Influence of L-ascorbic acid on Chronic Hypoxia-induced alteration of Cell Signaling Pathways on Cardiovascular System in Male Wistar Rats with or without Exposure to Heavy Metal Nickel.

Thesis submitted to
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For the award of the degree of

Doctor of Philosophy in

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By

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March 2020

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I hereby declare that this thesis entitled ***"Influence of L-ascorbic acid on Chronic Hypoxia-induced alteration of Cell Signaling pathways on Cardiovascular System in Male Wistar Rats with or without Exposure to Heavy Metal Nickel"*** is bonafide and genuine research work carried out by me under the supervision of Dr. Basavaraj Devaranavadagi, (Guide) Department of Biochemistry and Professor Kusal K. Das (Co-guide) Department of Physiology, Shri B. M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapur, 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

To my Parents

Mr. & Mrs. Chandrasekhar Reddy and Venkata Narayanamma

and

To my Friend and my Brother Mr. Niranjan Reddy

Whose affection, love, encouragement make me able to get such Success and Honor.

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LIST OF ABBREVIATIONS

%	per cent
µl	micro litre
µm	micro meter
µM/L	Micro Moles per Litre
4-HNE	4-Hydroxynonenal
ABC	Avidin-Biotin-Peroxidase Complex
ACh	Acetylcholine
AGE	Advanced glycation end products
Akt	Protein kinase B
ANOVA	Analysis of Variance
ANS	Autonomic nervous system
ARNT	Aryl-Hydrocarbon-Nuclear Receptor-Translocator
ATP	Adenosine 5'-Triphosphate
AV	Atrioventricular
b. wt	Body weight
BH4	Tetrahydrobiopterin
BP	Blood Pressure
Ca^{2+}	Calcium
CB	Carotid body
CCB	Calcium channel blocker
cells/cumm	cells per cubic millimetre
CFU	Colony-forming unit
CH	Chronic hypoxia
CHER	Cholesterol esterase
CHOD	Cholesterol oxidase
CK-2	Casein kinase 2
Cm	Centimetre
CM	Chylomicron
COPD	Chronic obstructive pulmonary disease
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on animals
$CuSO_4$	Copper sulphate
DBP	Diastolic blood pressure
DF	Degree of freedom
DNA	Deoxyribonucleic acid
ECA	External carotid artery
ECG	Electrocardiogram
ECP	Extracellular matrix
EDTA	Ethylenediaminetetracetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	endothelial Nitric oxide synthase
Epo	Erythropoietin
ERKs	Extracellular signal regulated kinases
ET-1	Endothelin-1
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide

FBS	Fasting blood sugar
Fe^{2+}	Ferrous ion
FFA	Free Fatty Acids
FGF-2	Fibroblast growth factor -2
FMN	Flavin mononucleotide
g/dl	grams/decilitre
GLUT-4	Glucose Transporter-4
GND	Ground
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
Hb	Haemoglobin
HCl	Hydrochloric acid
Hct	Hematocrit
HDL	High-density lipoprotein
HF	High frequency
HIFs	Hypoxia-Inducible Factors
HPE	Histopathological examination
HR	Heart rate
HRE	Hypoxia-responsive elements
HRP	Horseradish peroxidase
HRV	Heart rate variability
HVA	High voltage-activated
HVD	Hypoxic ventilatory decline
i.p	Intra peritoneal
ICA	Internal carotid artery
IF-kβ	Inhibitory factor-kappa beta
IH	Intermittent hypoxia
iNOS	inducible Nitric oxide synthase
ip	Intra peritoneal
JNK1	c-Jun N-terminal kinase 1
K^+	Potassium channels
Kg	Kilogram
Km	Correction factor
LA	Lumen area
LCCA	Left common carotid artery
LDL	Low-density lipoprotein
LF	Low frequency
m^2	meter square
MAP	Mean Arterial Pressure
MDA	Malondialdehyde
mg	milligram
mg/dl	milligram per decilitre
mg/kg	milligram per kilogram
mIU/L	Milli International Units per Litre
mm Hg	Millimetre of mercury
mm^3	cubic millimetre
MMPs	Matrix Metalloproteinases
N_2	Nitrogen
Na^+	Sodium
NADPH	Nicotinamide adenine dinucleotide phosphate

$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NE	Norepinephrine
NF-kβ	Nuclear factor-kappa beta
NIBP	Non-invasive blood pressure
NIDMM	Non-insulin dependent diabetes mellitus
$NiSO_4$	Nickel sulphate
nm	Nanometre
nNOS	neuronal Nitric oxide synthase
NO	Nitric Oxide
nu	normalized units
NWI	Normalized wall index
O_2	Oxygen
OD	Optical density
OS	Oxidative stress
OSA	Obstructive sleep apnoea
P com A	Posterior communicating artery
p38 MAPK	p38 mitogen-activated protein kinases
PCA	Posterior cerebral artery
PDGF	Platelet- derived growth factor
PEGME	Polyethylene- glycol- methyl ether
pg/ml	picogram per millimetre
PHDs	Prolyl hydroxylases
PI3-K	Phosphoinositide-3 kinase
PLGF	Placental growth factor
PO_2	Partial pressure of oxygen
PPA	Pterygopalatine artery
PSI	Pulmonosomatic Index
PUFAs	Polyunsaturated fatty acids
pVHL	von Hippel- Lindau tumour- suppressor protein
PVS	Polyvinyl sulfonic acid
RAAS	Renin Angiotensin Aldosterone System
RBC	Red Blood Cell
R_f	Respiratory frequency
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Respiratory rate
SA	Sinoatrial
SBP	Systolic blood pressure
SD	Standard deviation
sec	seconds
SNS	Sympathetic nervous system
SOD	Superoxide dismutase
SPSS	Statistical Package for the Social Sciences
STA	Superior thyroid artery
TC	Total cholesterol
TG	Triglycerides
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
TVA	Total vessel area

VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptors
VLDL	Very low- density lipoprotein
VSMCs	Vascular smooth muscle cells
WA	Wall area
WAT	White adipose tissue
$ZnSO_4$	Zinc Sulphate

ABSTRACT

Objective: To study the influence of L-ascorbic acid supplementation on the mechanisms of cardiovascular cell signaling pathways by either chronic hypoxia or heavy metal nickel exposure on the involvement of the common hypoxia signaling pathways in altered cardiovascular pathophysiology.

Methods: In the present study we included forty-two adult male Albino Wistar rats (Rattus Norvegicus) and randomly allocated into seven groups and named groups as control; L-ascorbic acid (50 mg / 100 gm. b.wt orally); chronic hypoxia (CH) (10% O_2, 90% N_2); $NiSO_4$ (2.0 mg/100gm b.wt, i.p, every alternate day); CH + $NiSO_4$; L-ascorbic acid + CH and L-ascorbic acid + $NiSO_4$ and respective interventions were given for 21 days. Before and after the intervention period weights of all the experimental animals were recorded and % body weight gain was calculated. Electrophysiological parameters like ECG, blood pressure (MAP) and pneumogram of animals were recorded in anaesthetized/conscious animals after the intervention period. Cardiovascular autonomic function was assessed by HRV analysis. Oxidative stress and antioxidant defence were assessed by estimating MDA, ascorbic acid, α-tocopherol in the serum and MDA in heart and lung tissue homogenates by spectrophotometric methods. Oxygen sensing molecular markers like VEGF, NOS3 and NO were estimated in the serum by ELISA and immunoblotting techniques. Cardiovascular remodeling was studied by assessing cardio-somatic index, and histopathological examination of H & E stained sections of the ventricles, intramyocardial coronary artery, and elastic artery. Further, to know the vascular remodeling normalized wall index (NWI) of coronary artery was calculated. In addition to these histopathological examinations of the lung was also done.

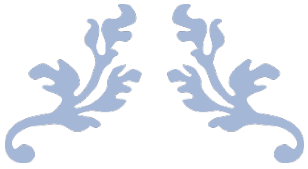
Results: Results suggest that chronic hypoxia (CH), $NiSO_4$ and CH + $NiSO_4$ impairs overall gravimetry of experimental animals, cardiac autonomic functions, vascular functions, induces

oxidative and nitrosative stress, up regulates oxygen sensing molecular markers like VEGF and NOS3 proteins. Further, chronic hypoxia and heavy metal nickel toxicity have adverse impact on histopathology of lung, cardiac and aortic tissues. Additionally, increased NWI values support the cardiovascular remodeling. All these results indicate a possible common link between nickel toxicity and hypoxia by modulate cell signal transduction with sympathetic overactivity, oxidative stress and alteredcardiovascular pathology.

Antioxidant vitamin L-ascorbic acid supplementation was able to 1) Reduce sympathetic over activity 2) reduce MAP 3) decrease oxidative stress 4) decreases nitrosative stress and 5) ameliorate cardiovascular remodeling resulting from chronic hypoxia and $NiSO_4$ exposure. These effects of L-ascorbic acid could be attributed to its antioxidant property. Thus, the results of the present study suggest a possible use of antioxidant vitamin L-ascorbic acid supplementation as an add-on therapy against hypoxia or nickel toxicity-related pathophysiology.

Conclusion: Present study shows that CH or nickel induce hypoxia cell signalling mechanisms through high production of reactive oxygen species and deprivation of antioxidant vitamins like L-ascorbic acid and alpha tocopherol, sympathetic over activity, cardiovascular, and pulmonary remodelling and hypertension. Results also indicate that heavy metal nickel induce cardiovascular pathophysiology is sensitive to oxygen hence alteration of cardiovascular anatomy and physiological function in the experimental animal by nickel or chronic hypoxia derive similar outcome. The results obtained in this study may have clinical value in humans and the effect of L-ascorbic acid on CH or nickel induced cardiovascular toxicities deserves further exploration by targeting the common transcriptional influences.

Key words: nickel, chronic hypoxia, cardiovascular pathophysiology, L-ascorbic acid



CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

Oxygen (O_2) contributes one fifth of the earth's atmosphere. O_2 is essential for animal life: used by mitochondria present in animal cells as a terminal electron acceptor in the oxidative phosphorylation reaction to produce ATP in electron transport chain (ETC). During oxidative phosphorylation, a higher risk of reactive oxygen species (ROS) generation will be there. So, these high levels of ROS may interfere with the biochemical and physiochemical properties of cellular macromolecules, leading to cell death. Hence, conservation of oxygen homeostasis is important for cell growth and survival (Semenza, 2003). Normal levels of oxygen in healthy tissues varies widely between organs and range between ~ 4.6 % O_2 to 9.4 % O_2 while O_2 concentration in tumor range on an average between 1–2 % O_2 or below (Muz et al., 2015). Pathophysiological conditions where the tissue oxygen levels lower than 3% is considered as hypoxia (McKeown, 2014). During hypoxia, Hypoxia inducible factor-1 (HIF-1α) accumulates within cells and induces transcription of its target genes such as erythropoietin, heme oxygenase, and vascular endothelial growth factor (VEGF) (Semenza, 2003, 2004). Margaritis et al has shown a link between hypoxia induced oxidative stress for the development of cardiovascular disease (Margaritis et al., 2017). Oliveira et al reviewed 13 manuscripts in their systematic reviewand concluded that hypoxia causes sympathetic predominance and the reduction in vagal activity (Oliveira et al., 2017). So, hypoxia play a vital role in the modulation of autonomic nervous system dysregulation, stress mediated cardiometabolic diseases and hypertension in patients experiencing hypoxic stress (Carnagarin et al., 2018; Fukuda et al., 1989; Xiang et al., 2015).

Most of the researchers has reported a strong link between environmental pollutants and various cardiovascular diseases (CVD) (Cosselman et al., 2015; Gold et al., 2000; Lippmann et al., 2006; Vallejo et al., 2006; Zhang, Chau et al., 2009). Amongst environmental heavy metal pollutants, a divalent cation nickel (Ni) has been most widely

used for various industrial applications like electroplating of metals, battery manufacturing, as a catalyst, electrical and electronic industries, metallurgy and metal alloys preparations (Barceloux and Barceloux, 1999). Nickel is a silver-white transition metal that belongs to the group VIII b of the periodic table. Nickel concentrations in ambient air is minimal (approximately 6–20 ng/m^3) but higher concentrations (up to 150 ng/m^3) are found in the air polluted by anthropogenic sources (Barceloux and Barceloux, 1999). Among the various nickel compounds, nickel sulfate contributes predominant nickel pollutant of ambient air. Nickel facilitates absorption of iron (Fe) in Fe^{3+} form in gastrointestinal tract (GIT) (Nielsen, 1980). Nickel is considered as essential trace element for several animal species, but in humans deficiency manifestations have not been clearly mentioned (Barceloux and Barceloux, 1999). In biological systems, absorption, distribution and clearance of nickel compounds mainly depend on their solubility in water and the route of exposure (Barceloux and Barceloux, 1999). The absorption and toxic manifestations of nickel compounds depend on the solubility and order of absorption as follows: nickel carbonyl > soluble nickel compounds (chloride, nitrate, sulfate) > insoluble nickel compounds (oxides, sulphides) (Sunderman et al., 1989). Studies revealed that divalent cation (Ni^{2+}) influences cardiovascular functions and causes aortic hyper contraction, but exact physiological mechanisms are yet to be known (Wani et al., 2018). As of now very few studies have been conducted to know the toxic effects of divalent cations like nickel on cardiovascular health.

L-ascorbic acid (vitamin C) is a water-soluble antioxidant and found to be the most effective circulatory antioxidant in human system (Frei et al.,1989). L-ascorbic acid or vitamin C prevents lipid peroxidation, oxidation of low-density lipoproteins and advance oxidation protein products (Balz Frei, 2004). The respiratory tract lining fluid (RTLF) contains a variety of endogenous antioxidant enzymes like superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. It also contains vitamins C, E and A and

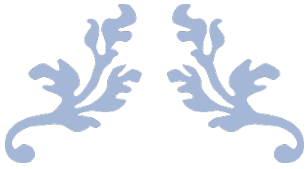
other exogenous antioxidant compounds (Cross et al., 1994). L-ascorbic acid may comprise the first line of defense system in RTLF against external pro-oxidative assaults (Kasprzak et al., 2011). It has been reported that intracellular depletion of ascorbic acid aggravated some heavy metal (nickel, cobalt etc.) induced carcinogenicity and acute toxicity (Wu et al., 2002). Maniyar et al have earlier shown changes in chemical behaviour of l-ascorbic acid alone or in combination of nickel sulfate at different pH solutions (Maniyar et al., 2012).Some of the clinical trials has proved intravenous pharmacological dosage of ascorbate was well tolerated by cancer patients with normal renal function and they also demonstrated that beneficial effect of ascorbate with standard of care chemotherapies (Hoffer et al., 2008; Monti et al., 2012; Riordan et al., 2005). Emadi et al has shown that high dose of L-ascorbic acid significantly improves the ventricular functions and reduces Intensive Care Unit (ICU) stay of patients undergone coronary artery bypass grafting surgery (Emadi et al., 2019)

It has been observed that both heavy metal nickel as well as chronic hypoxic exposure can activate the hypoxia-inducible pathway and facilitates selection of cells with increased transcriptional activity of hypoxia-inducible genes, which may be important in the nickel-induced carcinogenic process (Das et al., 2019). Like hypoxia, Ni (II) induces HIF-1 and therefore activates genes responsible for the up-regulation of glucose metabolism and glycolysis even in the presence of oxygen, the vascular endothelial growth factor (VEGF), and the tumor marker Cap43 (Salnikow et al., 2003). Recent studies indicate the ability of ascorbic acid to regulate factors that may influence gene expression, apoptosis and other cellular functions. In many studies L-ascorbic acid protects against cell death triggered by various stimuli and a major proportion of this protection has been linked with its antioxidant ability (Das et al., 2006, 2007; Gupta et al., 2006; Gupta et al., 2007). Studies of the anti-apoptotic activity of L-ascorbic acid have revealed its role in modulation of the immune system. Several studies reported the mechanisms by which L-ascorbic acid regulates the AP-

1 complex, including the Fos and Jun super families. Ascorbate treated cells exposed to UV-B irradiation led to a 50% decrease in JNK phosphorylation (which activated AP-1), therefore inhibiting the JNK/AP-1 signaling pathways (Duda-Chodak and Blaszczyk, 2008). As one of the common pathways to induce HIF-1 signaling mechanism by hypoxia and heavy metal (nickel) exposure is also via ROS generation hence the role of antioxidant like L-ascorbic acid to counteract this phenomenon is very much in research interest.

The results of some studies have indicated that nickel (a known free radical generator) exposure is responsible for a hypoxic cellular environment by depletion of iron from the cell(Function, 1979). Hypoxia signaling is one pathway that contributes to metal ion-induced carcinogenesis by disrupting cellular iron homeostasis though competition with iron transporters or iron-regulated enzymes. In-vitro studies of human and rodent cells showed that the HIF-1 signaling pathway is activated by exposure to carcinogenic nickel compounds. Nickel induced genes coding for glycolytic enzymes and glucose transporters, which are regulated by HIF-1 transcription factor, only in HIF-1 alpha-proficient cells. In addition, several other hypoxia-inducible genes were up-regulated by nickel in a HIF-dependent manner. Additionally, other genes were induced by nickel in an HIF-1-independent manner, suggesting that nickel exposure activates several signaling pathways. Assessing the induction of these pathways after exposure is essential to the understanding of cancer development related to nickel exposure (Wu et al., 2002a). Nickel sulfate can induce hypoxia-inducible genes and IL-8 production in human lung cells with or without interference with iron metabolism (Kang et al., 2006).

So, with the above background we understood that there are major gaps regarding the influence of L-ascorbic acid on longterm effects of hypoxia, heavy metal like nickel toxicity especially when it comes to linking cardiovascular and cellular responses with the physiological adaptation.



CHAPTER 2

REVIEW OF LITERATURE

2.1 Nickel:

Nickel is a silvery white metal that takes on a high polish. It is a transition metal, and is hard and ductile. It occurs most usually in combination with sulphur and iron in pentlandite, with sulphur in millerite, with arsenic in the mineral nickeline, and with arsenic and sulphur in nickel glance(Nestle, Speidel, and Speidel, 2002). Nickel is one of the five ferromagnetic elements. Nickel is also a naturally magnetostrictive material, meaning that in the presence of a magnetic field, the material undergoes a small change in length(Hathaway and Clark, 1993). The properties of nickel and its environmental distribution have been summarized by the US Agency for Toxic Substances and Disease Registry ("Toxicological Profile for Nickel," 2002). Nickel is used in a wide variety of metallurgical processes such as electroplating and alloy production as well as in nickel-cadmium batteries.

2.1.1 Nickel and its environmental Distribution:

Nickel normally present at very low levels in the environment hence very sensitive methods are needed to detect nickel in most environmental samples. Nickel in air is attached to small particles and it ranges from 1-86 ng/m^3. On the other hand the level of nickel in water is often as low as 10 ppb or less (except in industrial areas). Soil usually contains 4-80 ppm of nickel. Generally food is the major source of nickel exposure for general population. Nickel consumption through food is about 170 µg/day. Some foods contain high concentration of nickel e.g. tea, coffee, chocolate, soybeans, nuts, oatmeal, cabbage, spinach, potatoes etc ("Toxicological Profile for Nickel," 2002).

2.1.2 Pharmacokinetics of Nickel:

After nickel gets into the body, it can go all organs but it accumulate mainly in lungs, thyroid gland, adrenal gland followed by liver, kidney, heart, brain, spleen, testes and even in

bones (Bernacki, Zygowicz, and Sunderman, 1980). Nickel is not destroyed in the body, but its chemical form may be altered. The metabolism of nickel is most appropriately viewed in light of its binding to form ligands and its transport throughout the body. Much of the toxicity of nickel may be associated with its interference with the physiological processes of manganese, zinc, calcium, and magnesium (Nielsen, 1980). Nickel is a known hematotoxic, immunotoxic, hepatotoxic, pulmonotoxic, and nephrotoxic agent (Cameron et al., 2011; Das and Saha, 2010). Several heavy metals including nickel have been implicated in the increased risk of lung cancer(Anderman, 1994). The ability of nickel compounds to raise the intracellular concentration of nickel ions established the basis for its carcinogenic potential. Human and animal studies have demonstrated that exposure to nickel refinery dusts and tri-nickel-disulphide Ni_3S_2 increases the probability of lung and nasal cancer in workers. Nickel refinery dust and Ni_3S_2 are classified as human carcinogens (Group A) by the United States Environmental Protection Agency (US EPA) and Group 1 by the International Agency for Research on Cancer (Cameron et al., 2011).

2.1.3 Nickel Toxicity and Oxidative Stress:

The production of reactive oxygen species (ROS) is involved in the molecular mechanism of nickel toxicity and carcinogenicity (Ornoy, Rand, and Bischitz, 2010). There has been increasing interest in the role of free radicals and antioxidants in cancer and oxidative stress has been suggested to play a key role in carcinogenesis (Lu, 2007). Nickel also elicits increase nitric oxide production (Tian and Lawrence, 1996). Endogenous nitric oxide is a double-edged molecule, acting as an important physiological signalling molecule mediating various cellular functions; on the other hand it induces cytotoxic and mutagenic effects when present in excess (Kaynar et al., 2005). Over production of nitric oxide can react with the superoxide anion (O^{-}_2), which produces the peroxynitrite anion ($ONOO^{-}$). This reaction is one of the fastest in human biology (Land, 1990). Peroxy-nitrite is a powerful

oxidant and may trigger lipid peroxidation, inhibit mitochondrial electron transport, oxidize thiol compounds and oxidize and nitrate DNA (Kaynar et al., 2005). Nickel sulphate stimulates inducible nitric oxide synthase (iNOS) and inhibit endothelial nitric oxide synthase (e-NOS) activities(Das and Saha, 2010; Land, 1990).

2.1.4 Nickel Toxicity and Human Health:

A major amount of nickel enters our body via food and water intake, but inhalation exposure is most common in occupational workers. It has been reported that acute inhalation exposure of nickel in humans shows symptoms like headache, nausea, respiratory problems, and death(Das and Buchner, 2007; Rendall et al., 1994). Warner et al (1979) reported that there is no clinical evidence of developmental and reproductive toxicity in the women working in nickel refinery, but Chashschin et al (1994) reported reproductive and developmental effects of nickel in occupationally exposed populations (0.13-0.2 mg nickel/m3). Goyer et al (1991) reported that acute inhalation of nickel carbonyl leads to headache, nausea, vomiting, chest pain, hyperpnea, cyanosis, respiratory failure, and ultimately death if the exposure is severe. In the year 1994, Nicklin and Nielsen categorized asthmatic attack response caused by nickel inhalation as i) a rapid onset attack (antibody mediated Type I hypersensitivity) with broncho-spasm, ii) a late response reaction at 6-12 hours after exposure (antigen- antibody immune complex-mediated inflammatory reaction), and iii) a mixed or combined response. A study reported the increased blood reticulocytes in workers who drank water from a water fountain contaminated with nickel sulphate, nickel chloride, and boric acid (estimated dose of 7.1–35.7 mg Ni/kg) (“Toxicological Profile for Nickel,” 2002). Huang et al., conducted a survey of 50 peritoneal dialysis (PD) patients and 50 normal patients for urinary nickel concentration and analysed the possible association of urinary nickel concentrations with clinical outcomes and inflammatory biomarkers. Based on their study, it was found that nearly 50% of the patients undergoing PD had higher levels of

urinary nickel and also these patients had increased serum levels of high sensitivity C-reactive protein (Huang et al., 2017). In another research, Jouybari et al (2018) studied the role of toxic elements as biomarkers for breast cancer (BC), which showed a significant difference in the cadmium (Cd) and Ni statuses between healthy and BC patients, clearly indicating a direct and positive association between Cd and Ni concentrations and BC risk. Also, increased urinary total protein levels, β2-macroglobulin, retinal binding protein, and N-acetyl-β-D-glucosaminidase were reported in electroplating workers who consumed nickel contaminated water (Huang et al., 2017).

2.2. Hypoxia:

Hypoxia is a physiological or pathological state characterized by decreased oxygen levels in organs and tissues. Low oxygenation microenvironment plays a key role in embryogenesis and it may be normal physiological environment for some tissues of adults (e.g., bone marrow microdomains and thymus). But, commonly oxygen scarcity affects cellular functions and disrupts various biological processes including cell growth and differentiation, angiogenesis, metabolism, and pH homeostasis. Therefore, hypoxia is associated with various pathophysiological conditions including chronic obstructive pulmonary disease, pulmonary hypertension, congenital heart disease, cerebral ischemia, and cancer. The cell signalling mechanism to hypoxia is of great interest to many of the researchers because of the importance of oxygen sensing in various biological processes like angiogenesis, cellular survival/proliferation, energy metabolism, erythropoiesis, extra-cellular matrix function, invasion/metastasis, iron metabolism, pH regulation, multi-drug resistance and stem cell properties among others.

