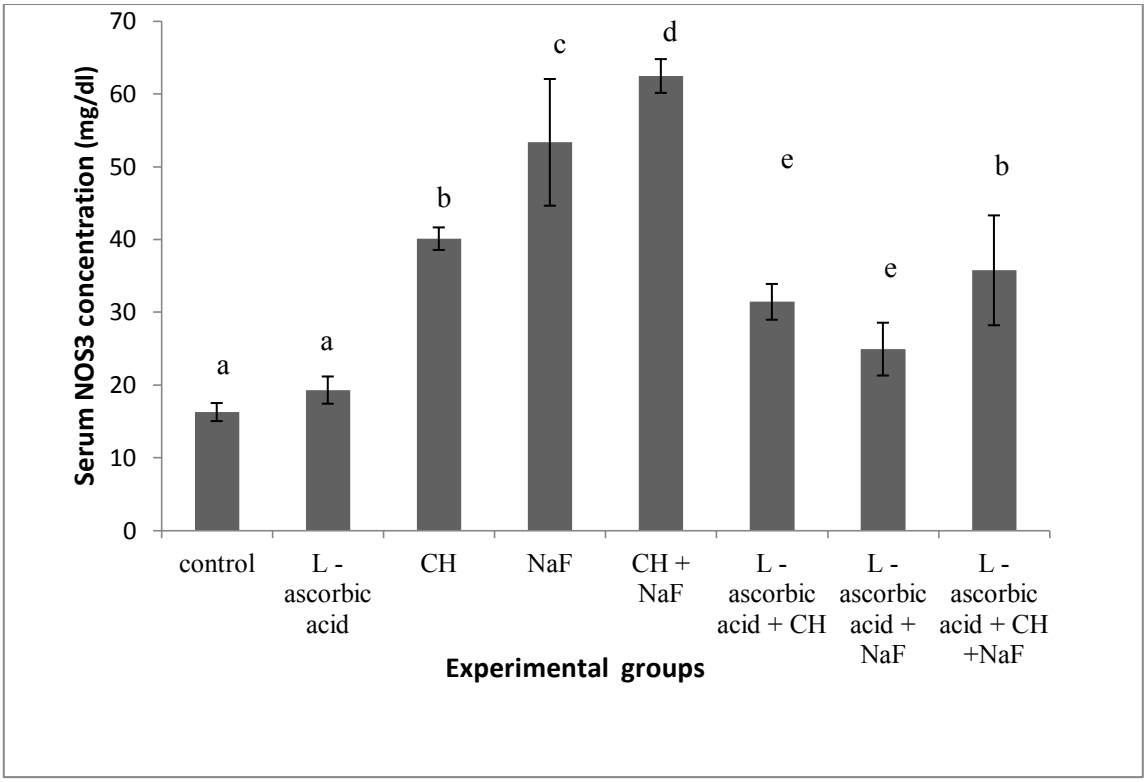


Figure 5.5.2: Comparison of serum NOS3 levels among experimental groups

Values are expressed as Mean ± SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison test. Values with different superscripts (a, b ,c ,d and e) are significantly different from each other ($p<0.05$), n=6 rats in each group

Figure 5.5.2 shows statistically significant increase in serum NOS3 concentration in CH , NaF, CH+ NaF groups as compared to respective controls. However simultaneous supplementation with L-ascorbic acid in L-ascorbic acid +CH, L-ascorbic acid + NaF and L-ascorbic acid +CH+ NaF groups showed significant improvements in serum NOS3 concentrations.

5.6 HISTOPATHOLOGY

5.6.1 Histopathology of Left ventricular myocardium

Figure 5.6.1 shows histopathology of Left ventricular myocardium. Hematoxylin and eosin stained sections of left ventricular myocardium in control and L-ascorbic acid groups showed normal left ventricular myocardium. CH showed hypertrophy of ventricular myocytes. But CH+ L-ascorbic acid group showed normal histology of left ventricular myocardium. NaF treated rats left ventricular myocardium appeared fibrotic and CH +NaF group rats showed focal degeneration in left ventricle. whereas in case of L- ascorbic acid supplementation in L-ascorbic acid +CH , L-ascorbic acid + NaF and L-ascorbic acid + CH + NaF groups show some significant improvements in architecture of left ventricular muscle fibers.

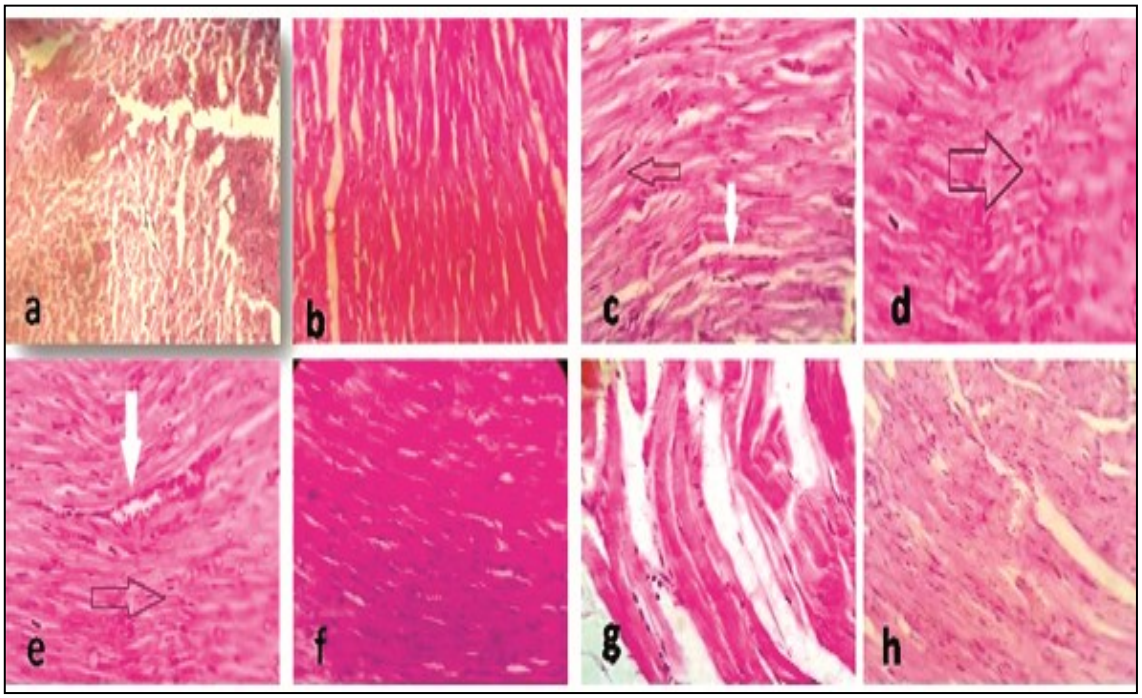


Figure 5.6.1: Histopathology of left ventricular myocardium in all groups of rats , (a) control (40x), (b) L-ascorbic acid (40x), (c) CH(40x), (d) NaF (40x), (e)L-ascorbic acid +CH(40x) ,(f) L-ascorbic acid + NaF (40x), (g) CH+ NaF (40x) and (h)L-ascorbic acid +CH + NaF (40x)