According to existing reports physiological oxygen (physoxia) conditions for human tissues and organs varies, always range between ~5% (e.g., brain) to ~10% (e.g., renal cortex) (Muz, de la Puente, Azab, and Azab, 2015).For some tissues, physoxia may normally fall

outside of this range, either below at ~1% (e.g., bone marrow and thymus) or above at ~14% (e.g., arterial blood) **(Figure 2.2)**

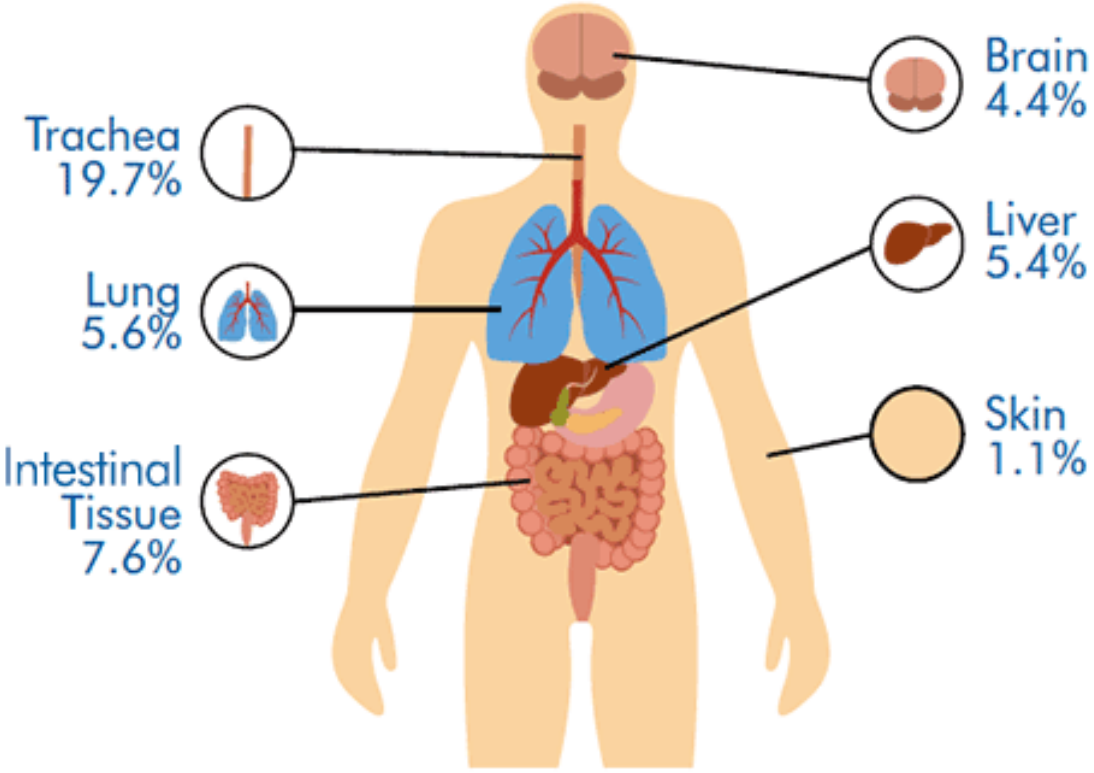


Figure 2.2 Normal median percentage of oxygen in major organs and Tissues (McKeown, 2014)

2.2.1 Hypoxia - Cell signalling:

Cellular hypoxia causes an initiation of hypoxia-response genes responsible for angiogenesis, oxygen transport, and metabolism(Harris, 2002). Hypoxia leads to alter intracellular chemical microenvironment by increasing calcium concentration ([Ca2+]i), 5-lipoxygenase, lipid peroxidation, cyclooxygenase (COX), constitutive nitric oxide synthase (cNOS), leukotriene B4 (LTB4), prostaglandin E2 (PGE_2), interleukins, tumor necrosis factor-α (TNF- α), caspases, complement activation heat shock protein 70 kDa (HSP-70), and

hypoxia-inducible factor-1α (HIF-1α) (Kiang et al., 2004; Moore et al., 1994). Chronic hypoxia stimulates both KLF6 and NF-κB gene expressions and it reduces KLF4 which further leads to an enhance iNOS expression (**Figure 2.2.1**). Moderate hypoxia is an important regulator to maintain pulmonary vascular tone (Madden, Vadula, and Kurup, 1992). Nevertheless, tissue hypoxia is also associated with a varied and extensive range of pathophysiological processes including vascular and degenerative diseases, chronic inflammation, and cancer (Valko, Morris, and Cronin, 2005).

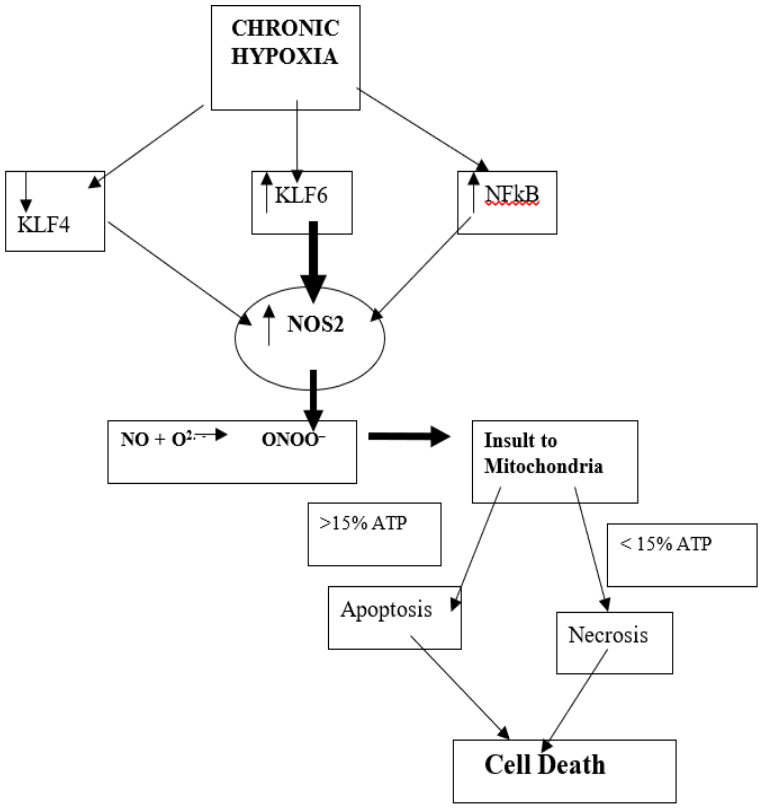


Figure 2.2.1: Graphic representation showing hypoxia-induced cellular abnormalities.

NF- kB, nuclear factor-kappa B; NO, nitric oxide; $ONOO^-$, peroxynitrate; KLF4, Kruppel-like factor 4; KLF6, Kruppel-like factor 6 (Das and Saha, 2015).

2.2.2 Hypoxia – Heavy Metal Toxicity –Oxygen Sensing Mechanism:

It has been observed that both heavy metal nickel as well as chronic hypoxic exposure can activate the hypoxia-inducible pathway and facilitates selection of cells with increased transcriptional activity of hypoxia-inducible genes, which may be important in the nickel-induced carcinogenic process. Like hypoxia, Ni(II) induces HIF-1 and therefore activates genes responsible for the up-regulation of glucose metabolism and glycolysis even in the presence of oxygen, the vascular endothelial growth factor, and the tumor marker Cap43(Chodak and Blaszczyk, 2008). The results of some studies have indicated that nickel (a known free radical generator) exposure is responsible for a hypoxic cellular environment by depletion of iron from the cell (Valko et al., 2005). Hypoxia signalling is one pathway that contributes to metal ion-induced carcinogenesis by disrupting cellular iron homeostasis though competition with iron transporters or iron-regulated enzymes(T. Davidson, Chen, Garrick, D'Angelo, and Costa, 2005). In-vitro studies of human and rodent cells by Salnikow et al showed that the HIF-1 signalling pathway is activated by exposure to carcinogenic nickel compounds (Salnikow et al., 2003). Nickel induced genes coding for glycolytic enzymes and glucose transporters, which are regulated by HIF-1 transcription factor, only in HIF-1 alpha-proficient cells(Maxwell and Salnikow, 2004; Salnikow et al., 2003). In addition, several other hypoxia-inducible genes were up-regulated by nickel in a HIF-dependent manner (Costa et al., 2005; T. L. Davidson et al., 2006; Maxwell and Salnikow, 2004; Salnikow et al., 2000). Additionally, other genes were induced by nickel in an HIF-1-independent manner, suggesting that nickel exposure activates several signalling pathways. Assessing the induction of these pathways after exposure is essential to the understanding of cancer development related to nickel exposure (Salnikow, Davidson, and Costa, 2002). The results of a further study by Salnikow et al (2004) indicated that nickel sulphate can induce

hypoxia-inducible genes and IL-8 production in human lung cells with or without interference with iron metabolism.

2.3 L-ascorbic acid (Vitamin C):

L-ascorbic acid is a water-soluble chain-breaking antioxidant, known to be the most effective aqueous phase antioxidant in human plasma(B. Frei, England, and Ames, 1989). Ascorbic acid also inhibits lipid peroxidation, oxidation of low-density lipoproteins and protein oxidation (Balz Frei, 2004). The respiratory tract lining fluid (RTLF) contains a variety of enzymatic antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase. It also contains non enzymatic vitamins C, E and A and other antioxidant compounds (Cross, van der Vliet, O'Neill, Louie, and Halliwell, 1994). Ascorbic acid may constitute the first line defence system in RTLF against external oxidants (Mudway et al., 2001). As intracellular ascorbate depletion enhances carcinogenicity and acute toxicity of nickel (Kasprzak et al., 2011). In previous studies it has been observed that there was an altered chemical behaviour of L-ascorbic acid alone or in combination of nickel sulphate in vitro at different pH solutions (Maniyar, Jargar, Das, Dhundasi, and Das, 2012).

Recent studies indicate the ability of ascorbic acid to regulate factors that may influence gene expression, apoptosis and other cellular functions (Wu, Tyml, and Wilson, 2002). In many studies it has been proved Vitamin C protects against cell death triggered by various stimuli and a major proportion of this protection has been linked with its antioxidant ability. Studies of the anti-apoptotic activity of Vitamin C have revealed its role in modulation of the immune system. Several studies reported the mechanisms by which Vitamin C regulates the AP-1 complex, including the Fos and Jun super families. Ascorbate treated cells exposed to UV-B irradiation led to a 50% decrease in JNK phosphorylation (which activated AP-1), therefore inhibiting the JNK/AP-1 signalling pathways(Wu et al.,

2002). The effect of simultaneously supplemented vitamin C on experimental nickel treatment show ascorbic acid is capable to reduce intestinal absorption of nickel. The mechanism involves that vitamin C is capable to reduce ferric iron to ferrous iron in the duodenum thus availability of divalent ferrous ion increases which competes with nickel also a divalent cat-ion for intestinal absorption (Das and Büchner, 2007).Recent reports indicate the capability of ascorbic acid as a regulatory factor may influence gene expression, apoptosis and other cellular functions of living system exposed to heavy metals (Das et al., 2015).

2.4 Hypoxia, Nickel (II) and L-ascorbic acid:

Hypoxia can stimulate a variety of systemic, local, and cellular responses. In mammalian systems, the systemic response includes the transcriptional up regulation of the gene encoding the peptide hormone erythropoietin (EPO). This cytokine increases the red blood cell count by stimulating erythropoiesis, thus increasing the efficiency of O_2 transport throughout the body. A second aspect of the systemic response to low oxygen tension is the increase in respiration rate that occurs through dopaminergic input to the carotid body. The up-regulation of dopamine is due to the hypoxia-induced transcriptional activation of tyrosine hydroxylase, the rate-limiting enzyme of catecholamine synthesis. Local areas of hypoxia can arise during embryogenesis; wound healing, and tumor growth. In these processes, hypoxic tissues up-regulate the transcription of genes encoding various angiogenic factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF), as well as vasodilators produced by enzymes, such as inducible nitric oxide synthase and heme oxygenase-1(Bos et al., 2005). The up-regulation of these factors results in an increased vascular bed density, vascular permeability, and oxygen availability to the starved tissues. At the cellular level, hypoxia can limit oxidative metabolism and thus decrease energy production. To adapt to a low-oxygen environment,

many cell-types convert to glycolysis for energy. This cellular response is mounted through the transcriptional activation of genes encoding glycolytic enzymes such as aldolase A, phosphoglycerate kinase 1, lactate dehydrogenase A, and phosphofructo kinase L and glucose transporters such as GLUT-1 (Minchenko, Kharkova, Hubenia, and Minchenko, 2013).

Several epidemiological studies well explained about the heavy metal induced toxicity and their carcinogenic manifestations. Various in vivo and in vitro studies explained about the diversity of heavy metal toxicities and their differential carcinogenic properties depending on their physiochemical properties and the route and extent of exposure (Cameron et al., 2011; Duda-Chodak and Blaszczyk, 2008; Lu, 2007). Recently many researchers are tracking the heavy metal induced cancer growth and progression by in depth understanding of cell signaling mechanisms and epigenetics (Galanis, Karapetsas, and Sandaltzopoulos, 2009; Leonard, Harris, and Shi, 2004; Nair, DeGheselle, Smeets, Van Kerkhove, and Cuypers, 2013; Salnikow et al., 2003; Tian and Lawrence, 1996; Valko et al., 2005; Valko, Rhodes, Moncol, Izakovic, and Mazur, 2006). These in depth understandings of metal induced carcinogenesis pathways will definitely helpful to develop protective therapeutic approaches. Many studies have reported that heavy metals cause tissue or organ damages by mimicking the cellular hypoxia through the inhibition of mitochondrial cytochrome C oxidase (Das et al., 2015; Das et al., 2019; Das and Saha, 2015; Leonard et al., 2004; Salnikow et al., 2000). Individual resistance to hypoxia provides an individual reaction of mitochondrial respiratory chain functioning, mitochondrial ion transport, properties of mitochondrial enzymes, and energy metabolism, mono-oxygenase system activity, biotransformation of genobiotics and drug metabolizing system (Tkachenko and Kurhalyuk, 2011).

2.5 Nickel Toxicity and Cardiovascular System:

It has been reported that increased air particles or pollutants can cause various

respiratory and cardiovascular problems. Even water and food toxicants make serious pathological impacts in living organisms. Due to revolutionized industrial applications contamination and intoxication of certain chemicals became serious problems to plants, human beings and wild life. Because of its industrial properties nickel became major pollutant in the environment. Jaiprakash et al (2017) has reported that polluted source of particulate matter contains high concentrations of nickel and other trace elements. Recent reports suggest that nickel toxicity may cause cardiovascular problems like hypertension and atherosclerosis (Chuang et al., 2013; Lippmann, Ito, Hwang, Maciejczyk and Chen, 2006; "Toxicological Profile for Nickel," 2002; Wani, Khan, and Basir, 2018). Some laboratory studies also proved that nickel exposure in high concentrations leads to cardiovascular problems (Beshir, Hafez, Shaheen, Shahy and El-sherif, 2019; Lippmann et al., 2006; Wani et al., 2018). Lippmann et al (2006) has reported significant cardiovascular risks in human beings. Chashschin et al (1994) reported various cardiovascular risk factors in the nickel refinery workers. Other reports proved that particulate matter containing metals enter into systemic circulation through pulmonary system causes various pathological alteration to cardiovascular system (Beshir et al., 2019). Yamawaki and Iwai (2006) had reported that inflammation in the lungs may cause systemic inflammation which may further lead to cardiovascular diseases.

2.6 Hypoxia and Cardiovascular System:

Diverse pathophysiological conditions cause cellular hypoxia in aerobic organisms. These alteration of oxygen concentrations at cellular or tissue level triggers various adaptive mechanisms from physiological adaptations to epigenetic regulations. Cardiovascular and pulmonary system plays a key role in maintaining oxygen homeostasis in biological system. So, cardiac autonomic system plays a key role while adapting to hypoxic conditions(Perini and Veicsteinas, 2003). Especially at high altitudes partial pressure of oxygen in the

atmosphere (PO_2) will be very low compared to sea level. Some pathological conditions like cardiopulmonary diseases or some chemical toxicity leads to hypoxia. All the conditions which disturbs the oxygen homeostasis leads to the stimulation of peripheral chemo receptors and influences the heart rate, cardiac output and pulmonary ventilation to restore oxygen levels (West, 2006).

Bernardi et al (2001) reported that breathing patterns and peripheral chemo and baro reflexes causes heart rate (HR) modulations when exposed to hypoxia (Halliwill, Morgan, and Charkoudian, 2003). At normoxic conditions beat to beat modulation of HR and blood pressure (BP) will be maintained by arterial baroreceptors (Floras, 1993). But under hypoxic conditions these peripheral chemoreceptors also regulate autonomic functions and alters baroreflex influence of HR and sympathetic activity which leads to increased HR, BP and sympathetic stimulations (Halliwill et al., 2003). The autonomic nervous system controls the heart beats through its sympathetic and parasympathetic discharging on the sinus node. Increased sympathetic activation leads to increase in HR, while increased parasympathetic or vagal activation leads to decreased heart rate (Floras, 1993). So, the combination of these humoral and neural mechanisms modulates HR. A widely used non-invasive technique to measure cardiac autonomic modulation by variation in R-R intervals is HR variability (HRV) (Akselrod et al., 1981; Malik et al., 1996).

CHAPTER 3

AIM and OBJECTIVES

3.1 AIM OF THE STUDY

The primary aim of the study was to evaluate the cardiovascular autonomic functions and hypoxia signalling molecules after chronic hypoxia/nickel exposure and their impact on cardiovascular remodelling and the role of an antioxidant vitamin L-ascorbic acid.

3.2 OBJECTIVES OF THE STUDY

1. To assess the ameliorating effect of L-ascorbic acid on chronic sustained hypoxia (10% oxygen) induced alterations of cardiac autonomic functions with or without exposure to heavy metal nickel.
2. To evaluate the ameliorating effect of L-ascorbic acid on chronic sustained hypoxia (10% oxygen) induced histopathological alterations of cardiac tissues, arteriolar system and lungs with or without exposure to heavy metal nickel.
3. To evaluate the ameliorating effect of L-ascorbic acid on chronic sustained hypoxia (10% oxygen) induced alterations of Hematological, biochemical parameters with or without exposure to heavy metal nickel.
4. To explore the effect of chronic sustained hypoxia and heavy metal nickel ($NiSO_4$) on oxygen sensing molecules like VEGF and NOS3 (e-NOS) with or without supplementation of L-ascorbic acid.

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 cardiovascular system in male albino rats with or without exposure to heavy metal nickel.

3.3.2 Alternate Hypothesis:

L-ascorbic acid supplementation may improve hypoxia-induced alterations of cardiovascular pathophysiology in male albino rats with or without exposure to heavy metal nickel.

CHAPTER 4

MATERIALS and METHODS

4. MATERIALS AND METHODS

4.1 Study design: Animal study

4.2 Inclusion criteria: Young adult male Wistar strain albino rats, aged 60-70 days, weighing 180 - 250 g were included in the study.

4.3 Exclusion criteria: Newborn, old age and diseased rats.

4.4 Ethical approval: The study was approved by the Institutional animal ethical committee (Ref: BLDE/BPC/641/2016-2017 dated 22.10.2016).

4.5 Source of Data: Laboratory- bred healthy adult male Wistar strain albino rats, aged 60-70 days and weighing 180-250 g were obtained from animal house of Shri B.M.Patil Medical College, Hospital and Research Centre, BLDE (DU), Vijayapura, 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 hr light/ 12 hr dark cycle with food and water made available ad libitum. The rats were randomly assigned to one of the seven groups as follows.

4.6 Study Protocol:

Experimental groups

Table 4.5: Random allocation of experimental animals into groups (n=6 rats in each group)

S.No	Group Name	Intervention for 21 days
1.	Control	Placebo; oral gavage only
2.	L-ascorbic acid	L-ascorbic acid (50 mg / 100 g. b.wt) by oral gave daily
3.	CH	Chronic Hypoxia (CH)
4.	$NiSO_4$	$NiSO_4$ (2.0 mg / 100 g.b.wt., i.p)/every alternate day; 10 doses
5.	CH + $NiSO_4$	Chronic Hypoxia (CH) + $NiSO_4$
6.	L-ascorbic acid + CH	L-ascorbic acid + Chronic Hypoxia (CH)
7.	L-ascorbic acid + $NiSO_4$	L-ascorbic acid + $NiSO_4$

CH, chronic hypoxia; $NiSO_4$, Nickel sulfate; b.wt, body weight; i.p, intra peritoneal.

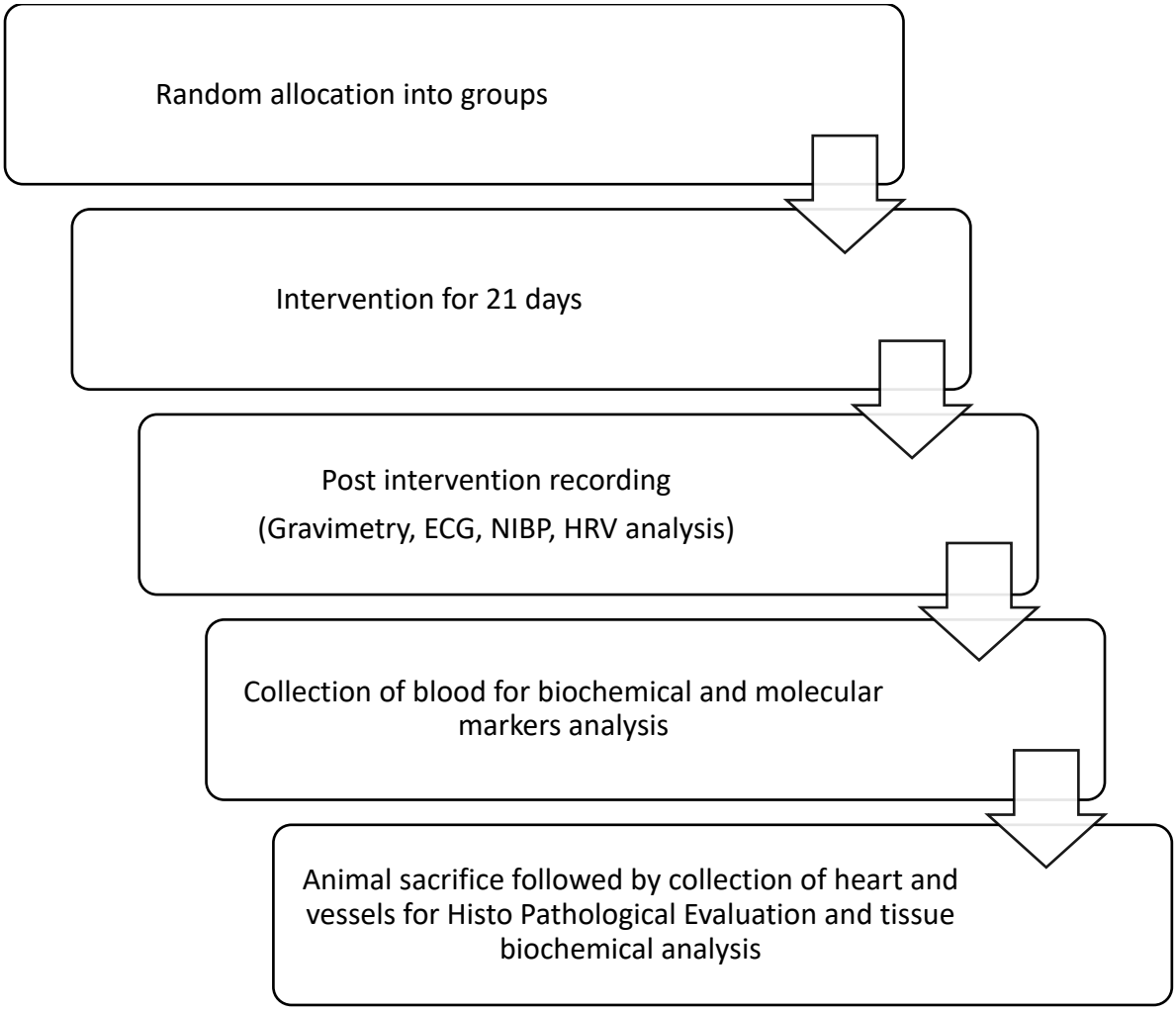


Figure 4.6: Summary of Experimental Protocol

4.7 Exposure of animals to chronic sustained hypoxia:

For chronic sustained hypoxia exposure caged rats (4 rats/cage) were placed in a 300-litre acrylic chamber and were exposed to inspired oxygen 10 % and nitrogen 90 % to induce normobaric hypoxia. The hypoxic environment was induced by an inflow of a mixture of room air and nitrogen. Carbon dioxide was absorbed by soda lime granules and excess humidity was removed by desiccator. The chamber was opened for 1 hour two times a week to clean the cages and to replenish food and water.

4.8. Method of data collection:

4.8.1. Gravimetry:

The body weight of all rats was recorded on day 1 of the experiment (initial body weight) and after 21 days of intervention (22nd day, final body weight) using electronic balance (Practum 1102-10IN, Sartorius Lab Instruments, Germany). Percentage change in body weight was calculated using the following formula. The rats of all groups were matched for weight at the onset of the experimental protocol.

$$Change\ in\ body\ weight\ (\%) = \frac{Final\ body\ weight - Initial\ body\ weight}{Initial\ body\ weight} \times 100$$

4.8.2. Electrophysiology

4.8.2.1 Recording of Pneumogram: The respiratory rate was assessed by using the respiratory pad transducer (BioPac Student lab system)after the intervention period (day 22nd).

4.8.2.2 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 recorded. Three values were obtained from each animal and the average of the three readings was considered. Mean arterial pressure (MAP) was calculated using the following formula.

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

4.8.2.3 Recording of ECG:

After the intervention period i.e on 22nd Day ECG was recorded using needle electrodes connected to windows based Biopac MP 45physiopacsystem though Biopac Student Lab 4.1 (BSL 4.1) software. All the recordings were performed in the morning hours following overnight fasting in anaesthetized animals (Ketamine, 60 mg/kg and Xylazine, 6 mg/kg). From the recorded ECG heart rate was also calculated.

4.8.2.4 Heart rate variability analysis:

From the recorded ECG (using Biopac Student Lab 4.1 software) RR intervals were exported to Kubios software for HRV analysis. HRV analysis was done by frequency domain method to assess the level of sympathetic activity, parasympathetic activity and sympathovagal balance.

Table 4.8.2.4: Components of the frequency domain method of HRV analysis

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

4.8.3 Collection of Blood:

All the animals were fasted overnight and blood was collected after 21 days intervention on day 22nd by cardiac puncture from anaesthetized rats in Ethylene Diamine Tetra Acetic Acid (EDTA) and plain tubeswith clot activator for further haematological and biochemical analysis respectively.

4.8.4 Hemogram:

Approximately 1 ml of blood was collected in commercially available EDTA tubes containing about 40 µl of potassium EDTA as an anticoagulant. The collected blood samples were analysed by an automated haematology cell counter (Sysmex K4500 Automated Haematology Analyzer) for determination of RBC count (million/mm^3), WBC count ($x10^3/\mu L$), Hemoglobin (g/dl).

4.8.5 Biochemistry

4.8.5.1 Lipid Profile

a. Serum total cholesterol (TC), Serum triglycerides (TG), and High-density lipoprotein (HDL) were analyzed using a commercial diagnostic kit (Erba Diagnostic Mannheim GmBH).
b. LDL levels were calculated using the Friedwald formula (Friedwald*et al*., 1972) as indicated below

- LDL (mg/dl) = Total cholesterol – HDL cholesterol - TG/5

4.8.5.1a Estimation of Serum Total Cholesterol:

Serum cholesterol was estimated by cholesterol oxidase – peroxidase enzymatic method (CHOD-POD) (Allain *et al*., 1974) using a commercial kit (Tranasia Bio-medicals Ltd, ERBA Diagnostics, Mannheim GmBH).

- **Principle**

Cholesterol esters were hydrolysed by cholesterol esterase to cholesterol and free fatty acids. Free cholesterol was oxidised by cholesterol oxidase to cholest-4-ene-3-one and hydrogen peroxide. This hydrogen peroxide combined with 4-aminoantipyrine to form chromophore (quinoneimine) dye which was measured at 505 nm.

- **Reagents**

1. Reagents
    - Good's buffer (50 mmol/L)
    - Phenol (5 mmol/L)
    - 4-aminoantipyrine (0.3 mmol/L)
    - Cholesterol esterase (≥200 U/L)
    - Cholesterol oxidase (≥50 U/L)
    - Peroxidase (≥3 k U/L)
2. Cholesterol Standard : 200 mg/dl

- **Procedure**

1. Three test tubes were taken and labelled as blank, standard and test. The procedure of the assay was as follows:

	Blank	Standard	Test
Sample	--	--	10 µl
Standard	--	10 µl	--
Reagent	1.0 ml	1.0 ml	1.0 ml
Distilled water	10 µl	--	--

2. Mixed well and incubated at 37^0C for 10 minutes.
3. The absorbance of the test and standard was read against blank at 505nm by UV visible spectrophotometer (Shimadzu, Model: UV 1800).

- **Calculation:**

Cholesterol (mg/dl) $= \frac{ODoftest}{ODofstandard} \times Concentrationofstandard\ (200\ mg/dl)$

4.8.5.1b Estimation of Serum Triglycerides:

The serum triglycerides were estimated by glycero phosphate oxidase peroxidase (GOD-POD) method (Werner et al., 1981) using a commercially available kit (ERBA Diagnostics, Mannheim GmBH).

- **Methodology**

This reagent is based on the method of Wako and the modification by McGowan et al. and Fossati *et al.* (Fossati and Prencipe, 1982; McGowan *et al*., 1983).

- **Principle**

Triglycerides were enzymatically hydrolyzed by lipase to glycerol and free fatty acids. The glycerol was subsequently measured by a coupled enzymatic reaction system. The glycerol released was phosphorylated to glycerol –3– phosphate by glycerol kinase. The glycerol –3– phosphate was oxidised by glycerol phosphate oxidase to produce dihydroxyacetone phosphate and hydrogen peroxide. Peroxidase catalyzed the reaction of hydrogen peroxide with 4-Amino antipyrine and 3, 5-Dichloro-2-hydroxybenzene sulfonate. The absorbance of chromogen formed was measured at 505 nm (500-540 nm).

$$\text{Triglycerides} + H_2O \xrightarrow{\text{LDL}} \text{Glycerol} + \text{Free Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow[Mg^{+2}]{\text{GK}} \text{Glycerol-3-Phosphate} + \text{ADP}$$

$$\text{Glycerol-3-Phosphate} + O_2 \xrightarrow{\text{GPO}} \text{DAP} + H_2O_2$$

$$H_2O_2 + \text{4AAP} + \text{3, 5 DHBS} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 2H_2O$$

LPL-Lipoprotein Lipase

GK - Glycerol Kinase

GPO - Glycerol Phosphate Oxidase

DAP - Dihydroxyacetone phosphate

ATP - Adenosine triphosphate

4-ATP - 4 Aminoantipyrine

DHBS - 3, 5-Dichloro-2-hydroxy benzene sulfonate

The intensity of chromagen (Quinoneimine) formed is proportional to the triglycerides concentration in the sample when measured at 505 nm (500-540 nm).

- **Reagent Composition:**

Active Ingredient	Concentration
ATP	2.5 mmol/L
Mg^{2+}	2.5 mmol/L
4 Aminoantipyrine	0.8 mmol/L
3,5-DHBS	1 mmol/L
Peroxidase	>2000 U/L
Glycerol Kinase	>550 U/L
GPO	>8000 U/L
Lipoprotein Lipase	>3500 U/L
Buffer (pH 7.0 $\pm$ 0.1 at 20^0 C)	53 mmol/L

Triglyceride Standard: 200 mg/dl (2.3 mmol/L)

Reagent reconstitution: The reagent bottle and AQUA - 4 (supplied in the kit) was allowed to attain room temperature (15-30^0 C). The amount of AQUA - 4 as indicated on the label was added to the contents of each vial. Swirled to dissolve and allowed to stand for 10 minutes at room temperature.

Sample: Serum or Plasma samples, free from hemolysis and collected after 12-16 hours fasting are suitable for triglyceride estimation.

- **Assay Procedure:**

Pipette into tube marked	Blank	Standard	Test
Working reagent	1000 µl	1000 µl	1000 µl
Distilled water	10 µl	-	-
Standard	-	10 µl	-
Test	-	-	10 µl

The above contents were mixed and incubated for 10 min at 37^0C. The absorbance of standard and each test was read at 505 nm on bichromatic analysers against reagent blank with UV visible spectrophotometer (Shimadzu, Model: UV 1800).

- **Calculations**

Triglycerides (mg/dl) = $\frac{Absorbance of Test}{Absorbance of Standard} \times Concentration of Standard\ (mg/dl)$

4.8.5.1c Estimation of HDL Direct

- **Principle**

The assay is based on a modified polyvinyl sulfonic acid (PVS) and polyethylene-glycol-methyl ether (PEGME) coupled classic precipitation method with the improvements in using optimized quantities of PVS/PEGME and selected detergents (Pisani *et al*., 1995). LDL, VLDL and chylomicron (CM) react with PVS and PEGME and the reaction results in inaccessibility of LDL, VLDL and chylomicron by cholesterol oxidase (CHOD) and cholesterol esterase (CHER). The enzyme selectively reacts with HDL to produce H_2O_2 which is detected through a Trinder reaction.

$$HDL \xrightarrow{CHOD,\ CHER} \text{Fatty acid} + H_2O_2$$

$$2H_2O_2 + \text{4-AA} + \text{TODB} \xrightarrow{\text{Peroxidase}} \text{Quinone} + 5H_2O$$

- **Reagent Composition**

R1

MES buffer
N, N-Bis (4-sulfobutyl)-3-methylaniline
Polyvinyl sulfonic acid
Polyethylene-glycol-methyl ester
$MgCl_2$

R2

MES buffer

Cholesterol esterase

Cholesterol oxidase

Peroxidase

4-aminopyrine

Detergent

R3 CAL

HDL/LDL calibrator

- **Reagent preparation**

Reagents R1 and R2 were liquid and ready to use.

Calibrator was reconstituted with 1ml of deionized water at 20-25^{0}C and mixed gently (avoid foaming) and allowed to stand for at least 30 minutes until complete reconstitution before use.

Sample: Serum or heparin plasma

- Assay Procedure

Pipette in Tube	**Reagent blank**	**Sample/Calibrator**
Reagent 1	750 µl	750 µl
D.D water	10 µl	10 µl
	Mix and incubate at 37^{0}C for 5 min.	
Add Reagent 2	250 µl	250 µl
	Mix and incubate at 37^{0}C for 5 min.	

The final absorbance was read at the specified wavelength against reagent blank by UV visible spectrophotometer (Shimadzu, Model: UV 1800).

CALCULATION

$$\text{HDL-C} = \frac{Abs.of Sample - Abs.of SampleBlank}{Abs.of Cal - Abs.of Cal.\ Blank} \times Concentration of Calibrator$$

4.8.5.1d Estimation of Serum LDL

LDL levels were estimated by calculation using the Friedwald formula (Friedwald*et al*., 1972)

- LDL mg/dl = Total cholesterol-HDL cholesterol-Triglyceride/5

4.8.6.2 OXIDATIVE STRESS ASSESSMENT:

4.8.6.2a Estimation of Serum and Tissue Malondialdehyde (MDA): By the method of Buege and Aust (Buege and Aust, 1978)

4.8.6.2b Estimation of Serum and Tissue ascorbic acid: By the method of Roe andKoether (1943)

4.8.6.2c Estimation of Serum α-tocopherol: By the modified method of Jargar et al (Jargar*et al*., 2012)

4.8.6.2a Estimation of Serum and Tissue Malondialdehyde(MDA):

By the method of Buege and Aust (Buege and Aust, 1978)

- **Introduction**

In the biological systems, malondialdehyde (MDA) is used as an indicator of oxidative stress. It is one of the end products of lipid peroxidation. It is formed by the degradation of polyunsaturated fatty acid (PUFA) by a free radical chain reaction. This compound is a reactive aldehyde and is one among the many reactive electrophile species causing oxidative stress.

- **Principle**

MDA formed by the breakdown of PUFA serves as a convenient index to determine the extent of lipid peroxidation. It reacts with thiobarbituric acid (TBA) giving a pink colour which is read at 535 nm.

- **Sample**: serum, saliva, tissue homogenate
- **Chemicals required**:
    1. Trichloroacetic acid (TCA)
    2. 2-Thiobarbituric acid (TBA)

3. Hydrochloric acid (HCl)
4. Malonaldehyde bis (dimethyl acetal)

- **Preparation:**

1. *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.

2. *MDA standard (stock-164 µg/ml)*

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

3. *MDA working standard (working- 1.64 µg/ml)*

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

- **The procedure for standardization:**

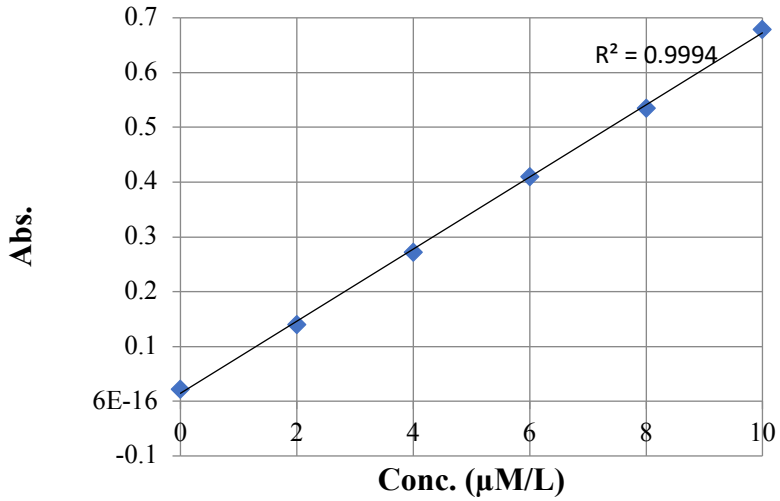
Standardisation (Range 2-10 µM/L)

The standardization was carried out referring to the table and all the reagents were added according to the values given in the table

S.No.	Vol. of MDA (ml)	Vol. of DW (ml)	Conc. of MDA (µM/L)	TBA-TCA-HCl (ml)	
B	0.0	1	0.0	1	Keep in boiling water bath for 15 min
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:

- Sample preparation:

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

Tissue Homogenate: 500 µl of 10% tissue homogenate was taken

(Tissue homogenate preparation: 10% tissue homogenate was prepared by adding 500 mg of tissue to 5ml of 0.1M phosphate buffer, homogenized for a few minutes, centrifuged and the supernatant was used in the estimation).

- **Procedure**

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

5. The concentration of MDA in the sample was determined by plotting the obtained absorbance against the standard graph. The optical density of pink colour formed was directly proportional to the concentration of MDA in the given sample.

- **Calculations**

The optical densities of the test samples were directly proportional to the concentration of MDA in the sample and calculated by plotting against the standard graph and multiplied by the respective dilution factors. The final concentration was expressed as μM/L.

4.8.6.2b Estimation of ascorbic acid

By Roe and Kuther method (Roe and Kuether, 1943)

ascorbic acid assay includes standardization of chemicals and estimation of vitamin C in the sample.

- **Standardization of ascorbic acid:**

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 500-520nm) spectrophotometrically.

- **Reagents**

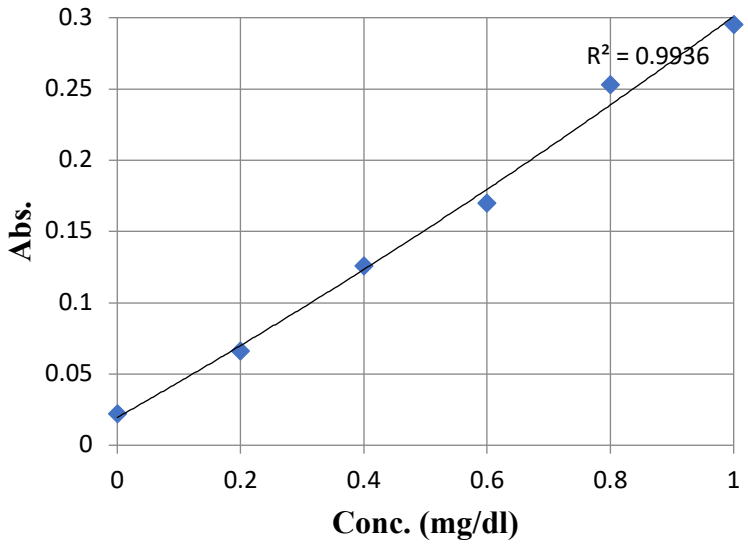
1. TCA (10%): 10gm of trichloroacetic acid (TCA) was dissolved in distilled water and the volume was made up to 100ml.
2. 2,4, dinitrophenylhydrazine (2,4, DNPH): 2gm of 2,4, dinitrophenylhydrazine was dissolved in 9N H_2SO_4 and volume was made up to 100ml.
3. Thiourea: 10gm of Thiourea was dissolved in 100ml of 50% ethanol

4. $CuSO_4$: 1.5 gm of $CuSO_4$ was dissolved in distilled water up to 100ml.
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:

S.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 56^0C 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	-	0.5	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.



Estimation of Vitamin C in the sample

- **Sample preparation:**

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

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

- **Procedure**

Following sample preparation, 500µl of supernatant was taken, 0.4ml of a colour reagent was added and placed 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**

Concentration of serum vitamin C

$$=\frac{ODofTest}{ODofStd} \times \frac{Conc.ofStd}{VolumeofTest} \times 100$$

=.....mg/dl

4.8.6.2c Estimation of Serum α-tocopherol

By Jameel Jargar and Das method (Jargar JG *et al*., 2012)

- **Reagents**

  Absolute ethanol (aldehyde free)

  N-propanol

  Xylene (extra pure)

  Ferric chloride (0.12%)

  Distilled water

  DL-α-tocopherol acetate

  2, 2'-Bipyridyl

- **Reagent preparation**

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

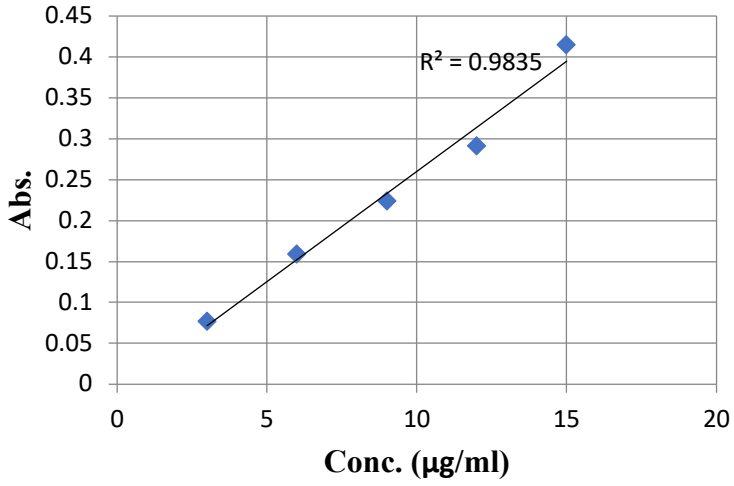
adjusted to 750µl using absolute ethanol. These solutions S1-S5 were equivalent to 4, 8, 12, 14, 16 and 20 µg/ml of α-tocopherol respectively using these solutions in the routine procedure as shown in the following table. The absorbance was read by using 200 µl of solutions prepared above including blank 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.

The Procedure of standardisation

S.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.

- **Standard Curve**



Analysis of serum α-tocopherol:

- Sample preparation: Allow 3ml of blood to clot in a centrifuge tube for 2 hours at room temperature and centrifuge at 3000rpm for 15 minutes to get the serum. 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 collected serum at 25^0C 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 Vit E (µg/ml)= $\frac{ODoftest-ODofBlank}{Slope} \times Dilutionfactor$

Slope = $\frac{y2-y1}{X2-X1}$, X and Y are concentration and absorbance of standard respectively

4.8.7 Molecular Markers

4.8.7a Estimation of Serum VEGF by ELISA method: Rat VEGF Elisa Kit (Chongqing Biospes Co., Ltd, Chongqing, China)

4.8.7b Estimation of Serum VEGF by western blotting method

4.8.7c Estimation of Serum NOS3: Rat NOS3 Elisa Kit (Chongqing Biospes Co., Ltd, Chongqing, China)

4.8.7d Estimation of Serum and Tissue Nitric Oxide (NO): By Greiss reaction

4.8.7a Estimation of Serum VEGF by ELISA Method

Serum VEGF was estimated by ELISA using Rat VEGF ELISA Kit (Catalog No.: BEK1228 Chongqing Biospes Co., Ltd, Chongqing, China) following the protocol given in the product manual.

- **Principle**

This kit is based on sandwich enzyme-linked immune-sorbent assay technique. 96 well plates pre-coated with Anti-VEGF polyclonal antibody were used. The biotin-conjugated anti-VEGF polyclonal 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 is proportional to the VEGF amount of sample captured in plate. O.D. absorbance was read at 450nm in a microplate reader, and then the concentration of VEGF can be calculated.

- **Range:** 15.6 pg/ml-1000 pg/ml
- **Sensitivity** <1 pg/ml

- **Kit components:**

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

- **Protocol**

Preparation of sample and reagents

1. ***Sample*:** The blood was collected in plain tubes and allowed to coagulate at room temperature (about 4 hours). Centrifuged at approximately 1500 x g for 15 min. The serum was aliquoted and stored at -20^{0}C.
2. ***Wash buffer:*** *T*he concentrated Wash buffer was diluted 25-fold (1:25) with distilled water.
3. ***Standard:*** Reconstitution of the lyophilized rat VEGF: the standard solution was prepared no more than 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 thoroughly.

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 thoroughly.

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 thoroughly. 0.3 ml form the 2nd was transferred to 3rd tube and mixed thoroughly and so on.

4. **Preparation of biotin-conjugated anti-rat VEGF antibody working solution:** Prepared no more than 2 hours before the experiment.

a. Calculation of 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 thoroughly 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 no more than 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**

Before adding to the wells the ABC working solution and TMB substrate are equilibrated at room temperature for at least 30 min.

1. Standard, test sample, and control wells are set on pre-coated plate respectively and their positions are recorded.

2. 0.1ml of 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml, 15.6 pg/ml standard solutions are aliquoted into standard wells.
3. 0.1ml of sample/standard diluents buffer was added into control (zero) well.
4. 0.1ml of properly diluted sample was added into test sample wells.
5. The plate is sealed with a cover and incubated at 37^0C for 90 min.
6. 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.
7. 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.
8. The plate was sealed with a cover and incubated at 37^0C for 60 min.
9. 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.
10. 0.1ml of ABC working solution was added into each well. The plate was covered and incubated at 37^0 C for 30 min.
11. 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.
12. 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.

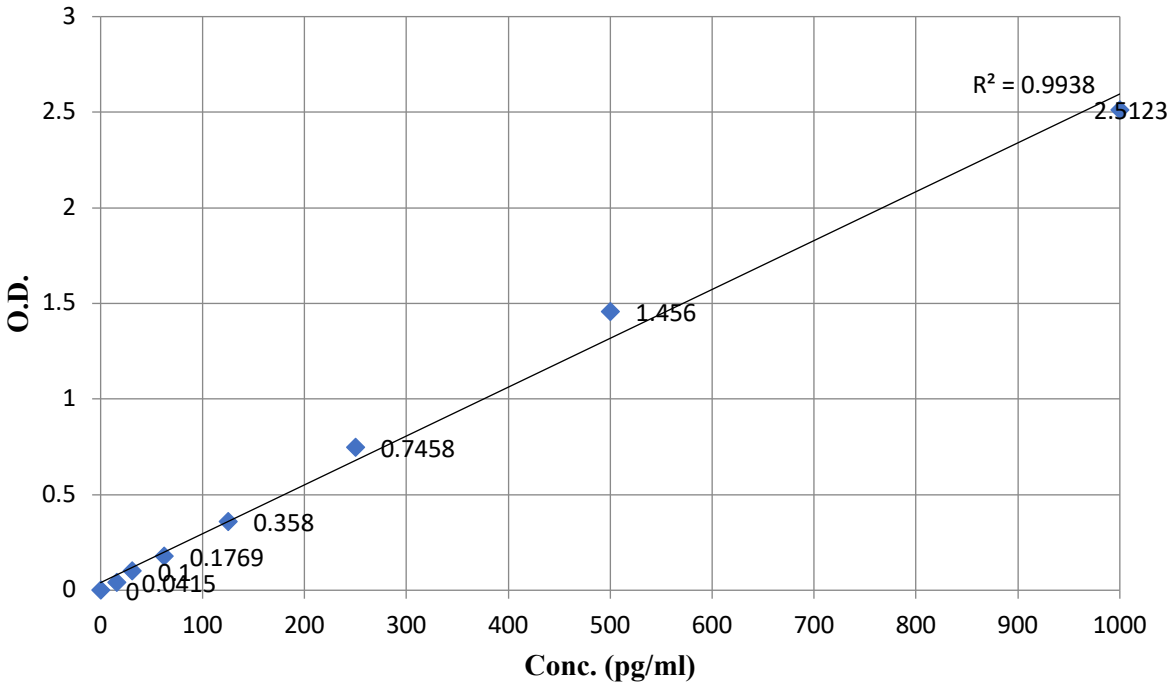
13. 0.1ml of stop solution was added into each well and mixed thoroughly. The colour changed to yellow immediately.
14. The O.D. absorbance was read at 450 nm in a micro plate reader (Model: Merilyzer EIAQUAN, Meril Diagnostics Pvt. Ltd.) within 30 min after adding the stop solution.

- **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 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
Y	OD_{450}	0	0.0415	0.1	0.1769	0.358	0.7458	1.456	2.5123



4.8.7b Estimation of serum VEGF by western blotting method:

Bio-Rad for Western BlottingProtocol

Key Solutions and Reagents

i. **Loading buffer: 2x Laemmli buffer**

4% SDS

10% 2-mercaptoethanol

20% glycerol

0.004% bromophenol blue

0.125 M Tris-HCl

Check the pH and adjust to pH 6.8 if necessary.

ii. **Running buffer: Tris/Glycine/SDS**

25 mM Tris

190 mM glycine

0.1% SDS

iii. **Transfer buffer**

25 mM Tris

190 mM glycine

20% methanol

Forproteinslargerthan80kD,SDSbeincludedatafinalconcentrationof0.1%.

iv. **PonceauSstainingbuffer** 0.2%(w/v)PonceauS 5% glacial aceticacid

v. **Tris-buffered saline with Tween 20 (TBST) buffer**

20 mM Tris, pH 7.5

150 mMNaCl

0.1% Tween 20

vi. **Blocking buffer**

3% bovine serum albumin (BSA) in TBST

Procedure

Sample Preparation

1. Take20µgofprotein from each serum sample and add an equal volume of 2x Laemmli sample buffer.
2. Boileachsampleinsamplebufferat95°Cfor5min.
3. Centrifugeat16,000xginamicrocentrifugefor1min.

Protein separation by gel electrophoresis

1. Load equal amounts of protein (20µg) into the wells of a mini (8.6x6.7cm) or midi (13.3x8.7cm) format SDS- PAGE gel, along with molecular weight markers.
2. Run the gel for 5 min at 50V.
3. Increase the voltage to 100–150V to finish the run in about 1hr.
4. Gel percentage selection depends on the size of the protein of interest. A 4–20% gradient gel separates proteins of all sizes verywell.

Transferring the protein from the gel to the membrane

1. Place the gel in1x transfer buffer for10–15min.
2. Assemble the transfer sandwich and make sure no air bubbles are trapped in the sandwich. The blot should be on the cathode and the gel on theanode.
3. Place the cassette in the transfer tank and place an ice block in the tank.
4. Transfer overnight in a cold room at a constant current of 10mA.

Note: Transfer can also be done at 100 V for 30 min–2 hr, but the method needs to be optimized for proteins of different sizes.

Antibody incubation

1. Briefly rinse the blot in water and stain it with Ponceau S solution to check the transfer quality.

2. Rinse off the Ponceau S stain with three washes with TBST.
3. Block in 3% BSA in TBST at room temperature for1 hr.
4. Incubate overnight in the primary antibody solution against the target protein at 4°C.

   Note: The antibody should be diluted in the blocking buffer according to the manufacturer's recommended ratio. Primary antibody may be applied to the blot for 1–3 hr at room temperature depending on antibody quality and performance.
5. Rinse the blot 3–5 times for 5 min withTBST.
6. Incubate in the HRP-conjugated secondary antibody solution for 1 hr at room temperature.
7. Rinse the blot 3–5 times for 5 min with TBST.