5.6.2 Histopathology of Lungs

Figure 5.6.2 shows histopathology of Lungs**.** Hematoxylin and eosin stained sections of lungs in control and L-ascorbic acid shows normal lung parenchyma with normal bronchial lumen, alveolar space and thin walled alveoli. Histopathology of the lung in CH, NaF and CH +NaF groups showed thickening of inter alveolar septae and alveolar spaces filled with eosinophilic fluid which causes reduction in alveolar space area and pulmonary edema.Whereas in case of L- ascorbic acid supplementation in L-ascorbic acid + CH , L-ascorbic acid + NaF and L-ascorbic acid + CH + NaF groups showed normal lung parenchyma.

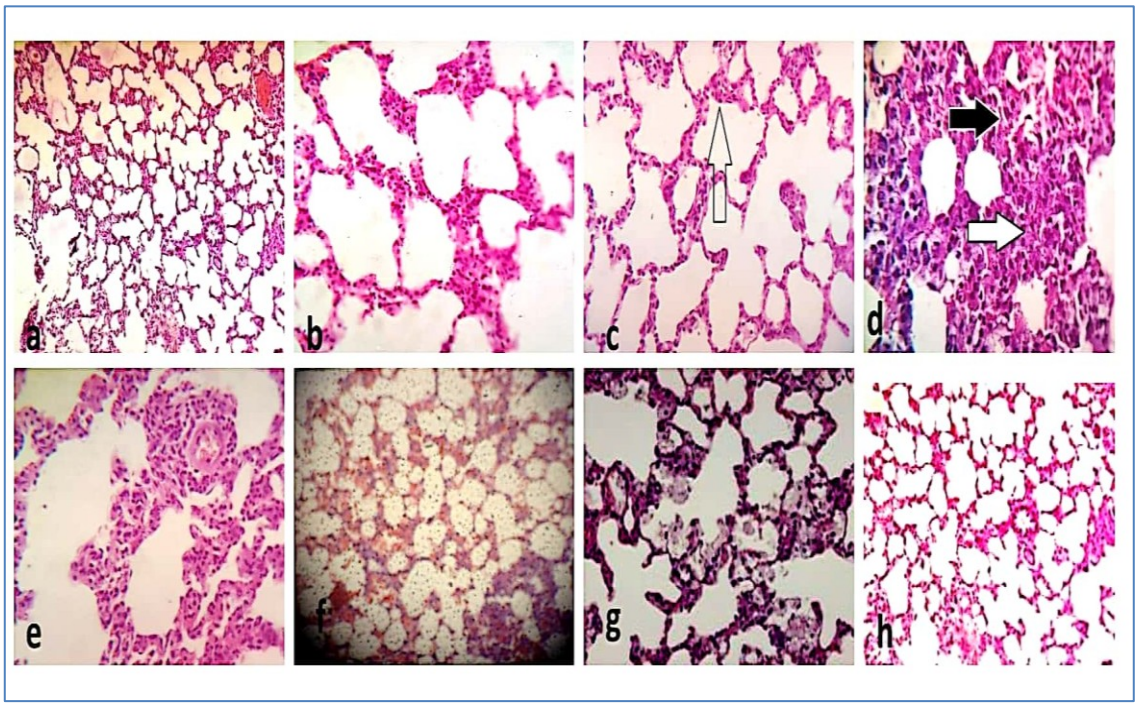


Figure 5.6.2: Histopathology of lungs in all groups of rats , (a) control (40x), (b) L-ascorbic acid (40x), (c) CH(40x), (d) NaF (40x), (e)L-ascorbic acid +CH(40x) ,(f) L-ascorbic acid + NaF (40x), (g) CH+ NaF (40x) and (h)L-ascorbic acid +CH + NaF (40x)

5.6.3 : Histopathology of Liver

Figure 5.6.3 shows histopathology of Liver. Hematoxylin and eosin stained sections of the liver in control and L-ascorbic acid groups showed normal architecture of liver.CH group showed inflammation and sinusoidal congestion. NaF group showed focal necrosis, mononuclear infiltrate and central vein congestion and CH + NaF also showed focal necrosis, mononuclear infiltrate and central vein congestion. Whereas in case of L-ascorbic acid supplementation hepatic architecture appeared normal in L-ascorbic acid + CH , L-ascorbic acid + NaF and L-ascorbic acid + CH + NaF groups.

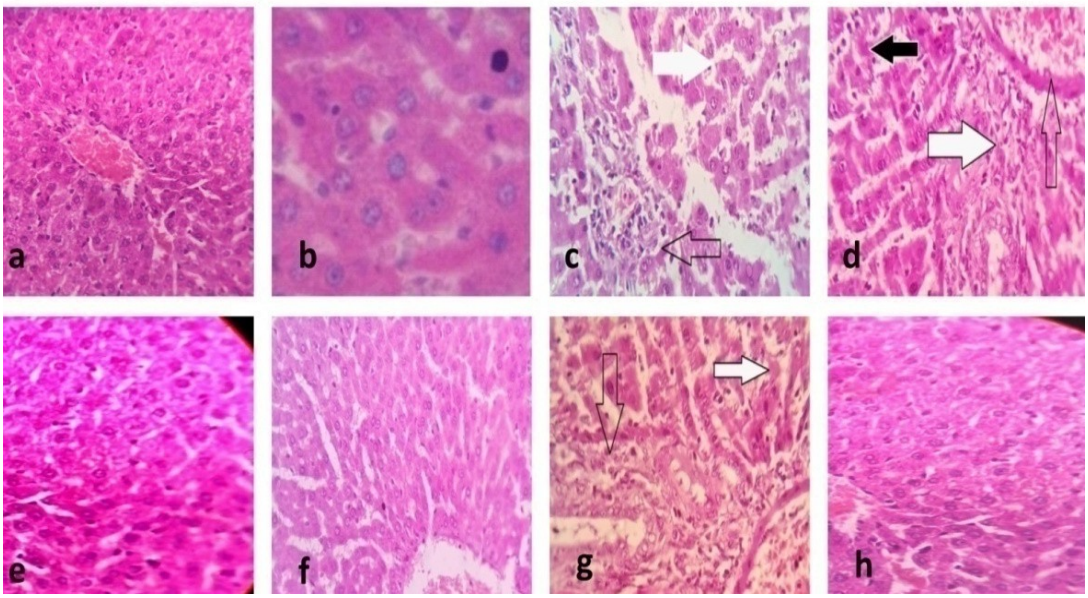


Figure 5.6.3: Histopathology of liver in all groups of rats , (a) control (40x), (b) L-ascorbic acid (40x), (c) CH(40x), (d) NaF (40x), (e)L-ascorbic acid +CH(40x) ,(f) L-ascorbic acid + NaF (40x), (g) CH+ NaF (40x) and (h)L-ascorbic acid +CH + NaF (40x)