Imaging and data analysis

1. Apply the chemiluminescent substrate to the blot according to the manufacturer's recommendation.
2. Capture the chemiluminescent signals using a CCD camera-based imager.
3. Use image analysis software to read the band intensity of the target proteins.

4.8.7c Estimation of Serum NOS3 by ELISA Method

Serum NOS3 was estimated by ELISA using Rat NOS3 ELISA Kit (Catalog No.: BYEK2703 Chongqing Biospes Co., Ltd, Chongqing, China) following the protocol given in the product manual.

- **Principle of the Assay**

This kit works on technique of sandwich enzyme-linked immune-sorbent assay. 96 well plates precoated 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 450nm in a microplate reader, and the concentration of NOS3 is calculated.

- **Range**: 3 - 120 pg/ml
- **Kit components:**

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

- **Protocol**

Preparation of sample and reagents

1. **Sample:** The collected blood is allowed to coagulate at room temperature for 10-20 min, and then centrifuged at the speed of 2000-3000rpm for 20 min to collect the supernatant.The supernatant is aliquoted and stored at -20^{0}C. Multiple freeze-thaw cycles were avoided.
2. **Wash buffer:** Concentrated wash buffer was diluted 30 fold (1:30) with distilled water.
3. **Standard:** Dilution of the standard: 10 standard wells were set on the pre-coated plates and 100µl of the standard was added to the 1st and 2nd well. 50µl of the standard diluent

buffer was then added to the above two wells and thoroughly mixed. 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 thoroughly. 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 thoroughly. 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 thoroughly. 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 thoroughly. 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.

- **Assay Procedure**

1. Equilibrate kit components for 15-30 min at room temperature
2. Standard, test sample and control (zero) were set in the wells on pre-coated plate respectively. Their positions were recorded. 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 (zero) well.
3. For 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 thoroughly.
4. The plate was covered with a plate sealer and incubated at 37^0 C for 30 min.
5. The sealer was removed and the plate was washed manually. 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 min, the contents of the plate were then aspirated. The plate was

clapped on absorbent filter paper. The same procedure was repeated four more times for a total of five washes.

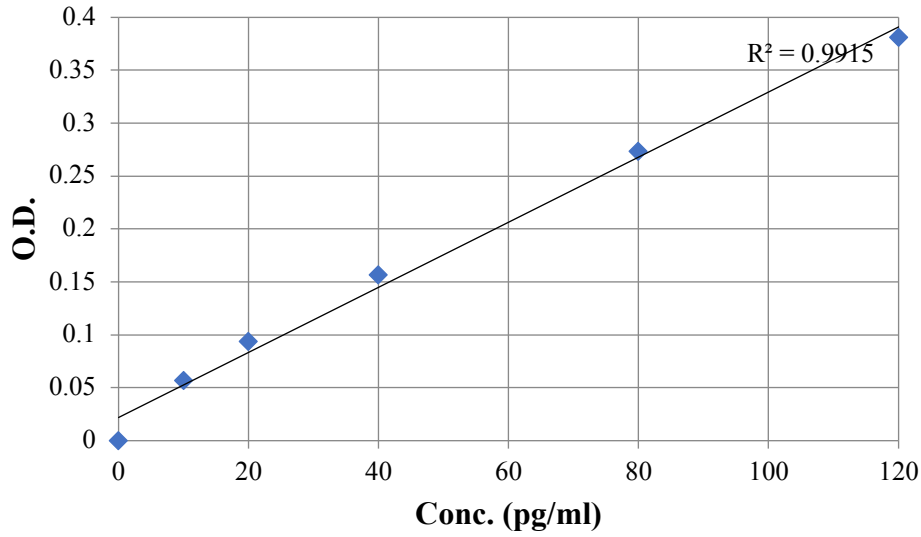
6. 50 µl of HRP conjugated anti-NOS3 antibody was added into each well (except control well).
7. The plate was covered with a plate sealer and incubated at 37^0 C for 30min.
8. The sealer was removed and the plate was washed.
9. 50 µl of TMB substrate A was added into each well, followed by 50 µl of 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.
10. 50 µl of Stop solution was added into each well and mixed thoroughly. The colour changed into yellow immediately.
11. The O.D. absorbance was read at 450nm in a microplate reader (Model: Merilyzer EIAQUAN, Meril Diagnostics Pvt. Ltd.) 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	0.0567	0.0935	0.1568	0.2737	0.3812



4.8.7c Estimation of Serum and Tissue Nitric Oxide concentration:

By Greiss Reaction (Moshage Han et al., 1995; Cortas and Wakid, 1990; Green et al., 1982)

- **Principle**

Nitrate, the stable product of nitric oxide was reduced to nitrite by cadmium reduction method after deproteination and coupling to N-naphthyl ethylene diamine. The coloured complex produced was measured at 540nm in a spectrophotometer.

- **Reagents**

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

Tissue homogenate preparation: 10% tissue homogenate was prepared by adding 500 mg of tissue to 5ml of normal saline, homogenized for a few minutes. To 1 ml of thistissue homogenate 2 ml of ethanol was added and mixed, centrifuged and the supernatant was used in the estimation.

a. *Deproteinization:*

In clean, dry centrifuge tube 0.5ml of serum or tissue homogenate was taken. To this 2.0 ml of 75 mmol/L $ZnSO_4$ solution was added and mixed. To this 2.5 ml of 55mmol/L of NaOH reagent was added and mixed well and centrifuged for 10 minutes. The supernatant was treated as a protein-free filtrate.

b. Activation of cadmium granules:

Cadmium granules that were previously stored in 0.1mol/L H_2SO_4 solution, were rinsed three times with distilled water at the time of assay. 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. The same procedure of activation of granules was followed each time.

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, 2.5-3 gm of freshly activated cadmium granules was added to each flask.
4. The contents of all the flasks were stirred to swirl the granules.
5. After 90 min the mixture in all three flasks was diluted to 4ml with distilled water.
6. 2 ml of this solution from respective flasks were pipette in 3 clean dry test tubes labelled B, S, T respectively.
7. 1ml of sulfanilamide followed by 1ml of N-napthyl ethylene diamine solution were added to each tube.
8. All the three tubes were shaken well and after 20 min OD of S, T was read against blank at 540 nm on a spectrophotometer.

- **Calculations**

$$\text{Nitric Oxide (µmol/L)} = \frac{ODofSample}{ODofStandard} \times conc.ofstandard \times DF$$

4.8.8 The sacrifice of animals and collection of tissues:

The animals were sacrificed after 21 days of intervention by an overdose of ketamine (150 mg/kg, ip) following the collection of blood. Rats were carefully dissected. Heart and lungs were separated. The individual organs were immediately weighed. The organosomatic index was calculated for each organ.

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

Part of the tissues was stored in 10% neutral buffered formalin for further histopathological examination (HPE) and the remaining was stored at -20^{o} C for tissue homogenate preparation. To study the vascular system, thoracic aorta (elastic artery) was also carefully dissected and stored in 10% neutral buffered formalin for further histopathological study.

4.8.9 Study of Cardiovascular and Pulmonary Remodelling:

4.8.9.1 Histopathological Examination:

The heart, lungs, and thoracic aorta were fixed in 10% neutral buffered formalin were embedded in paraffin blocks, sectioned with a microtome (0.7 µ thickness) and finally stained by Haematoxylin and Eosin and were subjected to histopathological examination.

4.8.9.2 Normalized Wall Index:

The heart particularly the ventricles and the vasculature (coronary artery and elastic artery) were evaluated for changes in the architecture to assess the effect of L-ascorbic acid supplementation on heavy metal nickel sulphate and chronic hypoxia induced cardiovascular remodelling. Also the histological images of the coronary artery were processed with Image J software (https://imagej.nih.gov/ij/). Lumen area (LA) and total vessel area (TVA) of the coronary artery were manually traced and values were obtained using the software. Wall area was calculated using the formula as depicted below.

Wall area (WA) = Total vessel area (TVA) - Lumen Area (LA)

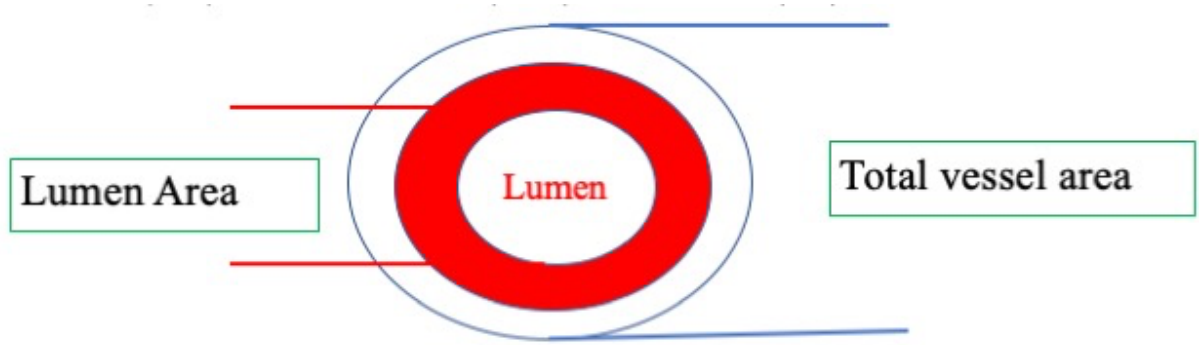


Figure 4.8.9.2 Schematic diagram depicting lumen area and total vessel area

Normalized wall index (NWI): Indicates the percentage of wall area of the total vessel area. NWI is considered a measure of arterial remodeling. Normalized wall index (NWI) for coronary artery was calculated as follows

NWI=Wall area/Total vessel area

4.9 STATISTICAL ANALYSIS

- SPSS 16.0 (SPSS Inc., Chicago, USA) was used for statistical analysis.
- All the parameters are presented as Mean $\pm$ SD. One-way analysis of variance (ANOVA) was used for statistical significance of data across multiple groups followed by post hoc test to determine the significant difference between groups.
- Pearson's correlation was done to establish a relationship between a pair of variables.
- p-value ≤ 0.05 was considered as statistically significant.

4.10 ETHICAL STATEMENT

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) (vide letter: BLDE/BPC/641/2016-17 dated 22.10.2016). All the experimental procedures were performed according to the guidelines laid by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

CHAPTER - 5

RESULTS

5.1 GRAVIMETRY

5.1 GRAVIMETRY

Table 5.1: Comparison of Body weight gain among experimental groups

Experimental Groups	Initial b.wt in gm.	Final b.wt in gm.	% b.wt Change
Control	194.94 ± 2.07^a	244.95 ± 5.09^a	25.65 ± 1.56^a
L-ascorbic acid	195.19 ± 4.55^a	247.38 ± 12.59^a	26.67 ± 3.60^a
CH	195.37 ± 4.34^a	214.30 ± 5.22^b	9.72 ± 3.01^b
$NiSO_4$	200.8 ± 2.23^a	221.24 ± 0.74^b	10.20 ± 1.41^b
CH+$NiSO_4$	198.6 ± 5.87^a	216.60 ± 5.30^b	9.16 ± 4.54^b
L-ascorbic acid + CH	196.6 ± 5.05^a	241.67 ± 5.61^a	22.94 ± 2.91^a
L-ascorbic acid + $NiSO_4$	$197.7 \pm 5.\ 12^a$	244.13 ± 9.89^a	23.61 ± 4.37^a
F value	1.433	24.73	25.27
p value	0.230	0.000*	0.000*

Values are expressed as mean ± SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests. Superscript a, and bindicates a significant difference between groups. *$p \leq 0.05$ was considered statistically significant.CH**,**chronic hypoxia; b.wt, body weight.

Table 5.1 shows experimental group comparative values of initial body weight (day 1), final body weight and % body weight changes. Weight of all group rats was almost similar so there was no statistical significant difference in Initial body weight. After 21 days of respective interventions final body weight was measured and values were compared by one way ANOVA followed by Tukey's Post Hoc test. Even though there was no statistical difference in the initial body weight of all group animals final body weight was significantly different between groups. Further % body weight gain was calculated and depicted in **Figure 5.1,** which shows a comparison of % body weight gain among experimental groups. Rats of all groups demonstrated an increase in body weight at the end of 21 days as indicated by % body weight gain. However, the % body weight gain was significantly lower in CH, $NiSO_4$ and CH + $NiSO_4$ groups compared to control and L-ascorbic acid supplemented groups. But

CH +L-ascorbic acid, $NiSO_4$ + L-ascorbic acid groups showed significant increase in body weight gain (%) when compared to CH and $NiSO_4$ groups respectively.

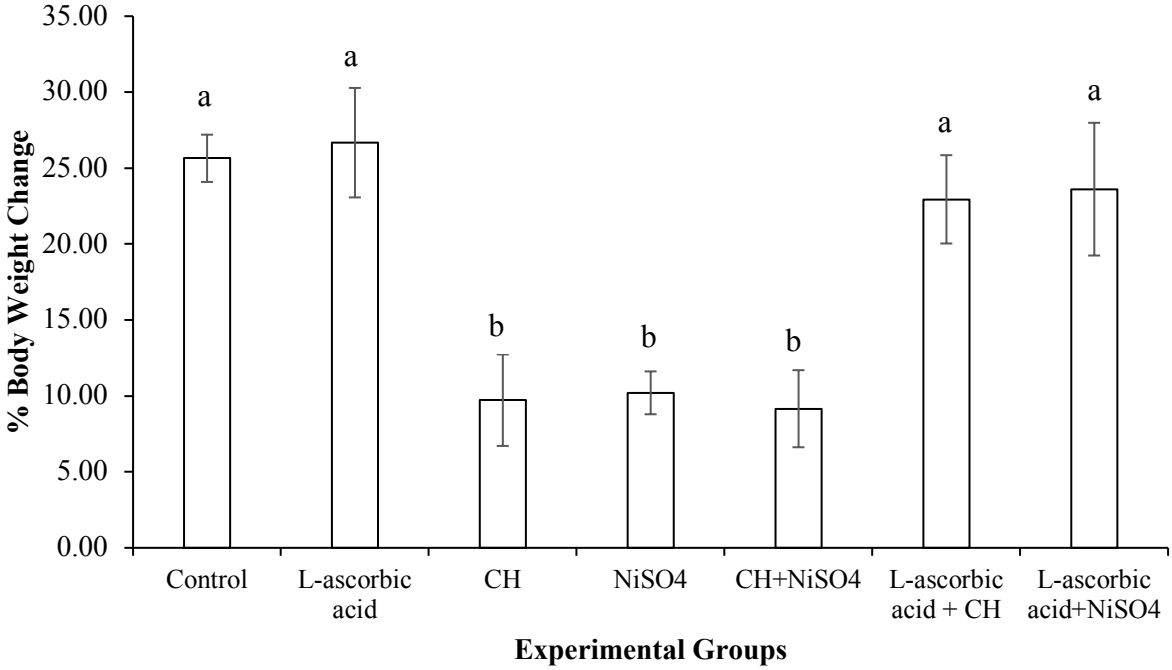


Figure 5.1 Comparison of % body weight change among groups

Superscript a, andb indicates a significant difference between groups. $p \leq 0.05$ was considered statistically significant. CH, chronic hypoxia.

5.2 ELECTROPHYSIOLOGY

5.2.1 Respiratory Rate

Figure 5.2.1 shows a comparison of respiratory rate (RR) among groups. The RR was increased in CH, $NiSO_4$ and CH + $NiSO_4$ groups when compared to control and L-ascorbic acid supplemented groups but this increase was not statistically significant.

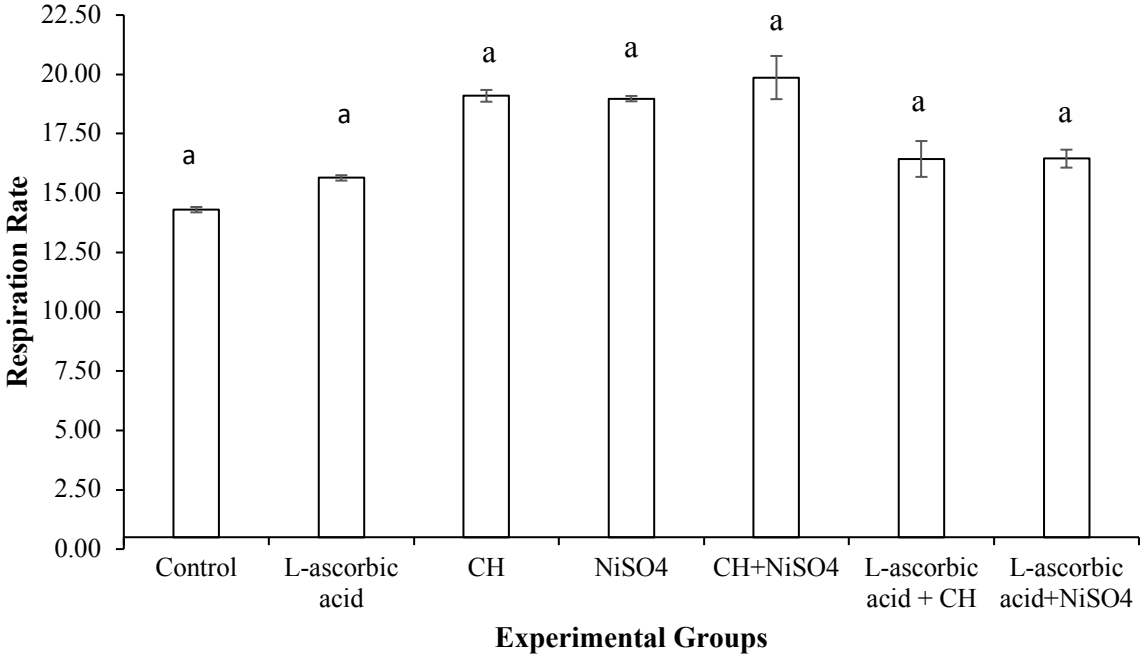


Figure 5.2.1 Comparison of Respiratory rate among groups

Superscript a, b and c indicates a significant difference between groups. $p \leq 0.05$ was considered statistically significant. CH, chronic hypoxia.

5.2.2 Heart Rate:

Figure 5.2.2 shows a comparison of small animal heart rate amongall experimental groups. CH, $NiSO_4$ and CH + $NiSO_4$ groups shown significant increase in heart rate when compared to control and L-ascorbic acid groups. But we had seen a significant decrease in heart rate of L-ascorbic acid + $NiSO_4$ when compared to $NiSO_4$ treated rats. L-ascorbic acid + CH did not show any significant change when compared to CH alone exposed rats.

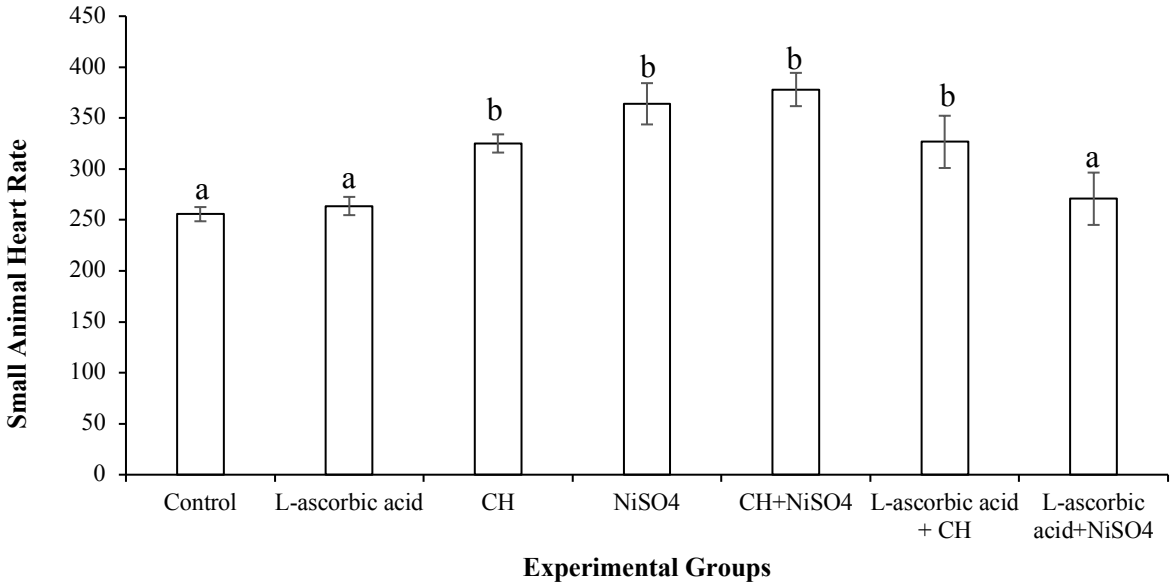


Figure 5.2.2 Comparison of Small Animal Heart Rate among Experimental Groups

Values were expressed as Mean ± SD. Oneway ANOVA followed by Post Hoc Tukey's multiple comparison test. Values with different superscripts (a, b) are significantly different from each other ($p<0.05$), n=6 rats in each group.CH, chronic hypoxia.

5.2.3 Mean Arterial Pressure (MAP):

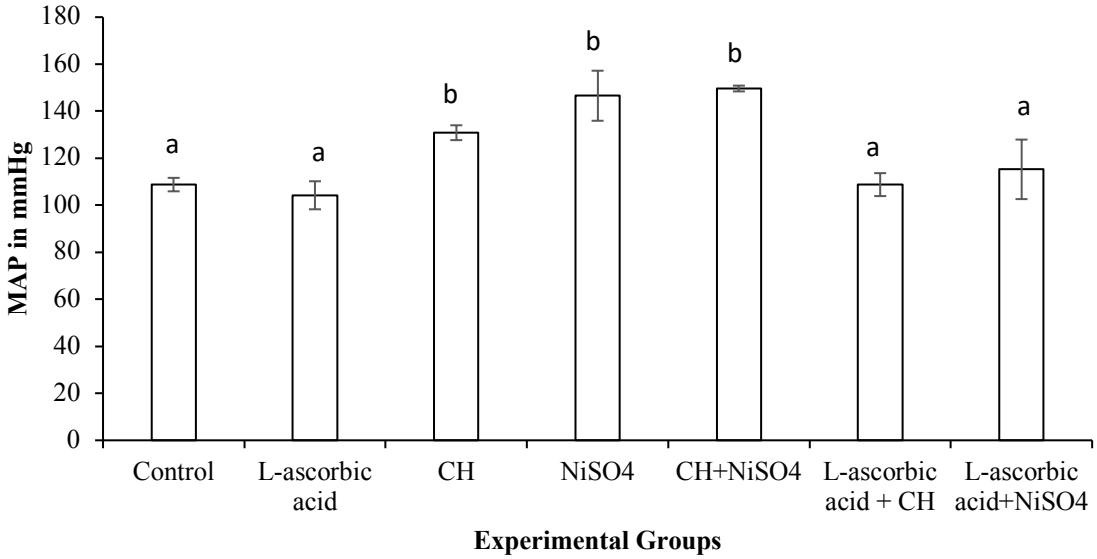


Figure 5.2.3 Comparison of MAP among Experimental Groups

Values were expressed as Mean ± SD. Oneway ANOVA followed by Post Hoc Tukey's multiple comparison test. Values with different superscripts (a, b) are significantly different from each other ($p<0.05$), n=6 rats in each group.CH,chronic hypoxia; MAP, mean arterial pressure.

Figure 5.2.3 shows statistical comparison of mean arterial pressure (MAP) among all experimental groups. We observed significant differences in the MAP values between groups. CH, $NiSO_4$ and CH + $NiSO_4$ groups demonstrated an increase in MAP. But L-ascorbic acid + CHand L-ascorbic acid + $NiSO_4$ groups demonstrated a decrease in MAP.

5.2.4 Heart Rate Variability: Frequency-Domain Analysis

Table 5.2.4: Comparison of Heart Rtae Variability (HRV) parameters among groups

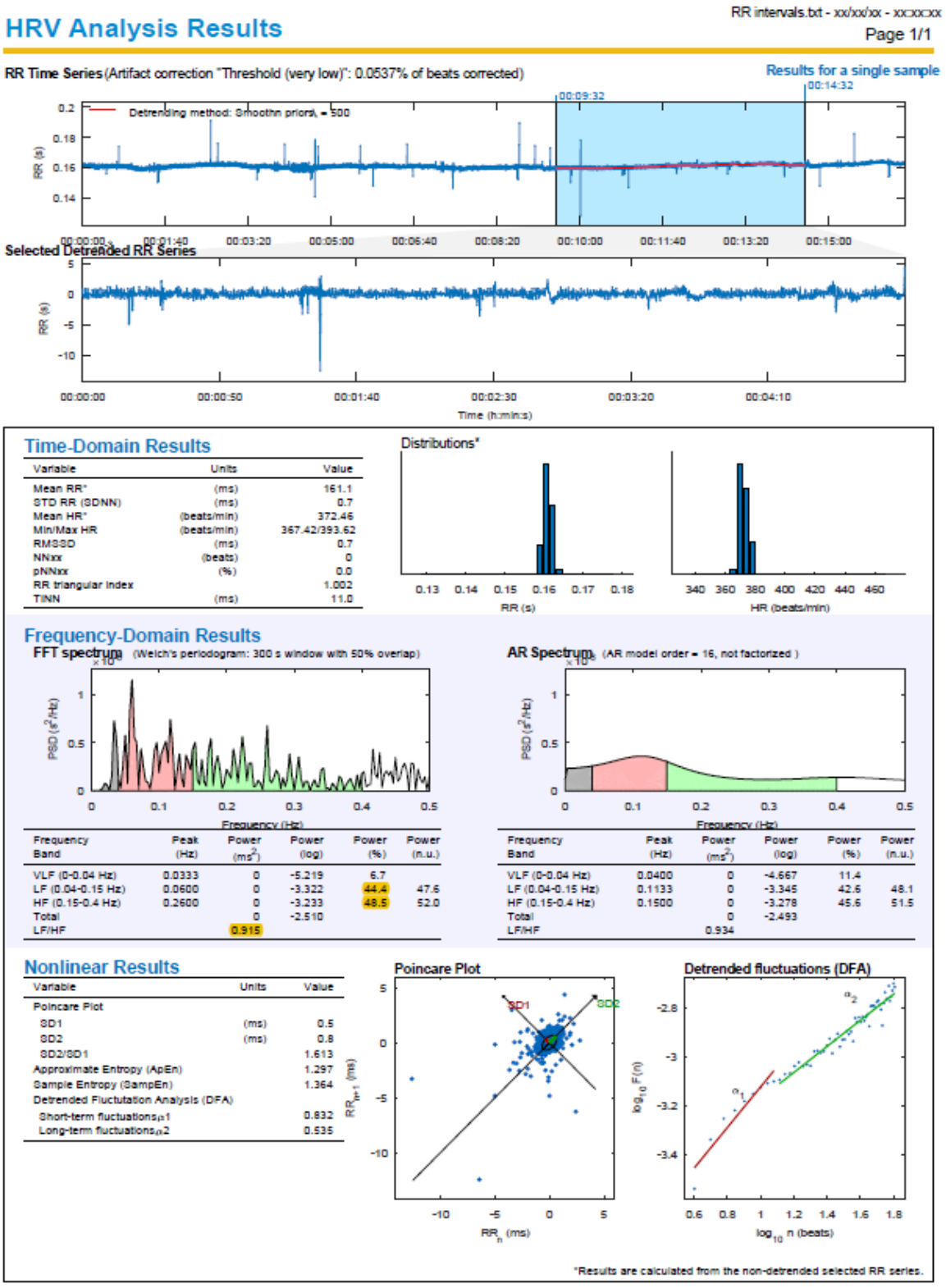
Experimental Groups	LF (nu)	HF (nu)	LF/HF Ratio
Control	49.87 ± 1.60^a	49.37 ± 1.53^a	1.01 ± 0.06^a
L-ascorbic acid	49.09 ± 1.10^a	49.05 ± 1.23^a	1.00 ± 0.02^a
CH	60.88 ± 1.42^b	41.22 ± 1.47^b	1.48 ± 0.03^b
$NiSO_4$	59.56 ± 1.77^b	41.47 ± 0.55^b	1.43 ± 0.02^b
CH + $NiSO_4$	57.80 ± 1.32^b	39.63 ± 5.07^b	1.38 ± 0.08^b
L-ascorbic acid + CH	50.37 ± 3.90^a	49.63 ± 3.90^a	1.02 ± 0.15^a
L-ascorbic acid + $NiSO_4$	53.33 ± 0.51^a	48.63 ± 1.22^a	1.16 ± 0.14^a
F value	48.93	22.17	41.83
p value	0.00^*	0.00^*	0.00^*

Values are expressed as Mean $\pm$ SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests. Superscript a, b and c indicates a significant difference between groups. $^*p \leq 0.05$ was considered statistically significant.CH, chronic hypoxia; LF, low frequency; HF,high frequency.

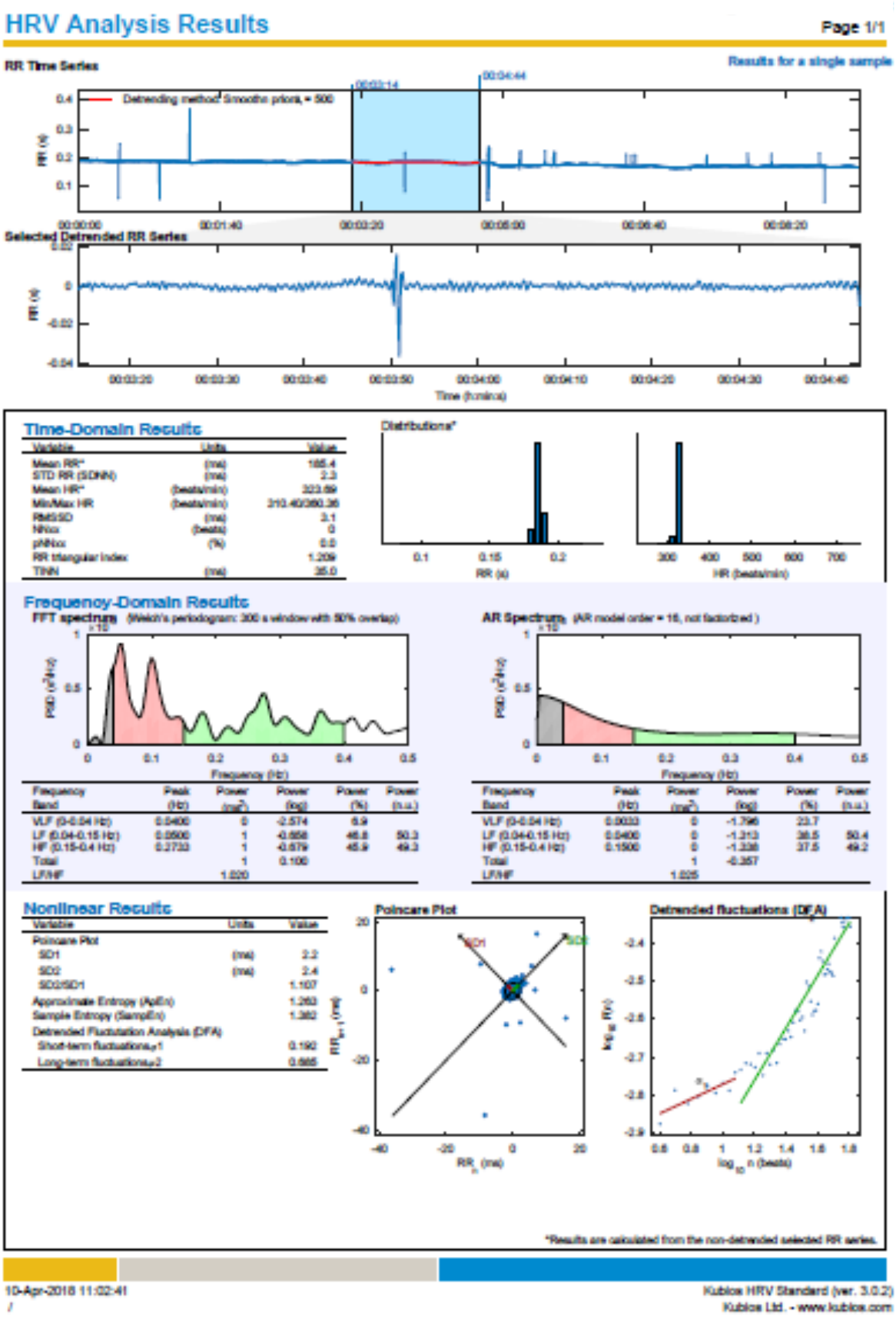
Table 5.2.4 shows frequency domain results of heart rate variability (HRV) among all experimental groups. Rats exposed to CH, $NiSO_4$and CH + $NiSO_4$ demonstrated increased sympathetic activity (LF), decreased parasympathetic activity (HF) and shift in the sympathovagal balance (LF/HF ratio) towards increased sympathetic activity when compared to control and L-ascorbic acid supplemented groups. CH + L-ascorbic acid and $NiSO_4$ + L-ascorbic acid group rats demonstrated a decrease in sympathetic activity and increase in the parasympathetic activity and decrease in sympathovagal balance when compared to CH and $NiSO_4$ groups respectively.

Analysis of Frequency-Domain Results of Rat 1 in each Group

CONTROL RAT 1



L-ASCORBIC ACID RAT 1

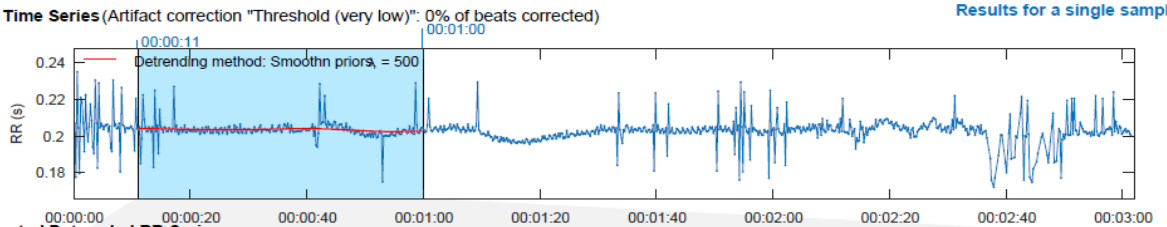


CHRONIC HYPOXIA (CH) RAT 1

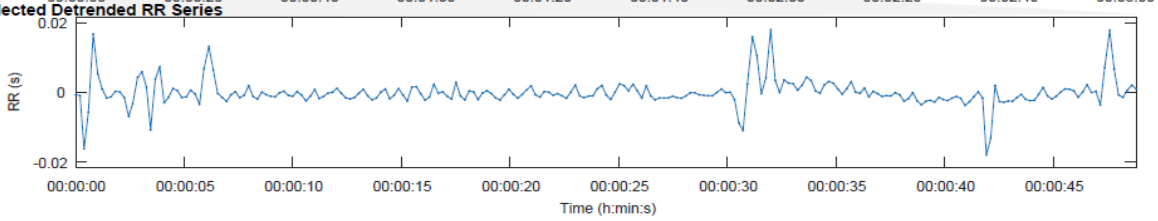
HRV Analysis Results

Results for a single sample

RR Time Series (Artifact correction "Threshold (very low)": 0% of beats corrected)



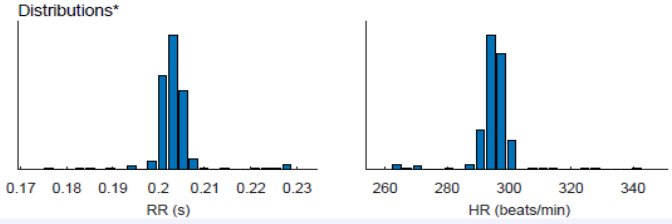
Selected Detrended RR Series



Time-Domain Results

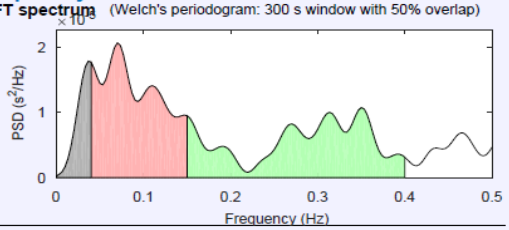
Variable	Units	Value
Mean RR*	(ms)	203.4
STD RR (SDNN)	(ms)	3.8
Mean HR*	(beats/min)	295.16
Min/Max HR	(beats/min)	280.34/308.42
RMSSD	(ms)	4.2
NNxx	(beats)	0
pNNxx	(%)	0.0
RR triangular index		1.131
TINN	(ms)	24.0

Distributions*



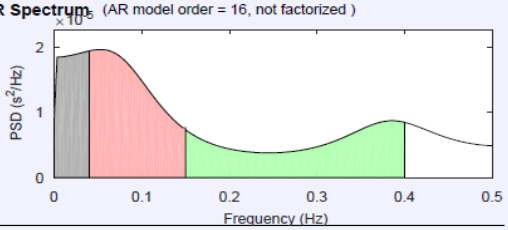
Frequency-Domain Results

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)	0.0367	0	-1.105	10.1	
LF (0.04-0.15 Hz)	0.0700	2	0.422	46.4	51.6
HF (0.15-0.4 Hz)	0.3500	1	0.353	43.3	48.2
Total		3	1.189		
LF/HF		1.072			

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

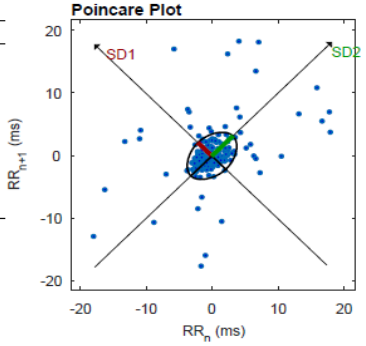


Frequency Band	Peak (Hz)	Power (ms^2)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0400	1	-0.304	19.6	
LF (0.04-0.15 Hz)	0.0533	2	0.491	43.3	53.8
HF (0.15-0.4 Hz)	0.3867	1	0.326	36.7	45.6
Total		4	1.328		
LF/HF		1.180			

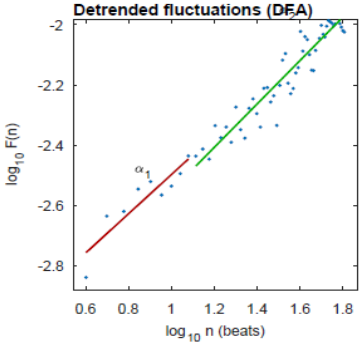
Nonlinear Results

Variable	Units	Value
Poincare Plot		
SD1	(ms)	3.0
SD2	(ms)	4.5
SD2/SD1		1.506
Approximate Entropy (ApEn)		0.977
Sample Entropy (SampEn)		1.160
Detrended Fluctutation Analysis (DFA)		
Short-term fluctuations $\alpha 1$		0.651
Long-term fluctuations $\alpha 2$		0.722

Poincare Plot



Detrended fluctuations (DFA)



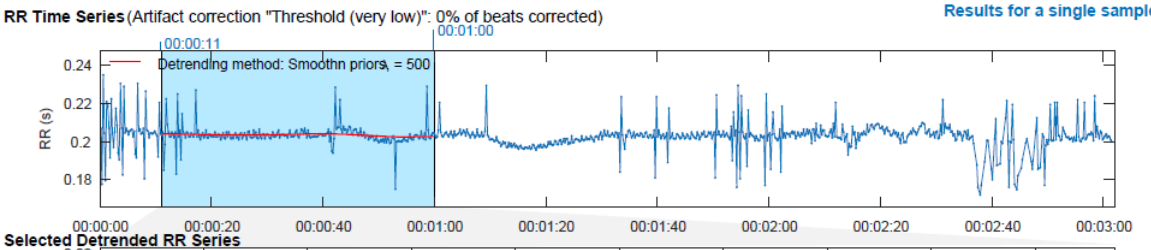
*Results are calculated from the non-detrended selected RR series.

$NiSO_4$ RAT 1

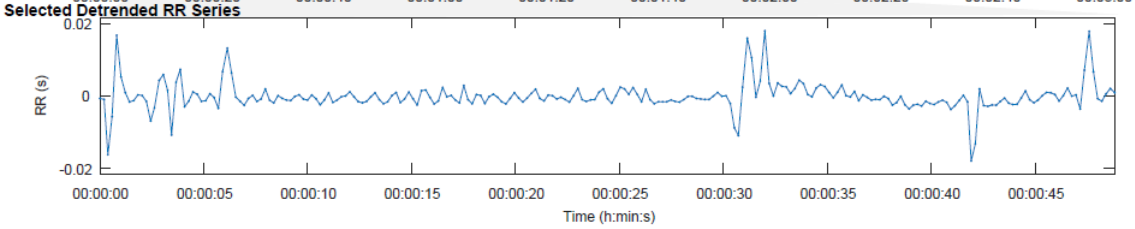
HRV Analysis Results

Results for a single sample

RR Time Series (Artifact correction "Threshold (very low)": 0% of beats corrected)



Selected Detrended RR Series



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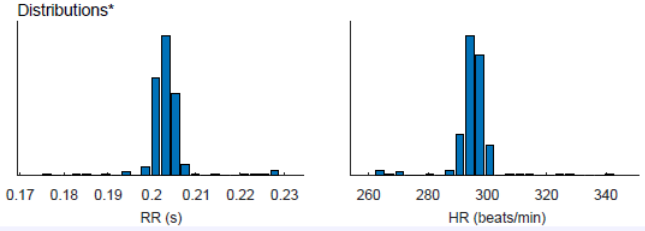
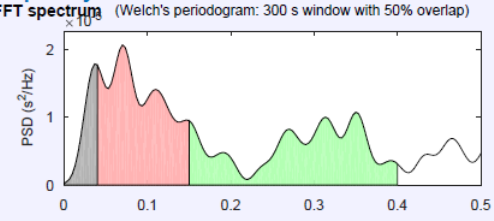
RR triangular index		1.131
TINN	(ms)	24.0

Distributions*



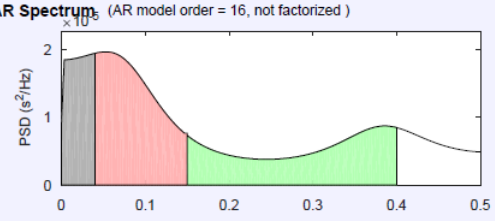
Frequency-Domain Results

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)	0.0367	0	-1.105	10.1	
LF (0.04-0.15 Hz)	0.0700	2	0.422	46.4	51.6
HF (0.15-0.4 Hz)	0.3500	1	0.353	43.3	48.2
Total		3	1.189		
LF/HF		1.072			

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

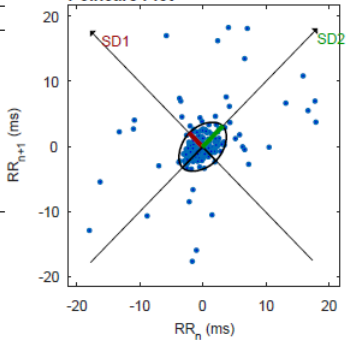


Frequency Band	Peak (Hz)	Power (ms^2)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0400	1	-0.304	19.6	
LF (0.04-0.15 Hz)	0.0533	2	0.491	43.3	53.8
HF (0.15-0.4 Hz)	0.3867	1	0.326	36.7	45.6
Total		4	1.328		
LF/HF		1.180			

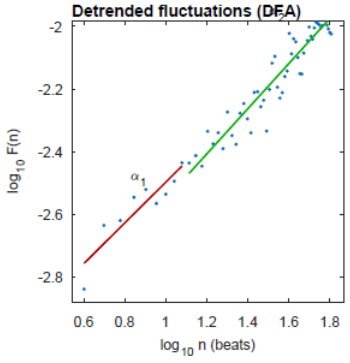
Nonlinear Results

Variable	Units	Value
Poincare Plot		
SD1	(ms)	3.0
SD2	(ms)	4.5
SD2/SD1		1.506
Approximate Entropy (ApEn)		0.977
Sample Entropy (SampEn)		1.160
Detrended Fluctutation Analysis (DFA)		
Short-term fluctuations $\alpha 1$		0.651
Long-term fluctuations $\alpha 2$		0.722

Poincare Plot



Detrended fluctuations (DFA)



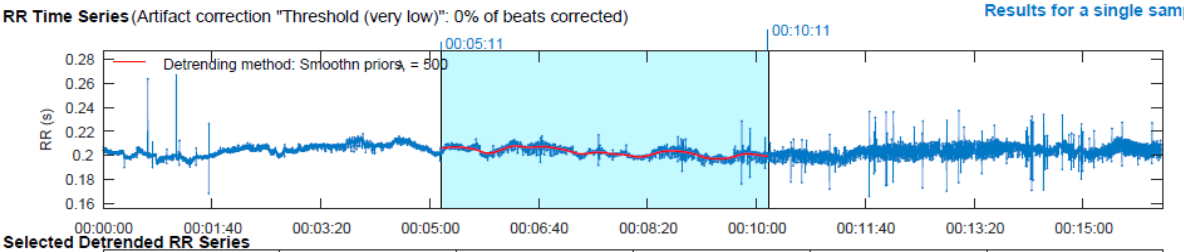
*Results are calculated from the non-detrended selected RR series.

CHRONIC HYPOXIA + $NiSO_4$ RAT 1

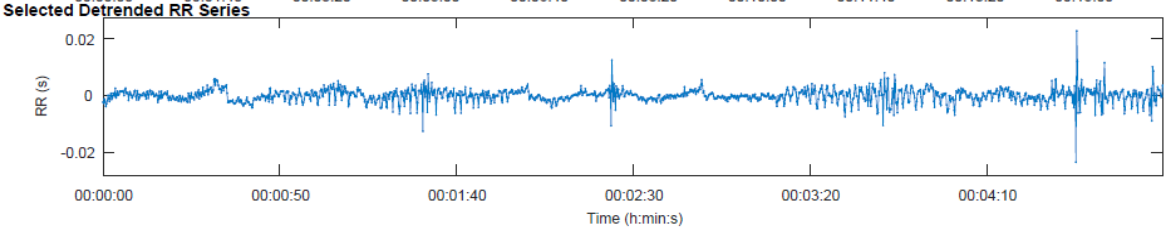
HRV Analysis Results

Results for a single sample

RR Time Series (Artifact correction "Threshold (very low)": 0% of beats corrected)



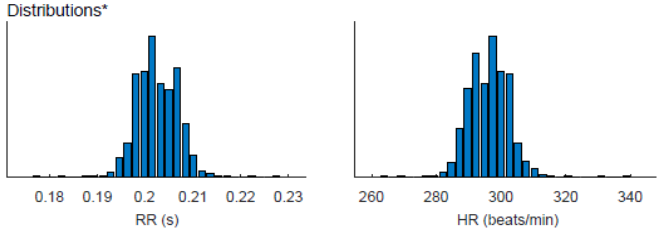
Selected Detrended RR Series



Time-Domain Results

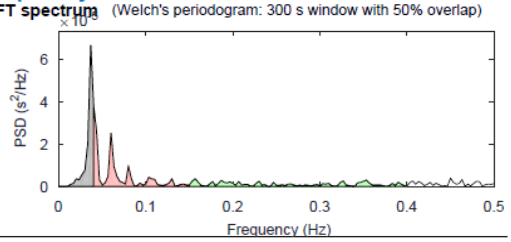
Variable	Units	Value
Mean RR*	(ms)	202.7
STD RR (SDNN)	(ms)	2.4
Mean HR*	(beats/min)	296.18
Min/Max HR	(beats/min)	284.63/307.87
RMSSD	(ms)	2.9
NNxx	(beats)	0
pNNxx	(%)	0.0
RR triangular index		1.695
TINN	(ms)	32.0

Distributions*



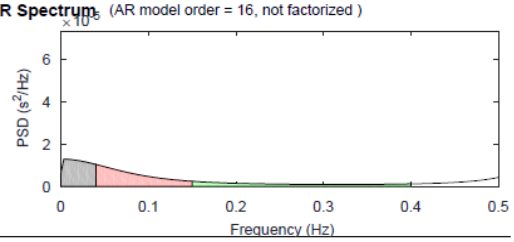
Frequency-Domain Results

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)	0.0367	0	-0.860	37.3	
LF (0.04-0.15 Hz)	0.0400	0	-0.771	40.8	65.1
HF (0.15-0.4 Hz)	0.1567	0	-1.396	21.8	34.9
Total		1	0.126		
LF/HF		1.867			

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

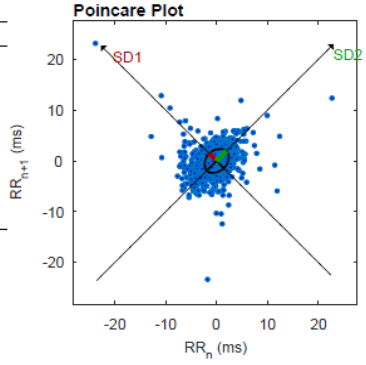


Frequency Band	Peak (Hz)	Power (ms^2)	Power (log)	Power (%)	Power (n.u.)
VLF (0-0.04 Hz)	0.0033	0	-0.763	34.2	
LF (0.04-0.15 Hz)	0.0400	1	-0.510	44.1	67.1
HF (0.15-0.4 Hz)	0.1500	0	-1.230	21.5	32.7
Total		1	0.308		
LF/HF		2.054			

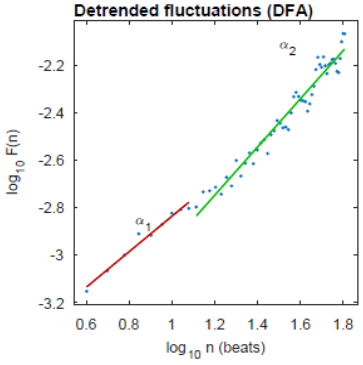
Nonlinear Results

Variable	Units	Value
Poincare Plot		
SD1	(ms)	2.0
SD2	(ms)	2.7
SD2/SD1		1.346
Approximate Entropy (ApEn)		1.403
Sample Entropy (SampEn)		1.498
Detrended Fluctutation Analysis (DFA)		
Short-term fluctuations $\alpha 1$		0.747
Long-term fluctuations $\alpha 2$		1.014