5.6.4 : Histopathology of kidneys

Figure 5.6.4 shows histopathology of kidneys. Hematoxylin and eosin stained sections of the kidneys in control and L-ascorbic acid groups showed normal renal cortex and glomerular tufts. CH group showed distorted epithelium in the renal cortex and glomerular distortion. Both NaF and CH + NaF groups showed congestion & inflammation of renal cortex with distorted epithelium. Where as in case of L- ascorbic acid supplementation in L-ascorbic acid + CH , L-ascorbic acid + NaF and L-ascorbic acid + CH + NaF groups show features of significant improvement and renal architecture appeared to be normal.

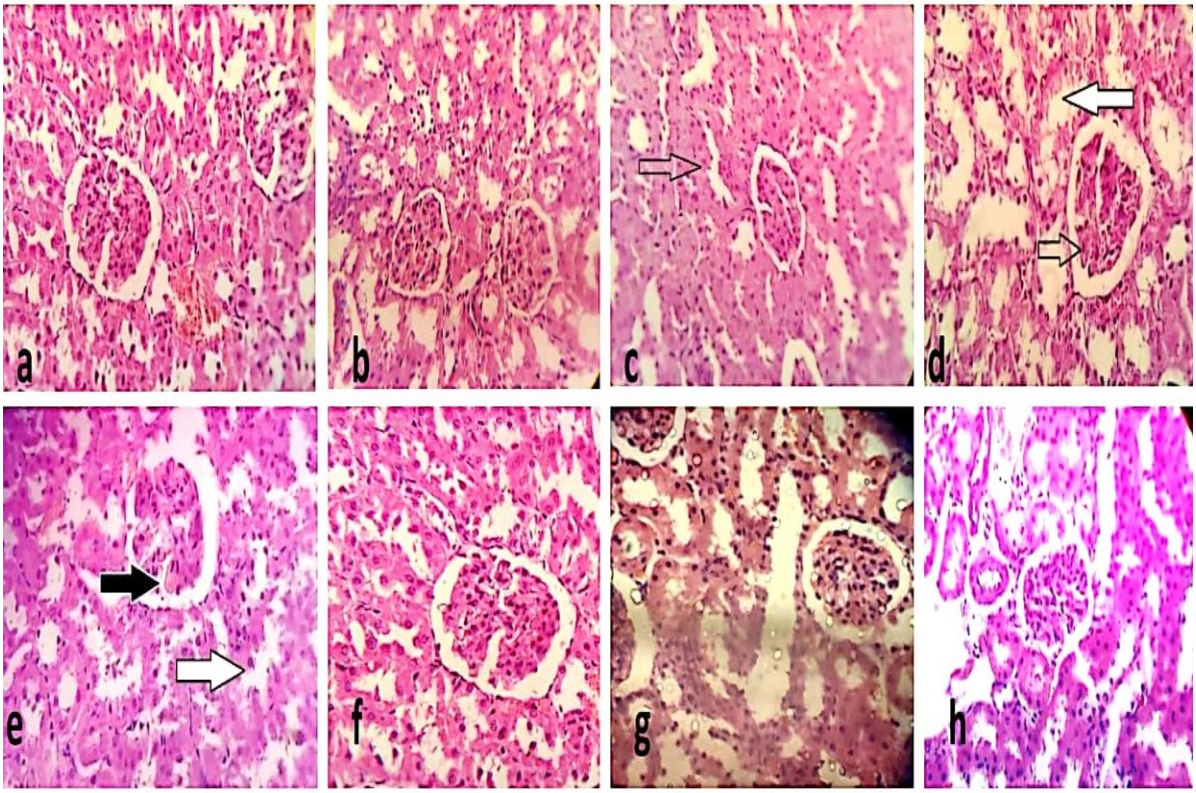


Figure 5.6.4: Histopathology of kidneys in all groups of rats , (a) control (40x), (b) L-ascorbic acid (40x), (c) CH(40x), (d) NaF (40x), (e)L-ascorbic acid +CH(40x) ,(f) L-ascorbic acid + NaF (40x), (g) CH+ NaF (40x) and (h)L-ascorbic acid +CH + NaF (40x)

DISCUSSION

6 : DISCUSSION

Protective role of L-ascorbic acid on cardiac autonomic functions, cardiopulmonary and hepatorenal dysfunctions were studied in CH, NaF and in combination of CH and NaF in experimental rats through evaluation of gravimetry, electrophysiology, serum oxidant and antioxidant status along with histopathology of heart, lung, liver and kidney.

6.1 : Gravimetry

Our study results are indicative of decrease in % of body weight gain and organosomatic index of heart, lungs , liver and kidneys in CH, NaF and CH + NaF groups when compared to control and L-ascorbic acid groups(supported by results of histopathology of lung, liver and kidney).Whereas histopathology of left ventricular myocardium shows features of hypertrophy in CH group rats . It may be due to increased load on the heart by sympathetic activation .It is also evident by increased heart rate and MAP values in CH group . This decrease in % of body weight gain and organosomatic index may be due to less food and water intake with altered growth rate. NaF may penetrate through blood-brain barrier and inhibit hunger center in hypothalamus (Vasant *et al.,* 2014) and NaF also induces breakdown of lipids and tissues proteins (Bouasla *et al*., 2014) . It may also lead to decrease in % of body weight gain and organosomatic index of heart, lungs , liver and kidneys.

NaF causes hyper secretion of HCl in stomach and induces death of epithelial cells in GI Tract. It may also lead to decreased water and food absorption (El-lethey *et al.,* 2010*).* Choudhury *et al* (2018) also observed such distortive changes in the epithelium of GI tract and loss of tissue proteins in NaF treated rabbits with decrease in % of body weight gain and degenerative changes .in the histopathology of liver . All these findings were found to be similar with our study results.