Poincare Plot

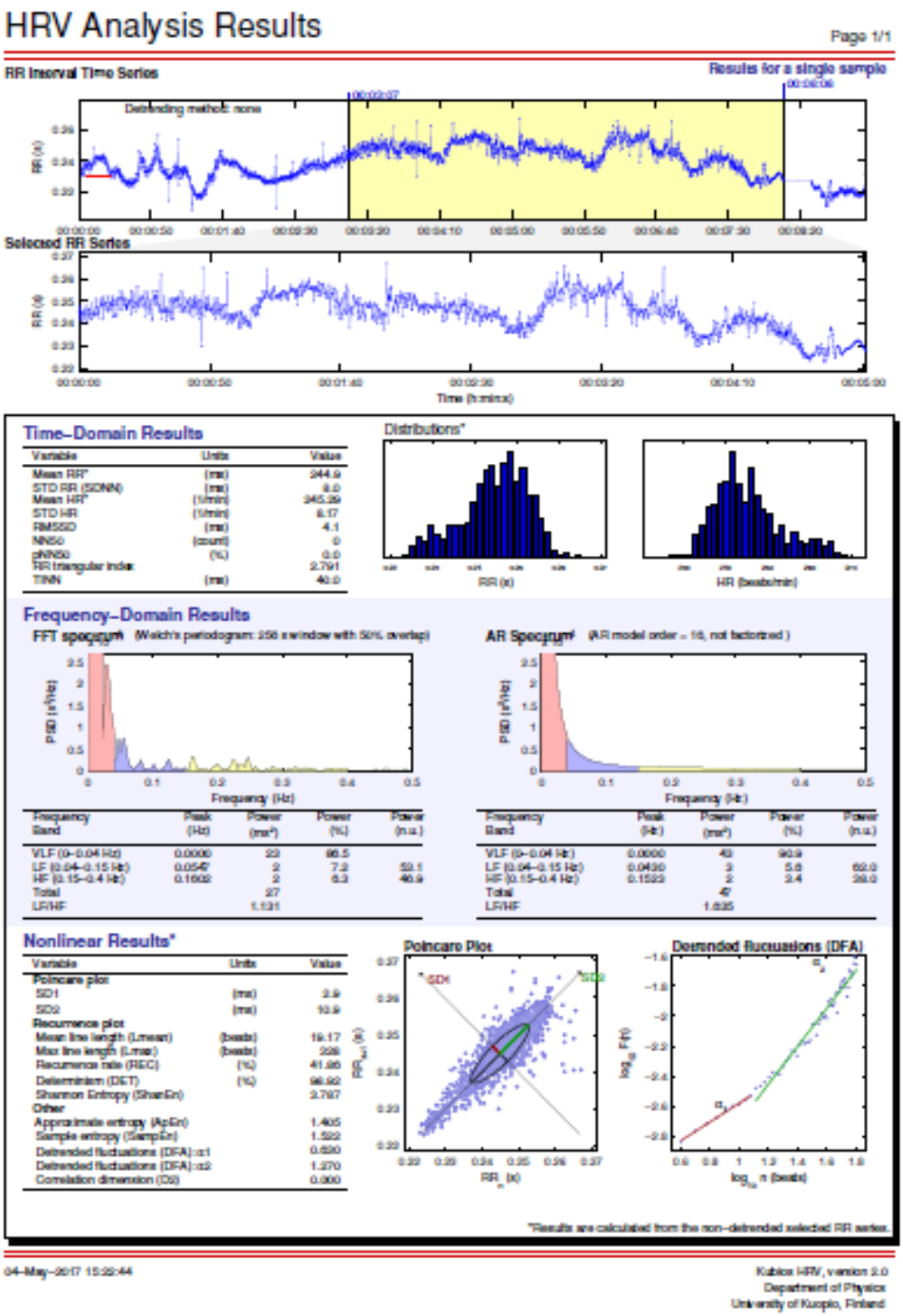


Detrended fluctuations (DFA)

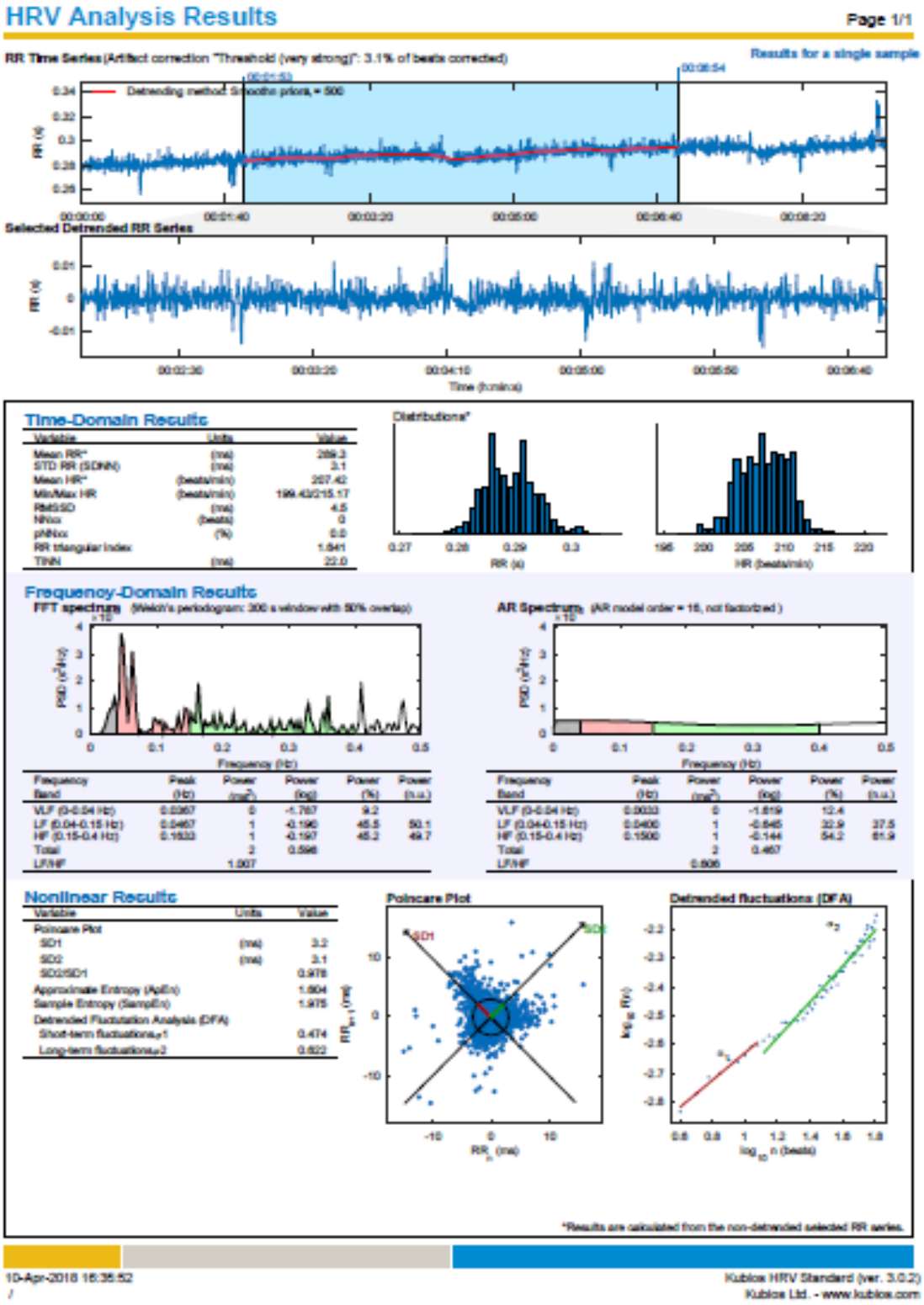


*Results are calculated from the non-detrended selected RR series.

L-ASCORBIC ACID + CHRONIC HYPOXIA RAT 1



L-ASCORBIC ACID + $NiSO_4$ RAT 1



5.3 HEMOGRAM

5.3 HEMOGRAM

Table 5.3: Comparison of haematological parameters among groups

Experimental Groups	RBC (million/cumm)	WBC ($x10^3/\mu L$)	Hb (g/dl)
Control	6.72± 0.71[a]	9.33± 0.81[a]	14.73± 0.55[a]
L-ascorbic acid	7.18± 0.45[a]	8.42± 0.47[a]	14.77± 0.69[a]
CH	8.94± 0.79[b]	9.26± 1.53[a]	15.33± 0.32[a]
$NiSO_4$	4.47± 0.88[c]	10.93± 3.52[a]	13.63± 0.65[a]
CH+$NiSO_4$	4.283± 0.94[c]	11.90± 0.66[a]	13.67± 1.10[a]
L-ascorbic acid + CH	6.957± 0.53[a]	9.31± 0.52[a]	13.80± 0.62[a]
L-ascorbic acid+$NiSO_4$	5.333± 0.46[c]	6.79± 0.30[a]	13.50± 0.97[a]
F value	41.99	8.74	7.20
p value	0.00*	0.00*	0.00*

Values are expressed as Mean±SD. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests. Superscript a, b, and c indicates a significant difference between groups. *$p \leq 0.05$was considered statistically significant. CH, chronic hypoxia; RBC,red blood cells; WBC,wight blood cells; Hb,hemoglobin.

Table 5.3 shows a comparison of haematological parameters among groups. RBC count, WBC count and haemoglobin (Hb).

We found significant increase of RBC count in CH group and significant decrease in $NiSO_4$ and CH + $NiSO_4$ 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 + $NiSO_4$ did not show any significant change when compared to $NiSO_4$ alone.We have not found any significant differences in WBC count, haemoglobin concentrations among experimental groups.

5.4 BIOCHEMISTRY

5.4.1 LIPID PROFILE

Table 5.4.1 represents a comparison of lipid profile among groups. After 21 days of intervention serum total cholesterol (TC), triglycerides (TGL), high density lipoproteins (HDL) and low density lipoproteins (LDL) were estimated and were found comparable between groups.

The results demonstrated significantly increased concentrations of TC, triglycerides and LDL in CH, $NiSO_4$ and CH + $NiSO_4$ groups when compared to control and L-ascorbic acid groups. But in rats belongs to L-ascorbic acid + CH and L-ascorbic acid + $NiSO_4$ groups demonstrated significant decrease in TC, TGL and LDL concentrations when compared to CH and $NiSO_4$ groups respectively. We have not found any significant variations in HDL concentrations among all experimental groups.

Table 5.4.1: Comparison of lipid profile among groups of experimental animals (n=6 in each group)

Experimental Groups	TC (mg/dl)	TGL (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Control	48.267 ± 1.83^a	51.60 ± 6.64^a	25.09 ± 3.78^a	12.86 ± 2.32^a
L-ascorbic acid	45.133 ± 5.15^a	48.47 ± 10.91^a	27.03 ± 3.01^a	8.41 ± 1.09^a
CH	65.423 ± 2.68^b	88.27 ± 4.48^b	26.26 ± 1.32^a	25.51 ± 2.70^b
$NiSO_4$	67.103 ± 3.88^b	84.29 ± 6.47^b	24.10 ± 3.80^a	26.15 ± 2.92^b
CH+$NiSO_4$	71.090 ± 6.70^b	94.95 ± 11.64^b	25.61 ± 4.56^a	26.49 ± 5.99^b
L-ascorbic acid + CH	52.843 ± 3.39^a	63.59 ± 4.08^a	24.30 ± 3.39^a	15.83 ± 0.35^a
L-ascorbic acid + $NiSO_4$	53.000 ± 3.16^a	56.92 ± 6.47^a	25.42 ± 4.08^a	14.19 ± 4.22^a
F value	50.66	45.38	0.638	33.12
p value	0.00^*	0.00^*	0.699	0.00^*

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 are significantly different from each other. $^*p \leq 0.05$ was considered statistically significant.CH, chronic hypoxia; TC, total cholesterol; TGL, triglycerides; HDL, high density lipoproteins; LDL, low density lipoproteins.

5.4.2 SERUM OXIDATIVE STRESS:

Table 5.4.2 showed significantly increased serum levels of lipid peroxidation product malondialdehyde (MDA) and decreased concentrations of antioxidant vitamins like ascorbic acid and α - tocopherol in CH, $NiSO_4$ and CH + $NiSO_4$ groups when compared to control and L-ascorbic acid groups. But L-ascorbic acid supplemented groups (L-ascorbic acid + CH and L-ascorbic acid + $NiSO_4$) significantly decreased MDA concentrations and increased levels of ascorbic acid when compared to CH and $NiSO_4$ groups respectively. L-ascorbic acid + CH group rats shown significantly increased concentrations of α – tocopherol when compared to CH group, but its concentrations were not significantly increased in L-ascorbic acid + $NiSO_4$ group when compared to $NiSO_4$ group.

Table 5.4.2: Comparison of oxidative stress parameters in serum among experimental groups (n=6 in each group)

Experimental Groups	MDA (µmol/L)	ascorbic acid (mg/dl)	α - tocopherol (µg/ml)
Control	1.66 ± 0.23^{a}	0.81 ± 0.04^{a}	2.86 ± 0.39^{a}
L-ascorbic acid	1.29 ± 0.10^{a}	1.29 ± 0.15^{b}	3.46 ± 0.85^{a}
CH	4.52 ± 0.94^{b}	0.55 ± 0.15^{c}	1.15 ± 0.32^{b}
$NiSO_4$	3.73 ± 0.43^{b}	0.62 ± 0.02^{c}	1.32 ± 0.30^{b}
CH + $NiSO_4$	6.95 ± 0.76^{c}	0.58 ± 0.09^{c}	1.11 ± 0.72^{b}
L-ascorbic acid + CH	2.11 ± 0.15^{a}	0.89 ± 0.05^{a}	2.05 ± 0.09^{a}
L-ascorbic acid + $NiSO_4$	1.38 ± 0.14^{a}	1.28 ± 0.13^{b}	1.39 ± 0.07^{b}
F value	104.60	69.41	20.46
p value	0.000^{*}	0.000^{*}	0.000^{*}

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

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

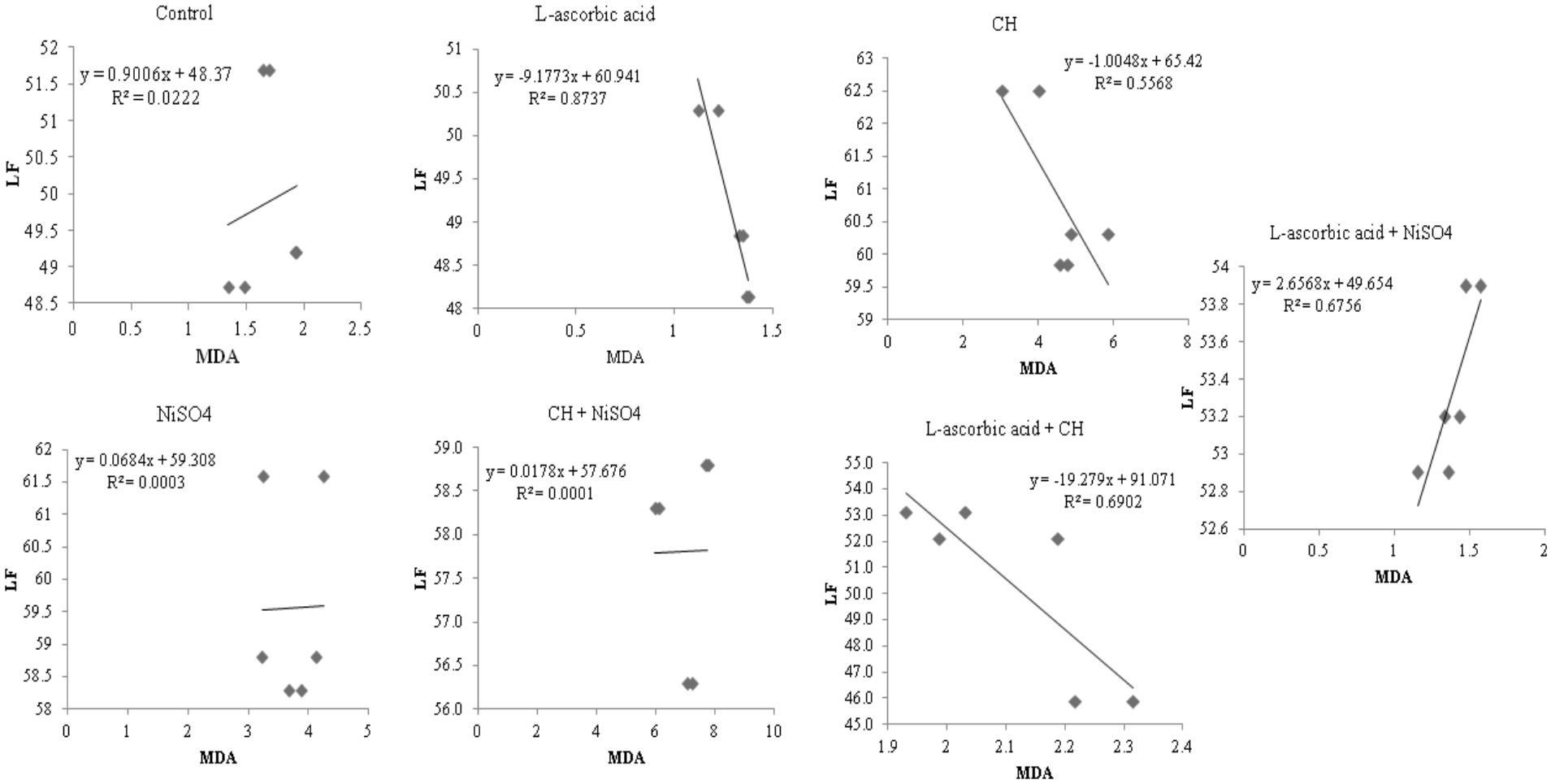


Figure 5.4.3a: Group wise correlation between LF and serum MDA (μmol/L)

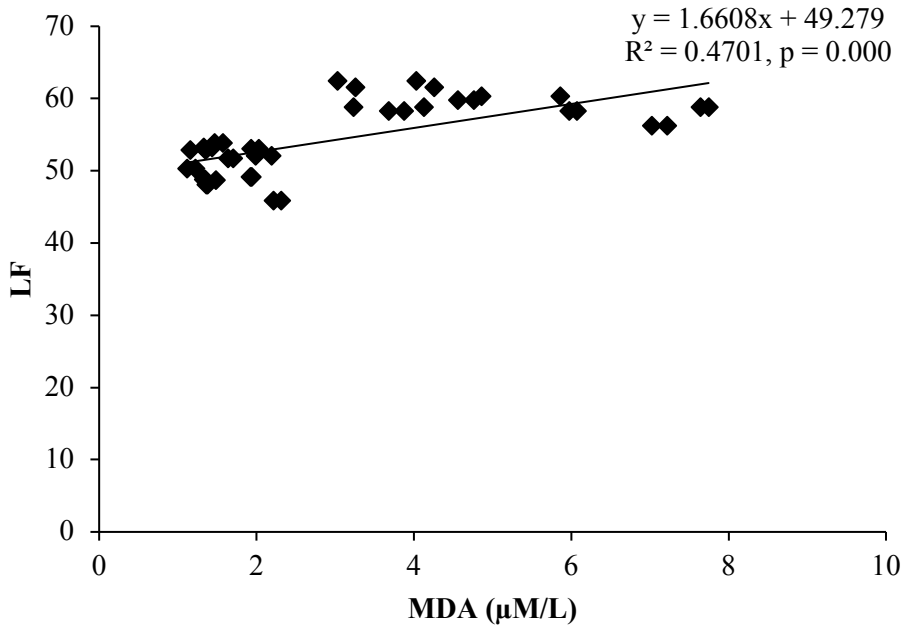


Figure 5.4.3b: Correlation between LF and serum MDA (µmol/L) among all groups.

Pearson's correlation was done to know the correlation between LF with serum malondialdehyde (MDA) a systemic oxidative stress parameter. We observed a statistically significant positive correlation between LF (nu) with serum MDA (**Figure 5.4.3a,** and **b)**.

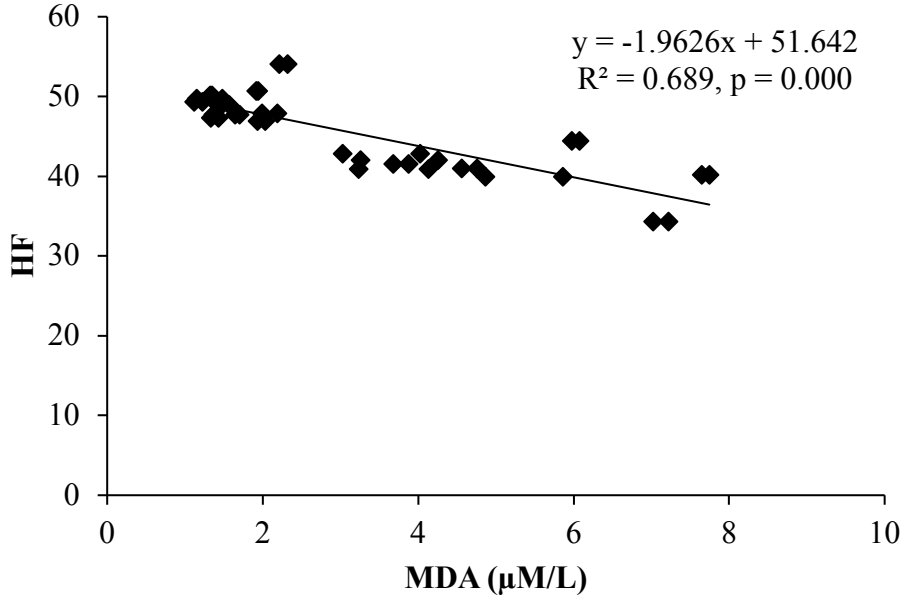


Figure 5.4.3c: Correlation between HF and serum MDA (µmol/L) among all groups.

Pearson's correlation was done to know the correlation between HF with serum malondialdehyde (MDA) a systemic oxidative stress parameter. We observed a statistically significant negative correlation between HF (nu) with serum MDA (**Figure 5.4.3b, c and d)**.

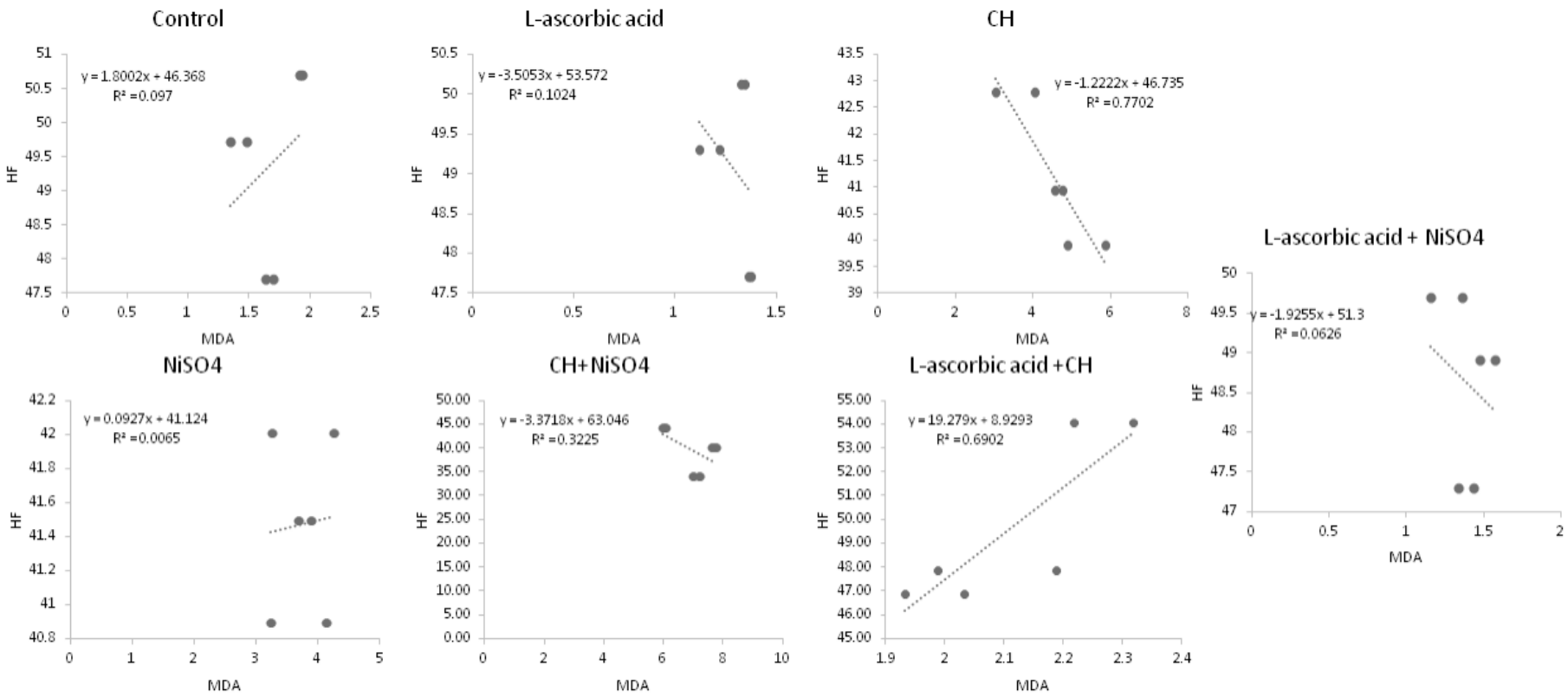


Figure 5.4.3d: Group wise Correlation between HF and serum MDA (µmol/L).

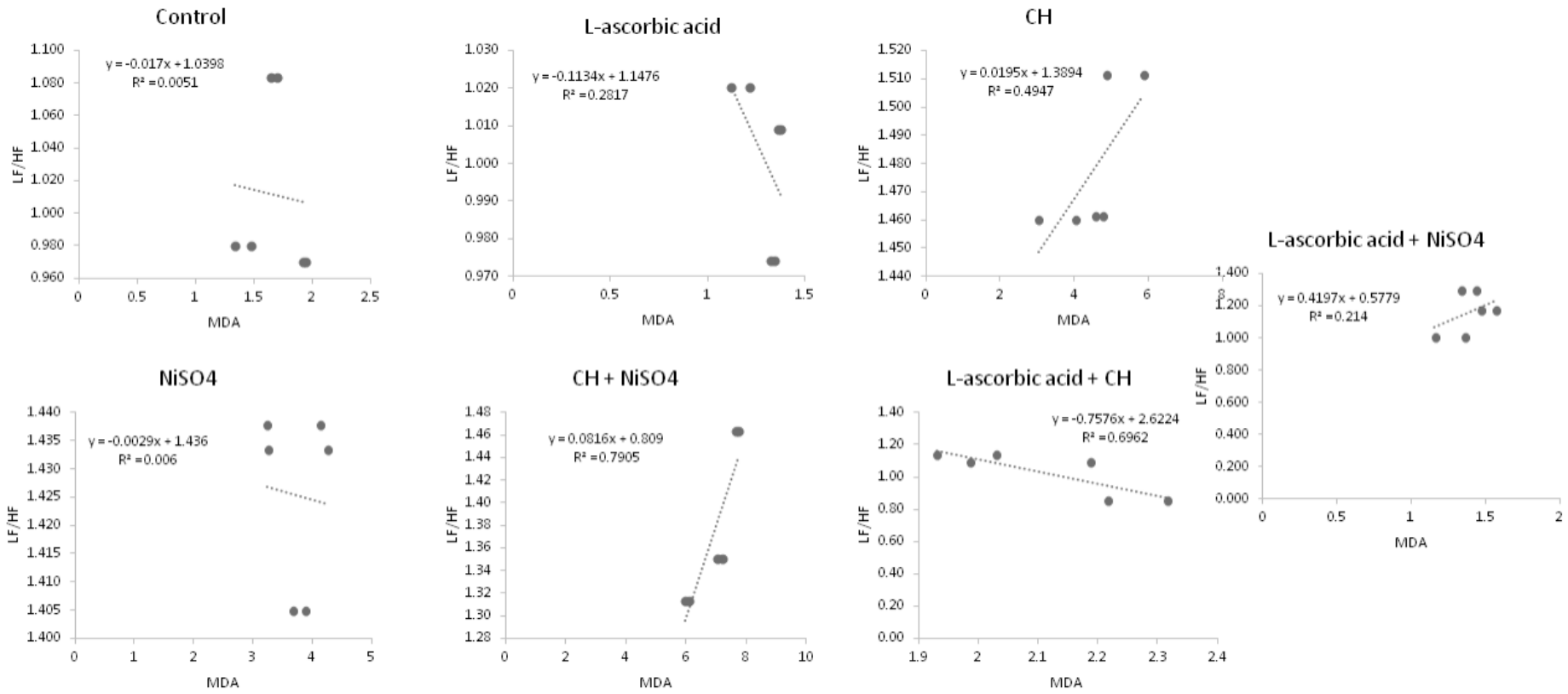


Figure 5.4.3e: Group wise Correlation between LF/HF and serum MDA (µmol/L).

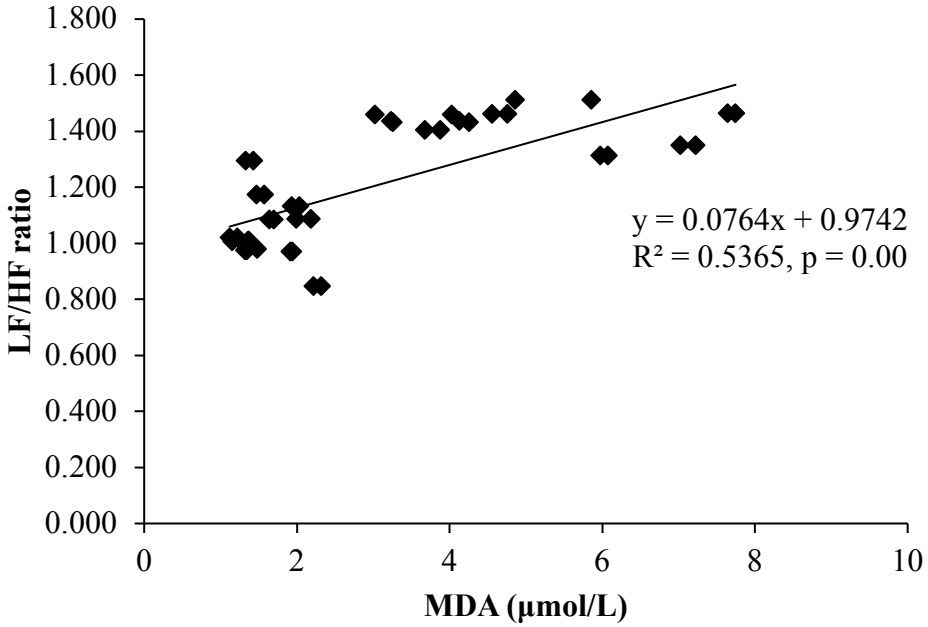


Figure 5.4.3f: Correlation between LF/HF and serum MDA (µmol/L) among all groups.

Pearson's correlation was done to know the correlation between LF/HF with serum malondialdehyde (MDA) a systemic oxidative stress parameter. We observed a statistically significant positive correlation between LF/HF with serum MDA (**Figure 5.4.3e,** and **f)**.

5.4.4 Tissue oxidative stress:

Table 5.4.4a and **b** shows heart and lung tissue MDA, NO and ascorbic acid concentrations of all experimental groups. Heart and lung tissue MDA, NO was significantly increased in CH, $NiSO_4$ and CH + $NiSO_4$ groups when compared to control and L-ascorbic acid groups. But supplementation with L-ascorbic acid has significantly decreased the MDA and NO concentations in L-ascorbic acid + CH and L-ascorbic acid + $NiSO_4$ group of rats respectively. Heart and lung tissue ascorbic acid concentrations were significantly decreased in CH, $NiSO_4$ and CH + $NiSO_4$ groups when compared to L-ascorbic acid group, and its levels were not significantly increased in L-ascorbic acid + CH and L-ascorbic acid + $NiSO_4$ groups.

Table 5.4.4a: Comparison of oxidative stress parameters in heart tissue homogenate among experimental groups(n=6 in each group)

Experimental Groups	H_MDA (μM/g of tissue)	H_NO (μM/g of tissue)	H_ascorbic acid (mg/g of tissue)
Control	3.483 ± 0.86^{a}	25.99 ± 5.858^{a}	4.76 ± 1.25^{a}
L-ascorbic acid	2.940 ± 1.07^{a}	23.61 ± 3.641^{a}	7.68 ± 1.93^{a}
CH	13.93 ± 0.29^{b}	85.46 ± 3.890^{b}	2.58 ± 1.00^{b}
$NiSO_4$	13.71 ± 0.66^{b}	82.25 ± 8.272^{b}	3.23 ± 1.64^{b}
$CH+NiSO_4$	12.97 ± 1.56^{b}	99.87 ± 6.852^{b}	1.41 ± 0.30^{b}
L-ascorbic acid + CH	7.78 ± 0.54^{c}	67.15 ± 4.320^{c}	3.02 ± 0.94^{b}
L-ascorbic acid + $NiSO_4$	6.99 ± 1.00^{c}	52.60 ± 8.188^{c}	5.12 ± 1.43^{b}
F value	191.04	175.03	27.19
p value	0.000^{*}	0.000^{*}	0.000^{*}

Values are expressed as Mean $\pm$ SD. One-way ANOVA followed by Post-Hoc Tukey's multiple comparison test. Values with different superscripts a, b, and c are significantly different from each other. $^{*}p \leq 0.05$ was considered statistically significant.CH, chronic hypoxia; H_MDA, heart malondialdehyde; H_NO, H_heart nitric oxide; H_ascorbic acid, heart ascorbic acid.

Table 5.4.4b: Comparison of oxidative stress parameters in lung tissue homogenate among experimental groups

Experimental Groups	L_MDA (μM/g of tissue)	L_NO (μM/g of tissue)	L_ ascorbic acid (mg/g of tissue)
Control	27.83 ± 3.07^{a}	15.89 ± 2.98^{a}	14.10 ± 4.148^{a}
L-ascorbic acid	25.19 ± 1.93^{a}	13.87 ± 1.45^{a}	16.40 ± 1.82^{a}
CH	43.82 ± 4.65^{b}	37.47 ± 3.96^{b}	5.86 ± 1.04^{b}
$NiSO_4$	56.47 ± 6.92^{b}	34.75 ± 3.30^{b}	4.95 ± 1.35^{b}
$CH + NiSO_4$	68.54 ± 2.95^{c}	38.61 ± 6.14^{b}	2.81 ± 1.13^{b}
L-Ascorbic acid + CH	28.90 ± 5.05^{a}	23.06 ± 2.75^{c}	6.23 ± 1.119^{b}
L-ascorbic acid + $NiSO_4$	30.00 ± 0.35^{a}	25.57 ± 1.25^{c}	6.53 ± 0.48^{b}
F value	126.39	64.03	51.19
p value	0.000^{*}	0.000^{*}	0.000^{*}

Values are expressed as Mean$\pm$SD. One-way ANOVA followed by Post-Hoc Tukey's multiple comparison test. Values with different superscripts a, b, and c are significantly different from each other. $p \leq 0.05$ was considered statistically significant. CH, chronic hypoxia; L_MDA, lung malondialdehyde; L_NO, L_heart nitric oxide; L_ascorbic acid, lung ascorbic acid.

5.5 MOLECULAR MARKERS

5.5 Molecular Markers:

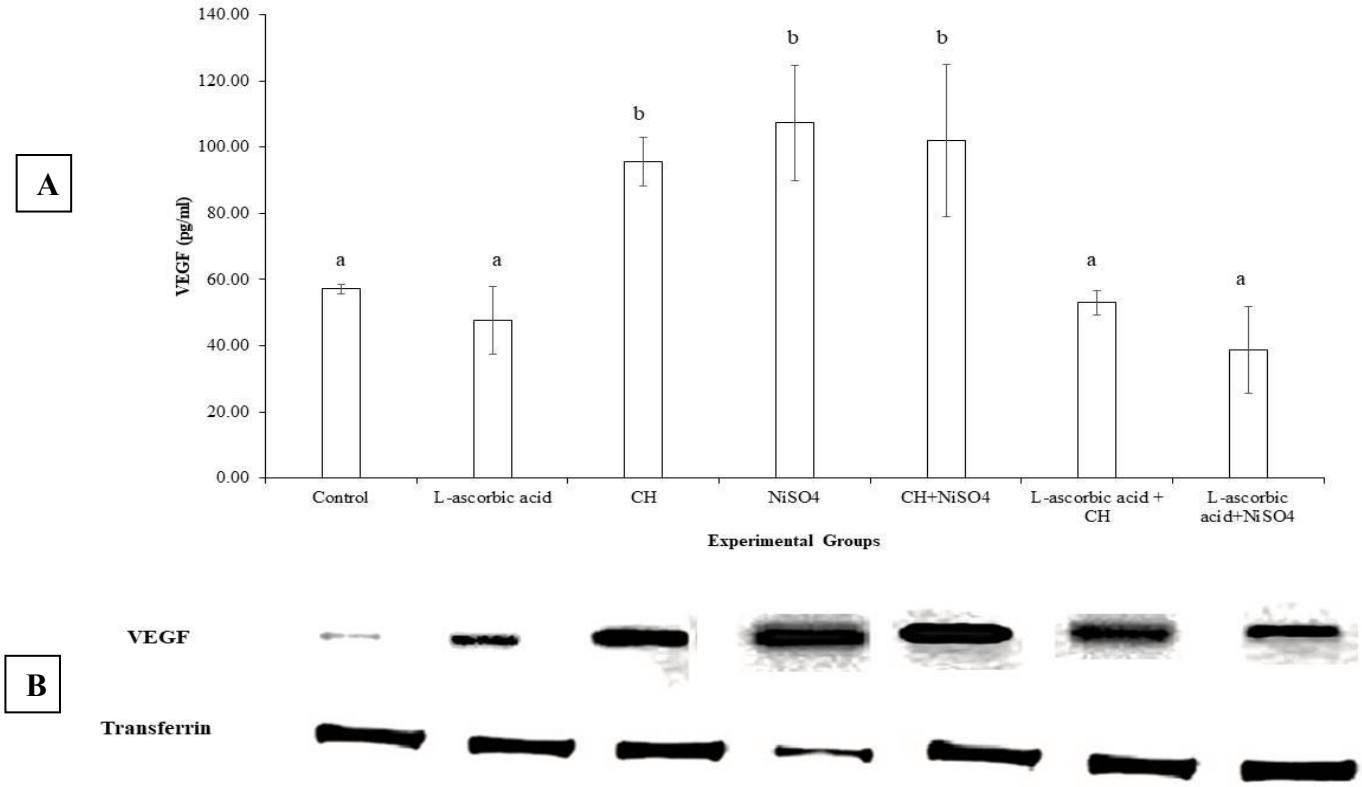


Figure 5.5 (A) Quantitative comparision of serum VEGF protein expression among Experimental Groups. **(B)** Qualitative analysis of protein levals of VEGF among control and experimental groups by western blotting. VEGF, vascular endothelial growth factor, CH, chronic hypoxia.

Serum VEGF of the control, L-ascorbic acid, Chronic hypoxia (CH), NiSO4, CH + $NiSO_4$, L-ascorbic acid + CH and L-ascorbic acid + $NiSO_4$was analysedquantitatively by ELISA method and qualitatively by western blotting using polyclonal antibody to rat VEGF that recognizes for detection of the 189, 165 and 121 amino acid splice variants of VEGF (1:1000). The blot was re-probed with anti-Transferrin (1:5000) antibody to confirm an equal loading of proteins.

Figure 5.5 A and **B** demonstrates increased serum VEGF (ELISA and Western blotting methods) and NO in CH, $NiSO_4$ and CH + $NiSO_4$ groups when compared to control and L-

ascorbic acid groups. In the L-ascorbic acid + CH and L-ascorbic acid + $NiSO_4$ group serum VEGF levels were decreased and are comparable with the control.

Table 5.5: Comparison of oxygen sensing molecular markers among experimental groups (n=6 in each group)

Experimental Groups		eNOS (NOS3) (pg/ml)	NO (μM/L)
Control		16.79 ± 2.93[a]	5.95 ± 0.69[a]
L-ascorbic acid		14.34 ± 4.35[a]	7.43 ± 1.53[a]
CH		35.91 ± 6.89[b]	15.19 ± 1.82[b]
$NiSO_4$		15.76 ± 5.09[a]	12.07 ± 1.88[b]
CH + $NiSO_4$		40.21 ± 8.18[b]	12.55 ± 2.35[b]
L-Ascorbic acid + CH		16.48 ± 4.87[a]	8.87 ± 0.57[a]
L-ascorbic acid + $NiSO_4$		17.36 ± 3.09[a]	10.10 ± 1.15[b]
ANOVA	F value	30.49	25.23
	p value	0.000*	0.000*

Values are expressed as Mean±SD. One-way ANOVA followed by Post Hoc Tukey'smultiple comparison test. Values with different superscripts a, b and c are significantly different from each other. $p \leq 0.05$ was considered statistically significant. CH, chronic hypoxia; VEGF, vascular endothelial growth factor; eNOS, endothelial nitric oxide synthase, NO, nitric oxide.

Table 5.5 shows serum NOS3 and NO levels and found that NOS3 (eNOS) concentrations were significantly increased in CH and CH + $NiSO_4$ groups when compared to control and L-ascorbic acid groups, but L-ascorbic acid + CH group significantly reduced the NOS3 levels when compared to CH group. However we have not found any significant changes of NOS3 levels between $NiSO_4$ and L-ascorbic acid + $NiSO_4$ groups. NO concentrations were significantly increased in CH, $NiSO_4$ and CH + $NiSO_4$ groups when compared to control. L-ascorbic acid supplemented group (L-ascorbic acid + CH) shown decreased NO but L-ascorbic acid + $NiSO_4$ group of rats did not shown any statistically significant chage.

5.6 CARDIOVASCULAR & PULMONARY REMODELLING

5.6 Cardiovascular Remodelling

5.6.1 Cardiosomatic Index

Table 5.6.1: Comparison of heart weight and cardiosomatic parameters among experimental groups (n=6 in each group)

Experimental Groups		Heart Weight (gm)	Final b.wt in gm.	Cardiosomatic Index
Control		0.87 ± 0.10^{a}	244.95 ± 5.09^{a}	0.357 ± 0.038^{a}
L-Ascorbic acid		0.89 ± 0.11^{a}	247.38 ± 12.59^{a}	0.360 ± 0.033^{a}
CH		0.97 ± 0.09^{a}	214.30 ± 5.22^{b}	0.454 ± 0.033^{b}
$NiSO_4$		1.10 ± 0.16^{b}	221.24 ± 0.74^{b}	0.497 ± 0.070^{b}
CH+$NiSO_4$		1.22 ± 0.11^{b}	216.60 ± 5.30^{b}	0.565 ± 0.059^{b}
L-Ascorbic acid + CH		0.99 ± 0.09^{a}	241.67 ± 5.61^{a}	0.412 ± 0.042^{b}
L-ascorbic acid + $NiSO_4$		1.02 ± 0.06^{b}	244.13 ± 9.89^{a}	0.419 ± 0.019^{b}
ANOVA	F value	24.73	7.99	16.700
	p value	0.000*	0.000*	0.000*

Values are expressed as Mean±SD. One-way ANOVA followed by Post Hoc Tukey's multiple comparison test. Values with different superscripts a, b and c are significantly different from each other. *$p \leq 0.05$ was considered statistically significant.CH, chronic hypoxia; b.wt, body weight.

Table 5.6.1 and **Figure 5.6.1** shows heart weight and cardiosomatic index of all experimental groups. Cardio somatic index was significantly increased in CH, $NiSO_4$ and CH + $NiSO_4$ groups when compared to control and L-ascorbic acid groups. However, in L-ascorbic acid + CH and L-ascorbic acid + $NiSO_4$ group cardiosomatic index was decreased when compared to CH and $NiSO_4$ groups respectively but the values were not statistically significant.

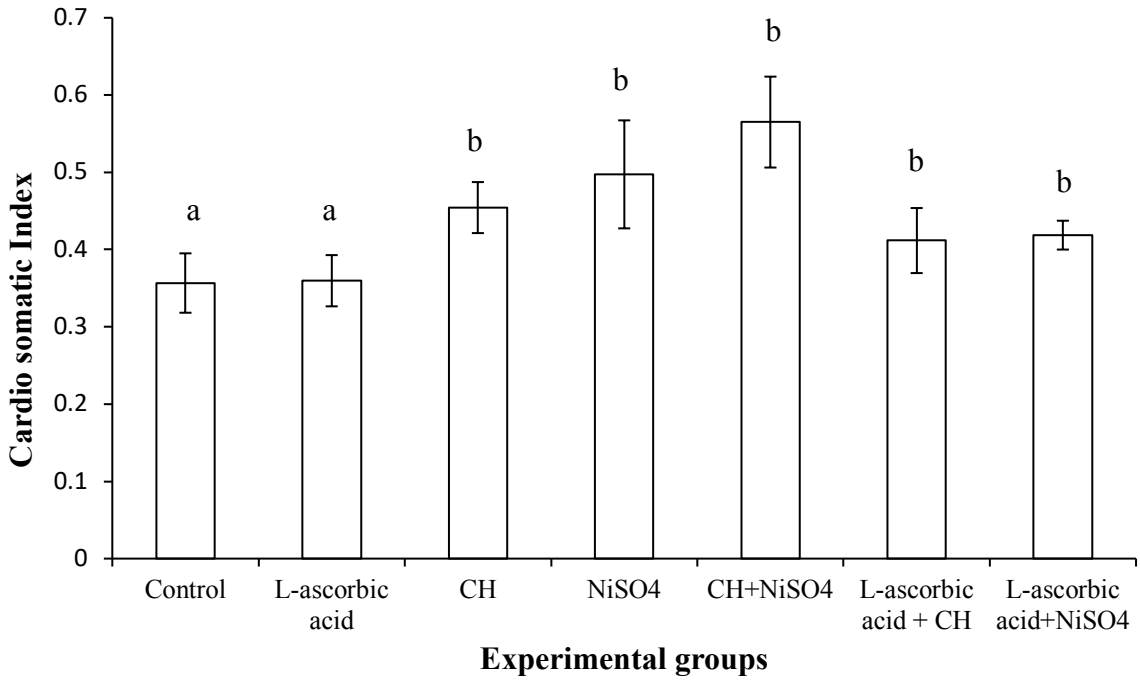


Figure 5.6.1 Comparision of Cardiosomatic Index among Experimental Groups

Values were expressed as Mean ± SD. Oneway ANOVA followed by Post Hoc Tukey's multiple comparison test. Values with different superscripts (a, b) are significantly different from each other ($p<0.05$), n=6 rats in each group.CH, chronic hypoxia.

5.6.2 Histopathological Examination of the Ventricles and Intramyocardial Coronary Artery:

Figure 5.6.2 shows pictures of themicroscopic examination of the myocardium stained with hematoxylin and eosin of all the experimental groups. Control and L-ascorbic acid groups gives the impression of normal myocardium. CH group rats showed mild hypertrophy of cardiac myocytes with nuclear enlargement and capillary congestion. Coronary artery demonstrated moderate arteriosclerosis and congestion. But CH+ L-ascorbic acid goup showed normal histology of myocardium with mild arteriosclerosis and dilatation of the coronary artery. $NiSO_4$ alone-treated rat tissue sections had shown focal myocardial hypertrophy, degeneration, capillary congestion and moderate arteriosclerosis of coronary arteries. Rats belongs to L-ascorbic acid + $NiSO_4$ show some histological improvements in

architecture by showing myocardium composed of branching and anastomosing striated muscle fibres arranged in parallel fashion, separated by capillaries and the features of arteriosclerosis, coronary arterial congestion are not seen.

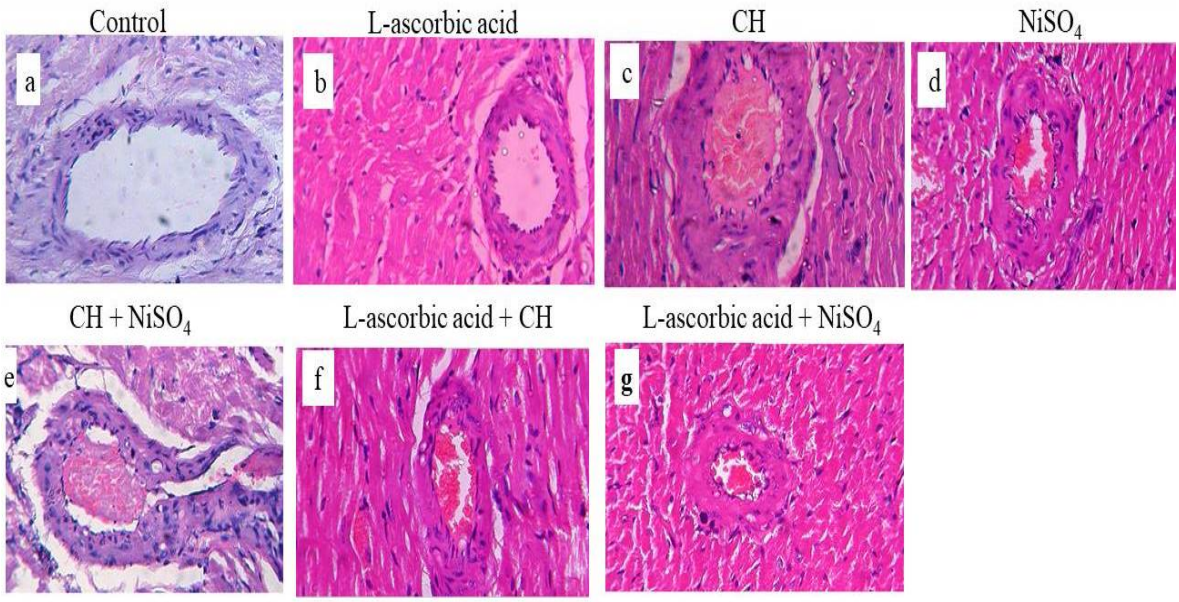


Figure 5.6.2 Photomicrograph of ventricular tissue with coronary artery stained with Haematoxylin & Eosin stain from a) control (x40); b) L-ascorbic acid (x40); c) CH (x40), d) $NiSO_4$ (x40); e) CH+ $NiSO_4$ (x40), f) L-ascorbic acid + CH (x40), g) L-ascorbic acid + $NiSO_4$ (x40). CH,chronic hypoxia.

5.6.3 Normalised Wall Index (NWI) of Coronary Artery

Normalised wall index (NWI) was calculated by using the following formula

NWI = Wall area/Total vessel area

Wall area (WA) = Total vessel area (TVA) - Lumen Area (LA)

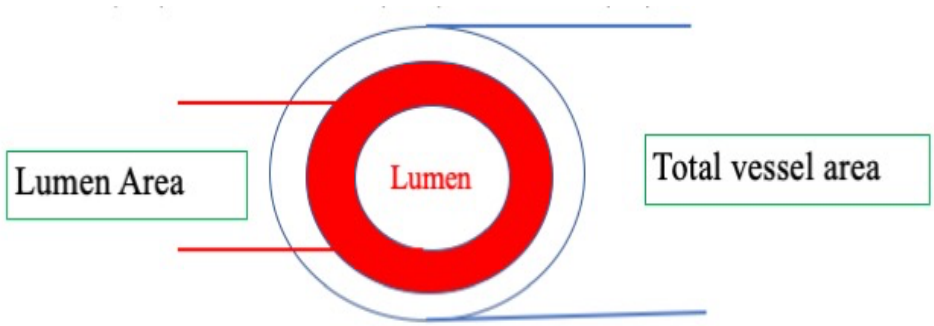


Figure 5.6.3 Schematic diagram depicting lumen area and total vessel area

Table 5.6.3: Comparison of NWI of coronary artery among experimental groups (n=6 in each group)

Experimental Groups		Total vessel area (TVA) (μm^2)	Lumen Area (LA) (μm^2)	Wall area (WA) (μm^2)	NWI
Control		135.92 ± 2.64^a	56.26 ± 2.30^a	79.66 ± 1.96^a	0.586 ± 0.011^a
L-ascorbic acid		135.76 ± 6.34^a	57.89 ± 1.39^a	77.87 ± 6.10^a	0.572 ± 0.020^a
CH		224.63 ± 4.93^b	65.82 ± 3.56^b	158.80 ± 7.28^b	0.706 ± 0.019^b
$NiSO_4$		245.66 ± 11.5^b	68.09 ± 2.77^b	177.57 ± 10.46^c	0.722 ± 0.012^b
CH + $NiSO_4$		266.46 ± 6.50^c	70.57 ± 1.75^b	195.88 ± 5.75^d	0.735 ± 0.035^b
L-Ascorbic acid + CH		135.11 ± 1.92^a	57.77 ± 5.58^a	77.34 ± 4.67^a	0.572 ± 0.037^a
L-ascorbic acid + $NiSO_4$		136.47 ± 2.52^a	55.46 ± 1.89^a	81.00 ± 3.72^a	0.593 ± 0.018^a
ANOVA	F value	583.75	26.09	449.49	85.10
	p value	*0.000	*0.000	*0.000	*0.000

Values are expressed as Mean±SD. One-way ANOVA followed by Post Hoc Tukey's multiple comparison test. Values with different superscripts a, b and c are significantly different from each other. *$p \leq 0.05$ was considered statistically significant. NWI, normalized wall index; CH, chronic hypoxia.