According to Basha *et al* (2011), antioxidant supplementation is effective in preventing NaF induced decrease in % of body weight gain and organosomatic index . Supplementation with L-ascorbic acid in L-ascorbic acid + CH , L-ascorbic acid + NaF and L-ascorbic acid + CH + NaF groups showed % of body weight gain and increased organosomatic index of heart, liver, lungs and kidneys due to protective actions of L-ascorbic acid in CH and NaF induced alteration of tissue protein breakdown and altered growth rate (Das SK *et al*., 2017).

6.2 : Electrophysiology

Protective role of L- ascorbic acid on cardiac autonomic functions in albino rats exposed to CH or/and NaF were studied by recording electrophysiological parameters.

Our study results are indicative of increase in heart rate and mean arterial pressure (MAP) in CH group of rats. Many components are responsible for increase in heart rate and MAP, the most determining component is sympathetic activation and it is also evident by HRV analysis . HRV analysis is the most valuable tool to assess the cardiac autonomic functions . In our study chronic hypoxia induced alterations of sympathovagal balance in experimental rats are indicative of cardiac autonomic dysfunctions with possible sympathetic dominance (Zajaczkowski *et al.,* 2018) .CH alters the neuronal effector pathways controlling the heartbeat. Oxidant tone and nitric oxide within the medulla alter sympathetic and vagal outflow. Increased MAP along with hypertrophy of left ventricular muscle mass (supported by results of histopathology of heart) in CH group of rats also corroborated with the findings of Ross *et al* (2010). Signalling pathways leading to hypertrophy are stimulated by growth factors in response to chronic hypoxia .Altered MAP and left ventricular structural changes in chronic hypoxic rats could be because of production of reactive oxygen species (ROS). CH induced sympathetic stimulation causes AT-11 via G-protein coupled receptor to generate ROS by NOX2 and activate MAPK resulting in ventricular hypertrophy (Li JM *et al*., 2002). ROS in oxidative stress also activates several genes via HIF-1α in cardio vascular system .HIF-1α activates eNOS and iNOS by VEGF and can increase availability of nitric oxide (Giordano, 2005). Hypertrophy of left ventricular muscle mass in CH group rats by ROS generation in oxidative stress can result in apoptosis and autophagy related cell death in left ventricular myocardium . But, L-ascorbic acid supplementation in (L-ascorbic acid + CH) group showed significant improvements in the heart rate and MAP parameters in rats. Our results on supplementation of L-ascorbic acid in cardiovascular electrophysiology of chronic hypoxic rats corroborated with the findings of Kane *et al* (2013).

The present study also showed parasympathetic dominance in NaF treated rats which clearly indicates altered autonomic functions. Decrease in heart rate, MAP in case of NaF treated rats in present study may be due to vasodepressor action of NaF by combined depression of the vasomotor center of the brain and of vascular smooth muscle (Leone *et al*, 1956). These observations on NaF treatment in present study was found to be contrary

to other previous study (Karademir *et al* ., 2011) .L -ascorbic acid also increases parasympathetic outflow at the level of nucleus ambiguous in NaF treated rats .Altered sympathovagal balance, Heart rate and MAP in CH, NaF and CH +NaF groups was significantly improved by L- Ascorbic acid supplementation in L-ascorbic acid + CH , L-ascorbic acid + NaF and L-ascorbic acid + CH + NaF groups. Our study observations are indicative of sympatho-vagal imbalance in CH, NaF and CH + NaF group of rats. Sympathetic dominance in CH group rats with subjugation of parasympathetic actions. Shifting of balance towards parasympathetic dominance was evident in NaF group of rats. Altered sympatho-vagal balance due to CH or/and NaF exposed group rats was remarkably revamped by L- ascorbic acid supplementation.

Improvement of all the electrophysiological parameters and ventricular histopathology in L-ascorbic acid supplementation on CH or/and NaF exposed rats indicate protective effects of L-ascorbic acid on cardiovascular pathophysiology. This could be due to potential antioxidant property of l-ascorbic acid against low oxygen microenvironment due to fluoride exposure *in vivo* in experimental rats.

6.3: Hemogram

Erythrocytes are the major (99%) constituent of all blood cells and erythrocytes play essential role in transport of oxygen in eukaryotes. Our study results show significant increase of erythrocyte count in CH and CH +NaF treated rats but erythrocyte count was significantly decreased in NaF treated group animals. Hypoxic activation of hypoxia inducible genes plays a key role to maintain oxygen homeostasis. Hypoxia inducible factor -1(HIF-1) is a principal transcription factor involved in production of erythropoietin from kidneys. Erythropoietin stimulates erythrocyte production from erythroid lineage of stem cells. The prime role of increased erythrocyte production is to supply oxygen at tissue level to overcome low oxygen microenvironment (Warnecke *et al*, 2004; Rivera *et al,* 1994). Earlier studies have suggested HIF -1 for Epo expression in renal tissues. Recently RNA interference studies had shown the role of HIF-2 in specifically regulating Epo expression in renal and other tissues like liver (Warnecke *et al* 2004). CH can lead to erythropoiesis by both HIF1 and HIF-2 mechanism of activation of Epo gene in renal tissues and HIF-2 is involved in erythropoiesis by hepatic tissue along with kidneys in more severe hypoxic conditions.

Many studies have shown the toxic effect of NaF on various hematological parameters either by increasing their destruction or by decreasing their production. However anemia induced in NaF toxicity is due to decrease in bioavailablity of precursors of heme synthesis .Plasma iron content and iron retention is decreased due to NaF toxicity but not due to inhibition of enzymes in heme synthesis (Mandal K D *et al.,* 2015*).*Our study results are indicative of decrease in erythrocyte count in NaF treated rats. It may be due to rupture of membranes of erythrocytes. NaF by increasing lipid peroxidation ,causes accumulation of calcium ions intracellularly and activation of calcium dependent kinases inducing apoptosis in erythrocytes (Agalakova NI *et al.,* 2011,2013). Klarl BA *et al (*2006) had shown that calcium induced activation of scramblase will lead to phosphatidylserine exposure on erythrocytes , phosphatidylserine receptors on macrophages recognize such erythrocytes and engulf them. It could also be due to inhibitory effect of NaF on erythropoiesis (Atmaca *et al.,* 2014). NaF induced injury to hematopoietic progenitor cell linage may also be a cause to decreased erythrocyte count in our study (Machalinska *et al.,* 2001). L-ascorbic acid combats ROS injury caused by NaF to erythroblasts and enhances differentiation of erythroblasts and extricates

erythroblasts from ROS damage (Gonzalez-Menendez *et al*., 2021). On treatment with L-ascorbic acid in CH group rats showed significant improvements in erythrocyte count. We have not observed any significant differences in WBC count, hemoglobin concentrations between experimental groups.

6.4 : Biochemical analysis

Oxidant status

Cell survival is entirely dependent on utilization of oxygen. By virtue of various physiological oxygen sensing mechanisms, cells are able to prevent adverse damage to some extent during hypoxic conditions. Chronic hypoxia induces oxidative stress in the cells. NaF toxicity also creates low oxygen micro environment at cellular level and stimulates various oxygen sensing molecular mechanisms. In our study CH and NaF induced oxidative stress causes imbalance between oxidant and antioxidants in cardiopulmonary and hepatorenal systems. Oxidative stress is characterised by increase in ROS generation of cardiopulmonary and hepatorenal systems. ROS include free radicals like super oxide, peroxy nitrite and hydroxyl radicals. ROS induces lipid peroxidation . Polyunsaturated fatty acids are oxidised in lipid bilayer from ruptured cell membranes .It can also cause oxidative damage to proteins and produce carbonyl compounds. Severe oxidative stress induced oxidative damage of genetic material along with protein, carbohydrates and lipids can lead to cell death. In our study NaF induced and/ or CH exposed rats show increase in serum MDA and serum nitrite concentration due to oxidative stress. MDA is an indicator of lipid peroxidation and an important biomarker of oxidative stress (Vasant RA *et al.,* 2012*)* .Increased MDA levels along with hypertrophy of left ventricular muscle mass (supported by results of histopathology of heart) in CH group of rats also corroborated with the findings of Ferdinal F *et al*(2019). Increase in nitrite concentration in NaF induced and CH rats may be due to over expression of NOS gene and increases production of nitric oxide (NO) (Hassan HA *et al.,* 2009; Nakamura T *et al*., 2007). ROS in oxidative stress also activates several genes via HIF-1α in the metabolically active tissues. HIF-1α activates eNOS and iNOS by VEGF and can increase availability of nitric oxide (Giordano, 2005). Increased levels of NO will react with the superoxide anion (O_2^-) and form peroxynitrite anion($ONOO^-$) helpful in preventing oxidative damage. Out of cardiopulmonary and hepatorenal systems, the most important organ for detoxication and metabolic pathways is liver .Hypoxia and NaF toxicity puts greater burden of oxidative stress on hepatocytes. Lu *et al*., (2017) had shown that NaF induced oxidative stress impairs function of liver and increases apoptotic activity in liver cells which is mediated by TNF -R1 signal pathway . Kidneys are also sensitive to hypoxia. Kidneys are the organs for fluoride excretion and most of it

is retained in the kidneys leading to nephrotoxicity (Song C *et al*., 2017). Our results on oxidative stress are also supported by histopathological findings of kidneys and lungs. L-ascorbic acid supplementation on MDA and NO levels are indicative of preventing oxidative and nitrosative damage in cardiopulmonary and hepatorenal systems.

Antioxidant status

Our study also shows decrease in serum SOD, vitamin C , hepatic vitamin C and vitamin E concentrations in CH exposed and/or NaF induced rats. SOD is vital as antioxidant in combating superoxide radicals in the tissues. It converts superoxide anion to O_2 and H_2O_2. H_2O_2.is converted to water and oxygen molecule by catalase (Yadav *et al*., 2015). Decrease in SOD activity in hypoxic rats may be due to attack of free radicals on enzyme SOD (Smita *et al.,*2015) . NaF decreases enzymatic activity of SOD by binding to its active site and SOD also fails to detoxify hydroxyl ions . So inhibition of antioxidant enzyme SOD leads to oxidative damage at mitochondrial level and increases ROS levels (Djurasevic *et al.,* 2008) . This increase in ROS is associated with cardiopulmonary and heaptorenal dysfunction. Vitamin C plays important role as antioxidant in preventing this ROS induced cardiopulmonary and heaptorenal dysfunction .Vitamin C combats superoxide radicals in tissues and also decreases superoxide production by inhibiting NADPH oxidase . Vitamin C also regenerates tetrahydrobiopterin (BH4) in tissues during oxidative stress. BH4 is important for NOS3 activity. NOS3 is essential in regulating nitric oxide levels during oxidative stress. Vitamin E with lipid peroxyl molecule is oxidized to α -tocopheroxyl molecule. α -tocopheroxyl is also recycled by Vitamin C . Oxidation of vitamin C by ROS can be linked with cardiopulmonary and heptorenal oxidative damage leading to many pathological conditions. Antioxidants like vitamin C and E protect against ROS damage (May and Harrison , 2013) . Vitamin C also prevents mitochondrial ROS generation . Transportation of vitamin C occurs in its oxidized form .Dehydroascorbic acid enters through hexose transporters (GLUT's) and then it is reduced back to vitamin C .Vitamin C is crucial in combating many pathological conditions associated with ROS mitochondrial injury by protecting its membrane and genome (Jin X *et al* .,2014). L-ascorbic acid supplementation in CH or/and NaF induced rats are indicative of preventing oxidative and nitrosative damage in cardiopulmonary and hepatorenal systems.

6.5 : Molecular markers

The evolutionary mechanisms through complex physiological framework ensures optimal delivery of oxygen to the cells needed for its survival. Cells are also mechanised to sense changes during low oxygen microenvironment. Urgently operating systemic responses to hypoxia are through operation of cardiovascular and respiratory reflexes (Auten and Davis , 2009). Cells also respond differently by regulating the gene expression of molecular targets in oxidative stress induced by chronic hypoxia (Michiels C, 2004). Oxidative stress is also the mode of action for NaF toxicity in both *in vitro* and *in vivo* conditions (Song C *et al*., 2017). The molecular responses developed during fluoride toxicity are same as that developed during hypoxic conditions. Increased ROS production in oxidative stress of cardiopulmonary and hepatorenal systems may trigger hypoxic cellular response by activating hypoxia inducible genes. Hypoxia-inducible factor-1 (HIF-1) is a transcription factor gene that regulates the adaptive response. It consists of a regulatory subunit HIF-1α, which accumulates under hypoxic conditions. During hypoxia, degradation of HIF-1α is suppressed, thus HIF-1α protein accumulates. During normoxic conditions also the HIF-1α gene is expressed but the HIF-1α protein undergoes degradation (Das *et al*.,2016). HIF-1α induces expression of many genes related with the glycolysis , angiogenesis ,erythropoiesis, inflammatory process ,apoptosis and remodelling. Out of the cluster of HIF-1 genes, expression of endothelial nitric oxide synthase (NOS3) and vascular endothelial growth factor (VEGF) are important in regulating nitric oxide levels . Serum VEGF and NOS3 levels were estimated in our study to assess oxygen sensing molecular mechanisms. Recent studies have described that fluoride being negatively charged creates hypoxic microenvironment within the cells by generating mROS. The basic mechanism of fluoride toxicity is by inhibiting mitochondrial proteins including mitochondrial respiratory chain complexes I-IV. Oxidative stress is because of leakage of cytochrome c and free radicals into the cytoplasm .Cellular respiration halts due to disruption of mitochondrial membrane (Yan *et al*.,2015; Lu *et al*., 2017). This mROS induced hypoxic microenvironment in fluoride toxicity is similar to chronic hypoxia induced oxidative stress ,leading to upregulation of VEGF gene through HIF-1α. (Rodriguez-Miguelez *et al*., 2015). We have also found increased VEGF concentrations in NaF treated and hypoxia exposed rats. There is lot of interplay between ROS, HIF- 1α , VEGF , NOS3 gene expression and NO production .HIF- 1α regulates both VEGF and NOS3 gene expression. VEGF triggers hypoxic

response and also leads to up regulation of NOS3 genes and enhances NO production. HIF-1 α directly stimulates NOS3 and increases NO production (Lau *et al*.,2013). We have also observed increased NOS3 and NO concentrations along with VEGF concentration in NaF treated and hypoxia exposed rats. Low Mean arterial pressure in NaF treated group rats may be due to increased nitric oxide production and its effect on Vascular tone. Increased expression of NOS3 and VEGF in fluoride induced and/ or hypoxia exposed rats increases angiogenesis in the vascular beds of cardiopulmonary and hepatorenal systems .It may be the adaptive mechanism in oxidative stress induced hypoxic signalling pathway (Vadivel A *et al*.,2014). 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 by Vitamin C to combat cellular hypoxia in cardiopulmonary and hepatorenal systems.

6.6 : Histopathology

6.6.1 Histopathology of left ventricle myocardium

Left ventricular myocardium showed altered architecture and function in CH and/or NaF exposed groups when compared to control group rats. CH group showed hypertrophy of ventricular myocytes, suggestive of structural impairment and left ventricular remodelling .Alterations in the arrangement of thin and thick filaments induce mechanical and hemodynamic changes .Increased oxidative stress in the left ventricle induces expression of genes , synthesis of proteins and alters cell size. Hypoxic signalling pathways induce formation of growth factors ,which also results in hypertrophy (Ferdinal *et al*., 2019; Li *et al.,* 2002) . Ventricular muscle fibrosis in NaF exposed rats is also suggestive of oxidative damage , resulting in pathogenesis. Degenerative changes were observed in the left ventricle of rats exposed to CH and NaF simultaneously. Fluoride induces toxicity on left ventricle by two different mechanisms, firstly by increasing ROS formation and then by inhibiting antioxidant enzymes resulting into oxidative and nitrosative stress (Nabavi *et al*., 2012). Antioxidants have shown to be vital in combating oxidative and nitrosative stress. L-ascorbic acid supplementation shows improvement in pathological alterations of left ventricular architecture induced by CH and NaF, it may be due potent antioxidant property of vitamin C by inhibiting ROS induced damage .

6.6.2 Histopathology of lungs

Histopathological evaluation revealed injury of pulmonary architecture in CH and/or NaF exposed groups when compared to control group rats. CH, NaF and CH +NaF groups showed thickening of inter alveolar septae and alveolar spaces filled with eosinophilic fluid which causes reduction in alveolar space area and pulmonary edema. Our study results are indicative of pathological changes associated with alveolar hypoxia. Alveolar epithelium can maintain its architecture and functional stability even under hypoxic conditions. Alveolar hypoxia may induce inflammation locally, later pulmonary alveolar macrophages and others can lead to systemic inflammatory process .Oxidative tissue damage results from the increased ROS generation in inflammatory process (Smita *et al*.,2015) . Abdel-Gawad *et al*., (2014) have shown in their electron microscope studies that chronic exposure of rats to fluoride resulted in myelin figures, which indicates disruptive changes in membrane lipids and protein of lung epithelium and also observed an increase of pulmonary alveolar macrophages. Pulmonary alveolar macrophages induces inflammatory process ,which is similar to hypoxia induced oxidative tissue damage in alveolar hypoxia. Hemmati *et al*., (2008) had shown that Vitamin C and E antioxidants protect lungs from oxidative damage. Our results on L-ascorbic acid supplementation showed improvements in pathological alterations of pulmonary architecture induced by CH and NaF.

6.6.3 Histopathology of hepatic tissue

Histopathological evaluation revealed altered hepatic architecture in CH and/or NaF exposed groups when compared to control group rats. CH group showed inflammation and sinusoidal congestion. Previously Das *et al* (2015) in their studies of chronic sustained hypoxia on liver of rats documented the presence of inflammation , sinusoidal congestion, focal necrosis and swollen hepatocytes. NaF induced toxicity lead to disruption of hepatic architecture and lead to mononuclear infiltration and hepatic necrosis (Prakash et al., 2018; Lu *et al.,*2017). Parihar *et al*.,(2013) in their studies had exposed rats to NaF for 30,60 and 90 days respectively and observed the toxic effects on histopathological changes of liver, in 30 days treated group there was focal necrosis and central vein dilation , in 60 days treated group ,some earlier areas containing heaptocytes got vacuolated whereas in 90 days treated group there was excessive hepatic cell necrosis and degeneration of hepatic cells with nucleus , In hypoxia exposure and NaF treated rats such changes have lead to hepatocellular damage. Hence hypoxia and NaF combined together induce greater pathological response and damage in liver when compared to hypoxia alone . Parihar *et al*., (2013) had shown that Vitamin C and E antioxidants with aloe vera supplementation prevented toxic effect of NaF on histopathology of liver. In our study vitamin C supplementation has prevented disruption of hepatic architecture and necrosis induced by CH and NaF.

6.6.4 Histopathology of kidneys

Histopathological evaluation of kidneys in control and L-ascorbic acid groups showed normal renal cortex and glomerular tufts. Whereas CH group showed distorted epithelium in the renal cortex and glomerular distortion. Matsumoto *et al*.,(2004) had shown in their studies that chronic hypoxia is a mediator of progression of tubular injury and increases apoptosis of epithelium in the proximal tubules due to oxidative stress. NaF and CH + NaF groups showed congestion & inflammation of renal cortex with distorted epithelium is also suggestive of hypoxic related tubular injury (Krishnamoorthy *et al.,* 2015).kidneys are the main organs for excretion of fluoride and it is retained by the kidneys causing pathological alterations associated with tubular degeneration by ROS generation. Supplementation of L-ascorbic acid in renal perfusion injury experiments improved kidney function and histopatholgical alterations by decreasing ROS levels and increasing SOD expression (Zhu *et al*.,2016).Our study results also show that supplementation of L-ascorbic acid remarkably improved structural and functional ability of renal architecture and renal architecture appeared to be normal .

SUMMARY

7.1: SUMMARY:

In our study, the protective role of L-ascorbic acid on cardiac autonomic functions, cardiopulmonary and hepatorenal dysfunctions were investigated in chronic hypoxia (CH) , sodium fluoride (NaF) and in combination of CH and NaF in experimental rats .Our research work also stressed on the molecular mechanisms of hypoxic cell signalling pathways involved in development of cardiovascular pathophysiology , oxidative and nitrosative stress in lungs and hepatorenal systems in CH or/and NaF toxicity induced rats. L –ascorbic acid supplementation in CH or/and NaF toxicity induced rats shown significant role in preventing oxidative and nitrosative damage in cardiopulmonary and hepatorenal system along with improvements in histopathology.

Male albino rats were randomly divided into 8 groups (n= 6/group),and groups were named accordingly .Group I(control) , group II (L-ascorbic acid ,50 mg / 100g. b.wt, orally), group III (chronic hypoxia, 10%O_2), group IV (NaF ;20 mg/kg b.wt /day ; ip), group V (NaF + chronic hypoxia, 10% O_2), group VI (L–ascorbic acid +chronic hypoxia, 10% O_2), group VII (L-ascorbic acid + NaF) and group VIII (L-ascorbic acid + NaF + chronic hypoxia, 10% O_2). The treatments were carried for 21 days.

Animals of all groups were weighed on the starting day of protocol and immediately after the end of 21st day. Percentage change of body weight gain and OSI was determined. Electrophysiological parameters like pneumogram, noninvasive blood pressure (NIBP) and ECG were recorded. HRV analysis was done to assess cardiac autonomic functions. Oxidant status were assessed by evaluating serum MDA and NO levels . Antioxidant status were assessed by evaluating serum SOD, vitamin E, vitamin C and hepatic Vitamin C. Oxygen sensing molecular markers like vascular endothelial growth factor (VEGF) and nitric oxide synthase 3 (NOS3) were also assessed. Histopathological evaluations were done to identify changes in myocardial tissue (ventricle), lungs, hepatic tissue and kidney.

Our results on gravimetry are indicative of decrease in % of body weight gain and organosomatic index of heart, lungs , liver and kidneys in CH, NaF and CH + NaF treated groups. Electrophysiological studies in CH group rats are indicative of altered sympathovagal balance and sympathetic dominance. Increase in heart rate and MAP in CH group is also indicative of sympathetic stimulation. Whereas NaF treated rats showed parasympathetic dominance along with decrease in heart rate and MAP. Imbalance in oxidants and antioxidant status in CH, NaF and CH + NaF groups was due to upregulation of NOS3 and VEGF through HIF-1α. signalling pathway .Histopathological evaluations were done to identify changes in myocardial tissue (ventricle), lungs, hepatic tissue and kidney. It is evident from the above results that fluoride toxicity and chronic hypoxia induce oxidative and nitrosative stress leading to cardiac autonomic dysfunctions and also causing cardiopulmonary and hepatorenal pathophysiology.

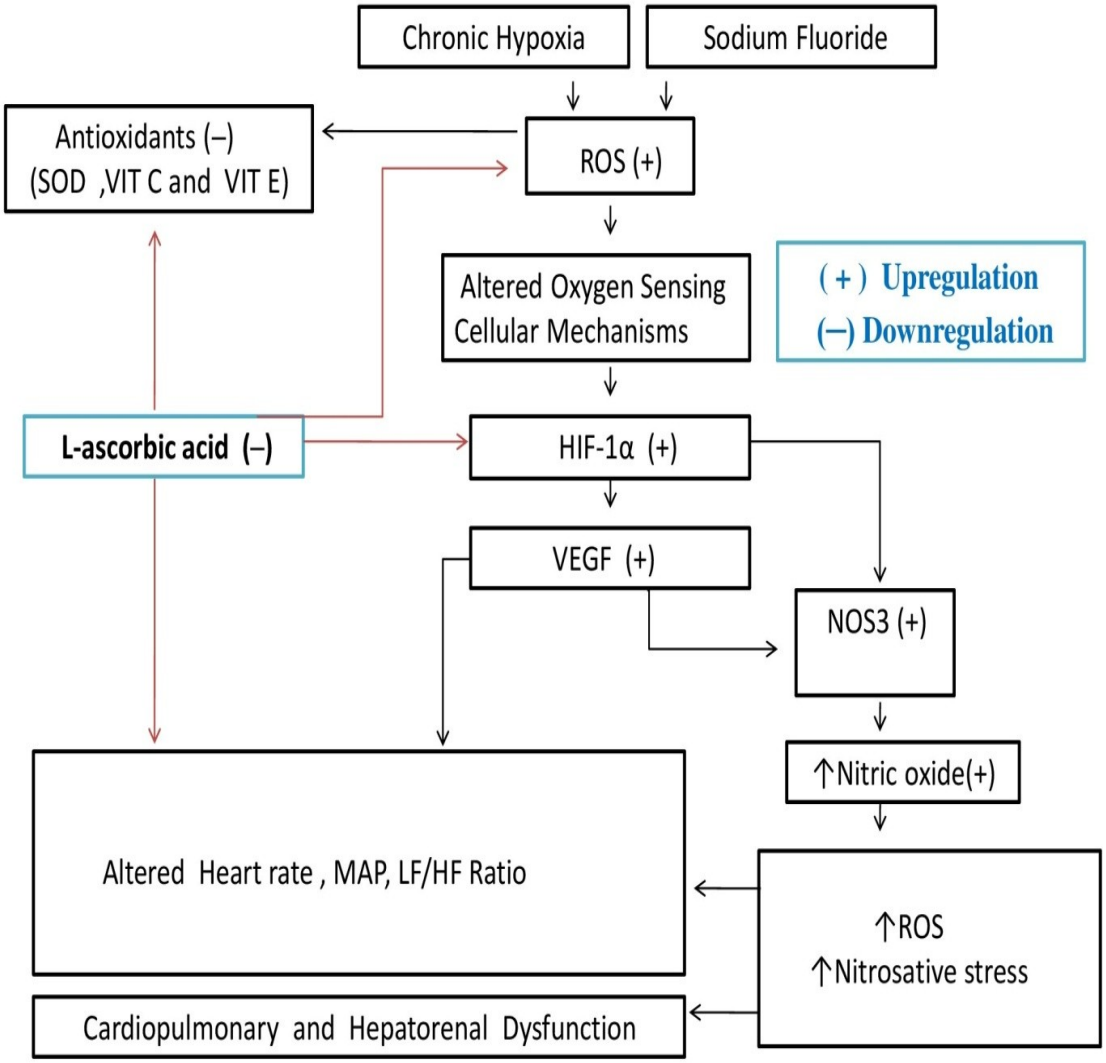
Animal groups treated with L-ascorbic acid showed improvements in % of body weight gain, OSI of heart, lung, liver and kidney. Improvements were also observed in altered heart rate, MAP, cardiac autonomic functions. It has also led to decrease in oxidative stress and nitrosative stress in chronic hypoxia and NaF treated groups. This could be due to potential antioxidant property of L- ascorbic acid.

CONCLUSION

7.2: CONCLUSION :

Results from our study shows that chronic hypoxia (CH) exposure and sodium fluoride(NaF) toxicity leads to cardiac autonomic dysfunctions, causes oxidative and nitrosative stress in cardiopulmonary and hepatorenal systems, enhances nitric oxide production by up regulation of VEGF & NOS3 genes leading to apoptosis. The supplementation of L-ascorbic acid has ameliorating effects on cardiac autonomic functions in chronic hypoxia (CH) induced male albino rats exposed to sodium fluoride (NaF). L-ascorbic acid supplementation is also salubrious to combat both chronic hypoxia (CH) and sodium fluoride (NaF) induced apoptotic cell signalling pathways leading to cellular adaptability.

GRAPHICAL ABSTRACT



LIMITATIONS OF THE STUDY :

- We were unable to perform quantification of gene expression of HIF1-α and VEGF by real-time quantification PCR.
- Study of Fluoride induced point mutations and expression of genes using cDNA microarray technique would have been better to understand the detailed molecular basis .
- Invitro studies using hypoxia susceptible cell lines with western blot analysis could be better to study the details of mediators involved in signalling pathways.

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ANNEXURE I
PLAGIARISM VERIFICATION CERTIFICATE

BLDE (DEEMED TO BE UNIVERSITY)

PLAGIARISM VERIFICATION CERTIFICATE

Annexure-1

1.Name of Student : Jaya simha Reddy..... Reg. No 13PHD006

2. Title of the Thesis: Influence of Antioxidant Vitamin (L-Ascorbic Acid) on Hypoxia Induced Oxidative and Nitrosative Stress in Physiological System of Male Albino Rats Exposed to Sodium Fluoride.

3. Department: Physiology

4. Name of Guide & Designation: **Prof Kusal K Das, Distinguished Chair Professor**

5. Name of Co Guide & Designation: **Dr Raju H Taklikar Professor and HOD**

The above thesis was verified for similarity detection. The report is as follows:

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The similarity index is above accepted norms, because of the following reasons:

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The thesis may be considered for submission to the University. The software report is attached.

Signature of Guide
Name & Designation
Prof. Kusal K. Das PhD
Laboratory of Vascular Physiology & Medicine
Department of Physiology
BLDE(DU) Shri B M Patil Medical College
Vijayapur-586103 Karnataka India

Signature of Co-Guide
Name & Designation
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Dept. Of. Physiology
Navodaya Medical College,
Raichur

Signature of Student

Verified by (Signature)
Name & Designation
9/7/2021
S.S. Hiremath
Librarian
B.L.D.E. Deemed to be University
Shri B. M. Patil Medical College,
Vijayapur.

ANNEXURE II

INSTITUTIONAL ANIMAL ETHICAL CLEARANCE CERTIFICATE

NAVODAYA MEDICAL COLLEGE
THE CAMPUS OF POSSIBILITIES

Ref. NMC/RCR/IAEC/2016-17/02

Date: 18-01-2017

To,

Mr. Jaya Simha Reddy
Assistant Professor
Department of physiology
Navodaya medical college Raichur,
Karnataka, India

Ethical clearance is hereby issued to your project titled "Influence of Antioxidant Vitamin (L-Ascorbic acid) on Hypoxia Induced Oxidative and Nitrosative stress in Physiological System of Male Albino Rats Exposed to Sodium Fluoride" under the guidance of Dr. Kusal K. Das, on submission after incorporating the suggestions as discussed by members of Institutional Animal Ethics committee during the meeting held on 13-01-2017

18/1/2017

Dr. Venkatesh M.Patil
Member Secretary, IAEC
Navodaya medical College, Raichur

Prof & Head
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ANNEXURE III

PRESENTATIONS

1. " L -ascorbic acid supplementation ameliorates oxidative and nitrosative stress in hypoxia induced male albino rats exposed to sodium fluoride" at 64th Annual National Conference of Association of Physiologists and Pharmacologists of India from 29th November to 1st December 2018 organised by Kasturba Medical College , Manipal.

2. " L-ascorbic acid supplementation ameliorates sodium fluoride induced alteration of cardiac autonomic functions in albino rats" at 2nd National conference in physiology at ATPCON-2018 conducted from 15th to 16 September 2018 by ESIC medical college, Hyderabad .

ANNEXURE IV

PUBLICATIONS

1. **Reddy JS**, R Reddy RC, Taklikar RH, Das KK. L-ascorbic Acid Supplementation Ameliorates Sodium Fluoride Induced Alteration of Cardiac Autonomic Functions in Hypoxic Rats. *Indian Journal of Public Health Research and Development*. 2019 ;10(6):165-170. http://dx.doi.org/10.5958/0976-5506.2019.01259.2. (Scopus)

2. **Reddy JS**, R Reddy RC, Taklikar RH, Das KK. L-ascorbic Acid Supplementation Ameliorates Sodium Fluoride Induced Oxidative and Nitrosative Hepatic Damage in Hypoxic Rats. *Indian Journal of Forensic Medicine&Toxicology*.2020:14(4);9289-9296. https://doi.org/10.37506/ijfmt.v14i4. (Scopus)