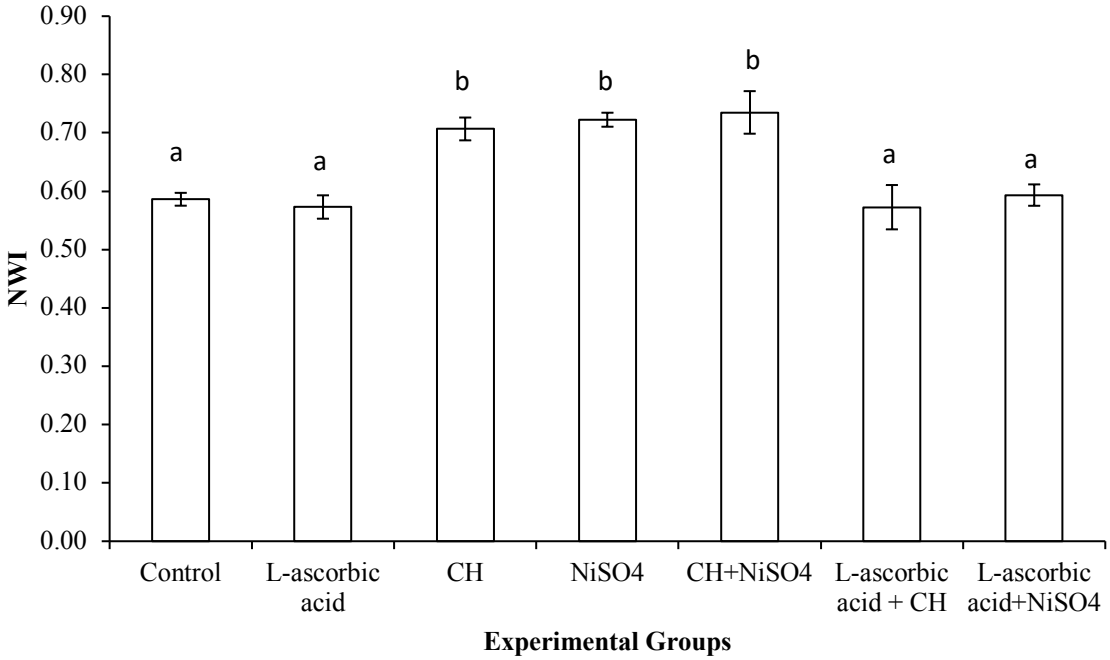


Figure 5.6.4 Comparison of NWI of Coronary Artery among Experimental Groups

Values were expressed as Mean ± SD. Oneway ANOVA followed by Post Hoc Tukey's multiple comparison test. Values with different superscripts (a, b) are significantly different from each other ($p<0.05$), n=6 rats in each group.CH, chronic hypoxia; NWI, normalised wall index.

Table 5.6.3 and **Figure 5.6.4** shows NWI values of coronary artery of all groups of experimental rats. CH, $NiSO_4$ and CH + $NiSO_4$ group rats showed higher NWI values when compared to control and L-ascorbic acid groups. But, L-ascorbic acid supplemented groups (L-ascorbic acid + CH and L-ascorbic acid + $NiSO_4$) showed significant decrease of NWI when compared to CH and $NiSO_4$ groups respectively.

5.6.4 Correlation between Coronary artery Normalised Wall Index (NWI) and serum nitric oxide (NO) concentrations among experimental groups:

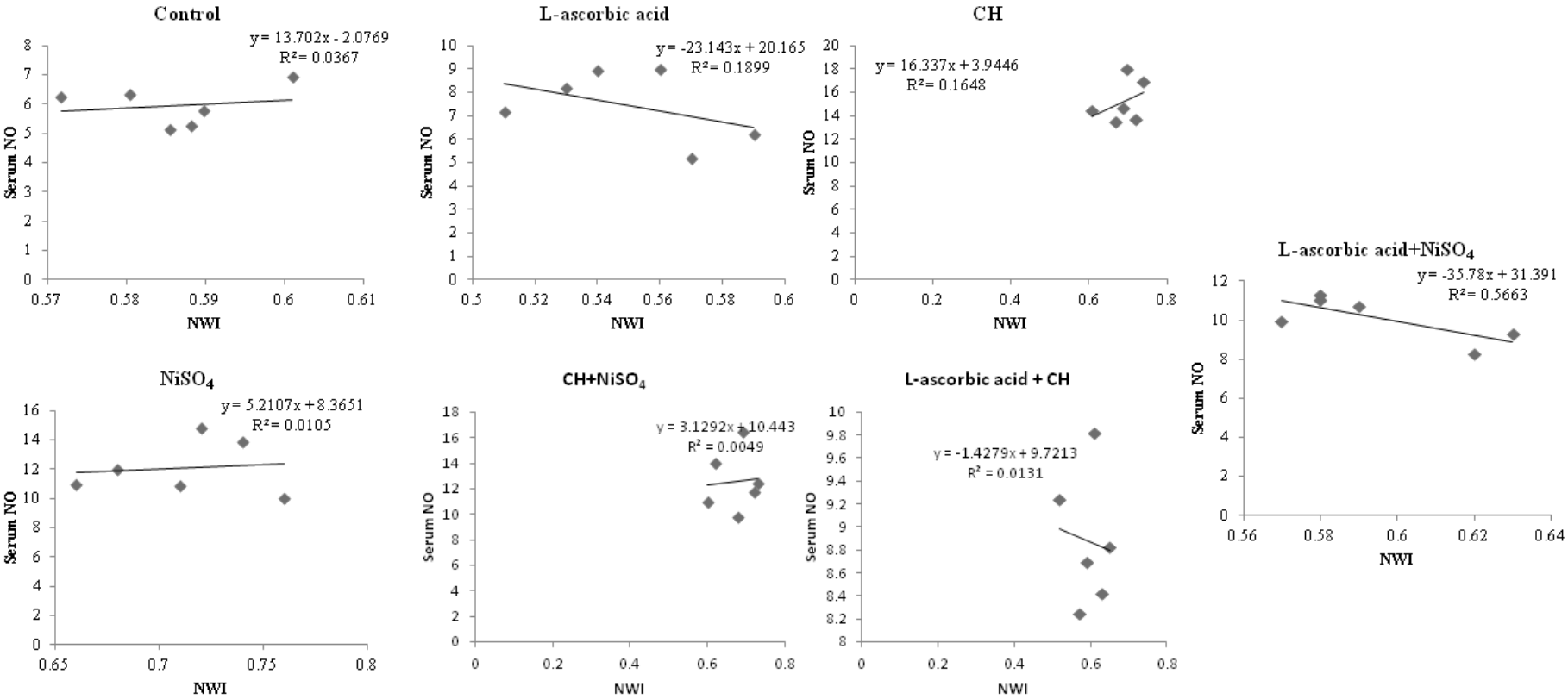


Figure 5.6.4a Group wise Correlation between NWI and serum NO (µM/L).

Pearson's correlation was done to know the correlation between NWI with serum NO. We observed a statistically significant positive correlation between NWI and serum NO (Figure 5.6.4a and b)

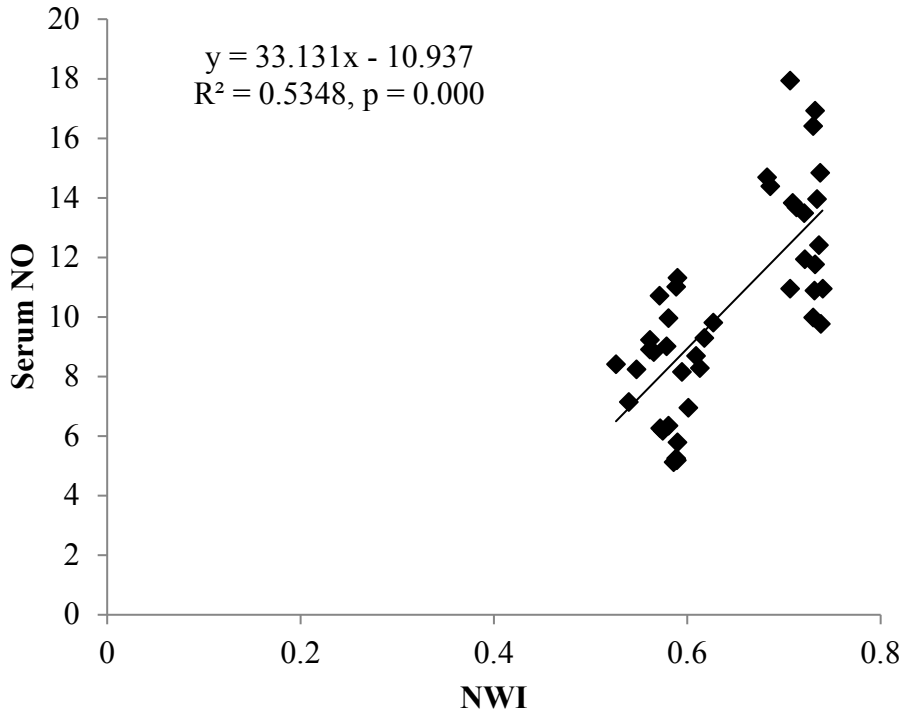


Figure 5.6.4a Correlation between NWI and serum NO (μM/L) among experimental groups

5.6.5 Correlation between Coronary artery Normalised Wall Index (NWI) and serum NOS3 concentartions among experimental groups:

Pearson's correlation was done to know the correlation between NWI with serum NOS3 concentrations. We observed a statistically significant positive correlation between NWI and serum NOS3 (**Figure 5.6.5a and b**).

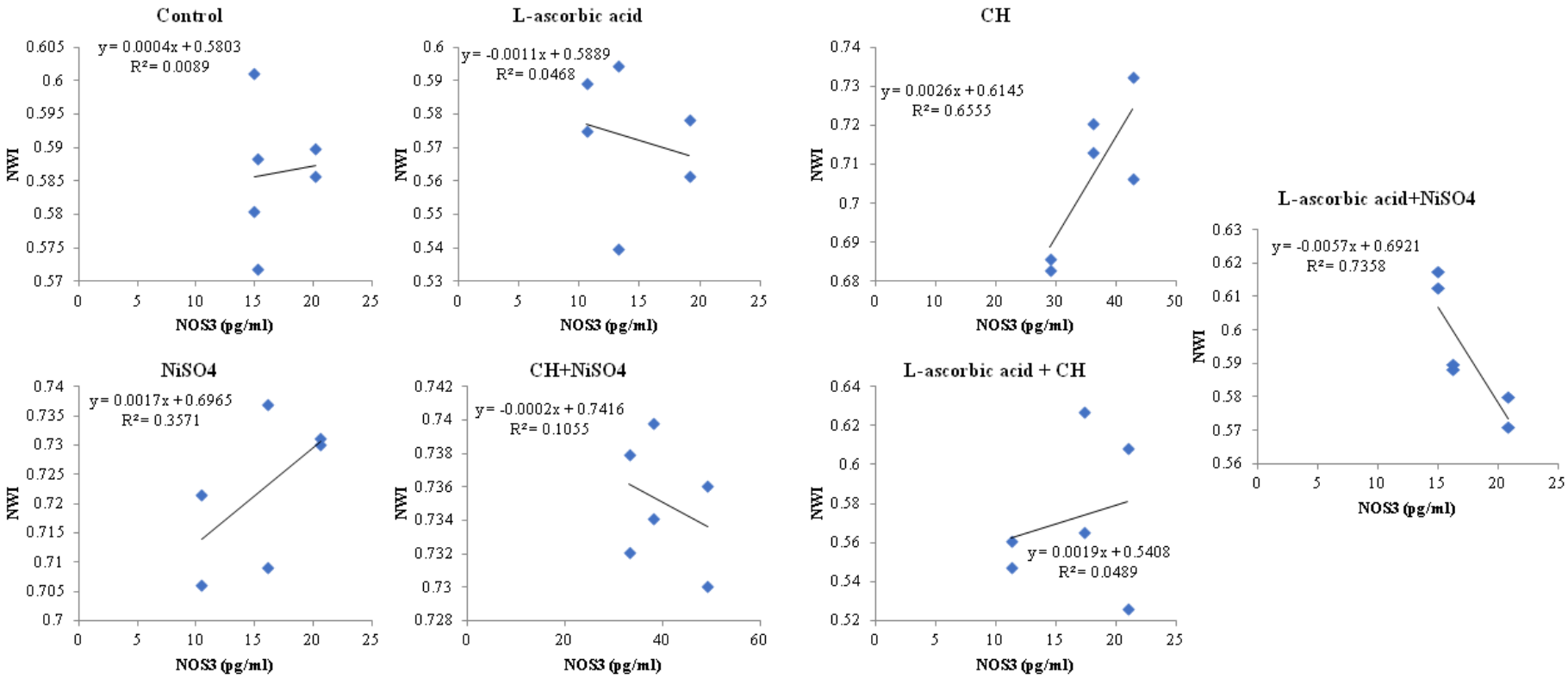


Figure 5.6.5a Group wise Correlation between NWI and serum NOS3 (pg/ml)

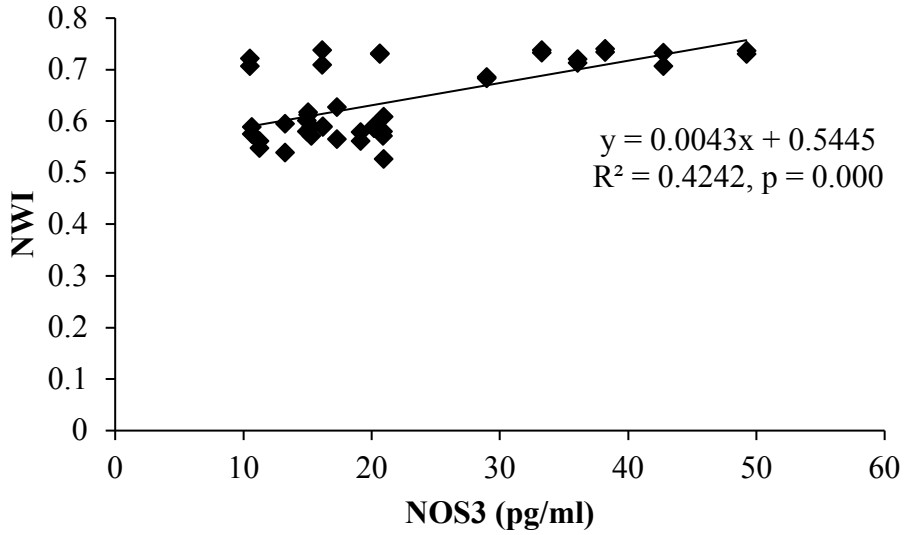


Figure 5.6.5b Correlation between NWI and serum NOS3 (pg/ml) among experimental groups

5.6.5 Histopathology of Elastic Artery

Figure 5.6.5 shows thoracic aortic tissue sections of all experimental groups. Control and L-ascorbic acid treated rats showed normal histological structures and not seen any features of arteriosclerosis, atherosclerosis, arteritis, calcification, aneurysm and dysplasia.CH group demonstrated moderate thickening of tunica intima, hyperplastic smooth muscle cells of tunica media and an overall increase in wall thickness of the elastic artery. CH+L-ascorbic acid group demonstrated only mild thickening of tunica intima and aortic dilation. $NiSO_4$ alone-treated rats aortic tissue sections show mild thickening of tunica intima, medial sclerosis with hyperplastic smooth muscle cells.Tissues sections of rats simultaneously treated with $NiSO_4$ + L-ascorbic acid show near normal architecture with only mild thickening of tunica media. CH + $NiSO_4$ severe thickening of tunica intima and media was observed.

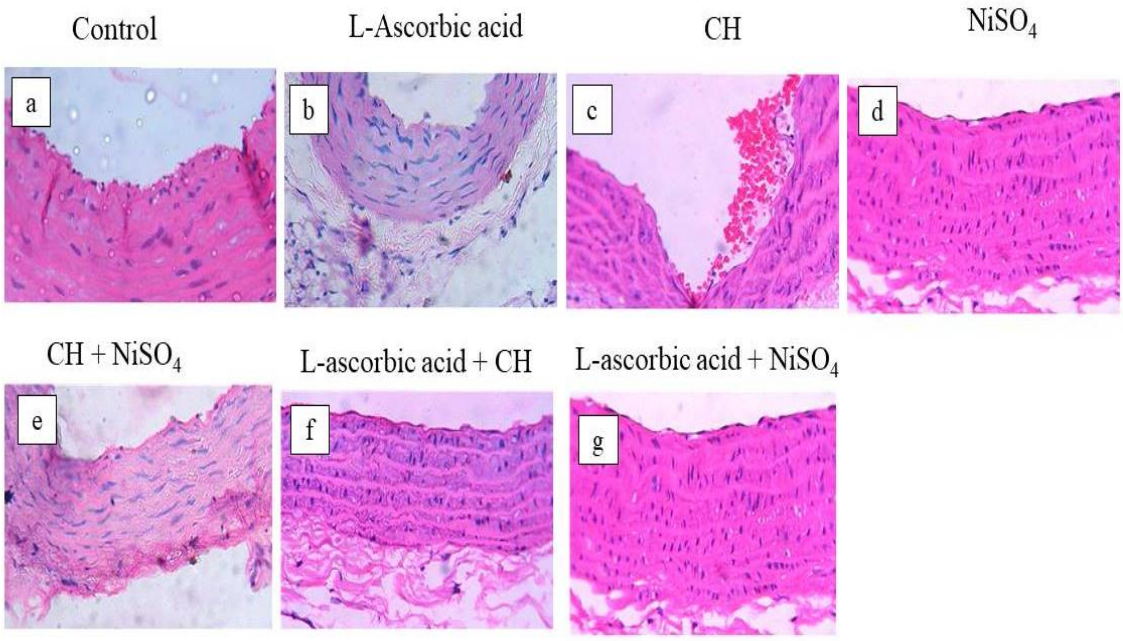


Figure 5.6.5 Photomicrograph of Elastic artery stained with Haematoxylin & Eosin stain from a) control (x40), b) L-ascorbic acid (x40), c) CH (x40); d) $NiSO_4$ (x40), e) CH + $NiSO_4$ (x40); g) L-ascorbic acid + CH (x40), g) L-ascorbic acid + $NiSO_4$ (x40). CH, chronic hypoxia.

5.6.6 Pulmonosomatic Index (PSI)

Table 5.6.6: Comparison of lungs weight and pulmonosomatic index parameters among experimental groups (n=6 in each group)

Experimental Groups		Lungs Weight (gm)	Final b.wt in gm.	Pulmonosomatic Index
Control		2.230 ± 0.039^a	244.95 ± 5.09^a	0.911 ± 0.03^a
L-Ascorbic acid		2.09 ± 0.047^a	247.38 ± 12.59^a	0.848 ± 0.061^a
CH		2.68 ± 0.146^b	214.30 ± 5.22^b	1.252 ± 0.042^b
$NiSO_4$		2.38 ± 0.206^a	221.24 ± 0.74^b	1.076 ± 0.093^b
CH+$NiSO_4$		2.61 ± 0.387^b	216.60 ± 5.30^b	1.203 ± 0.153^b
L-Ascorbic acid + CH		2.17 ± 0.035^a	241.67 ± 5.61^a	0.900 ± 0.020^a
L-ascorbic acid + $NiSO_4$		2.31 ± 0.135^a	244.13 ± 9.89^a	0.946 ± 0.023^b
ANOVA	F value	8.704	7.99	26.72
	p value	0.000*	0.000*	0.000*

Values are expressed as Mean $\pm$ SD. One-way ANOVA followed by Post Hoc Tukey's multiple comparison test. Values with different superscripts a, b and c are significantly different from each other. $p \leq 0.05$ was considered statistically significant. CH, chronic hypoxia; b.wt, body weight.

Table 5.6.6 and **Figure 5.6.6** shows significantly increased pulmonosomatic index of CH, $NiSO_4$ and CH + $NiSO_4$ groups when compared to control and L-ascorbic acid supplemented groups. But L-ascorbic acid + CH group showed significant decrease in pulmonosomatic index when compared to CH, $NiSO_4$ and CH + $NiSO_4$ groups. However we have not observed any statistically significant difference in rats treated with L-ascorbic acid + $NiSO_4$.

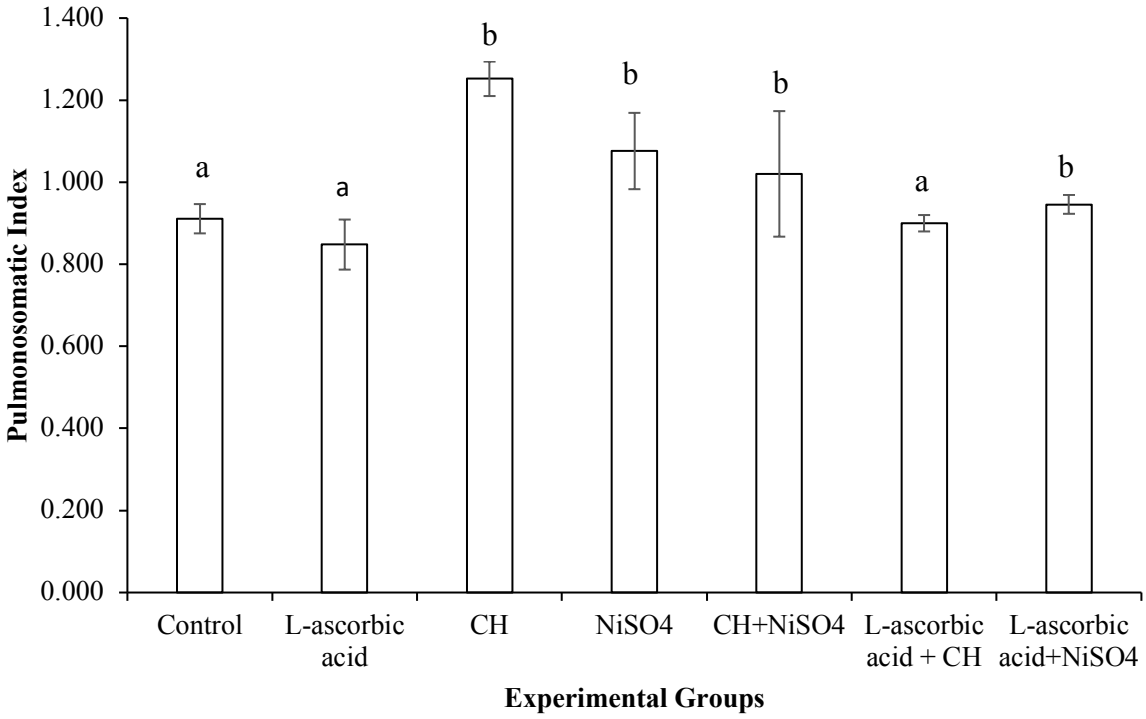


Figure 5.6.6 Comparision of Pulmonosomatic Index among Experimental Groups

Values were expressed as Mean $\pm$ SD. Oneway ANOVA followed by Post Hoc Tukey's multiple comparison test. Values with different superscripts (a, b) are significantly different from each other ($p<0.05$), n=6 rats in each group. CH, chronic hypoxia.

5.6.7 Histopathology of Lungs:

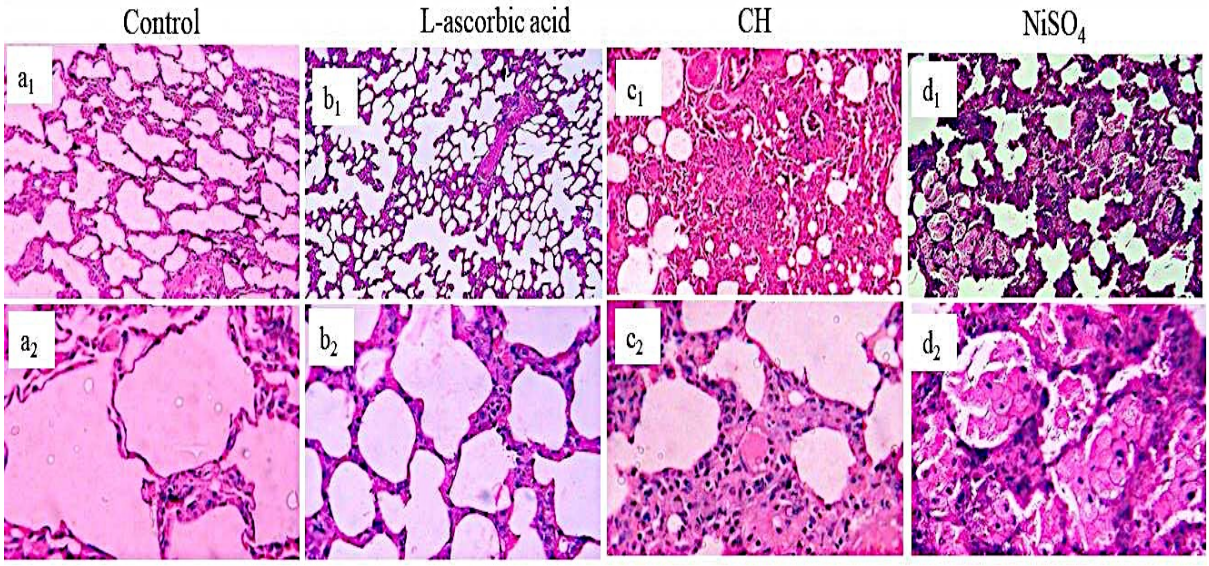


Figure 5.6.7a: Photomicrograph of lung tissue stained with Haematoxylin& Eosin stain from a_1) control (x10), a_2) control (x40); b_1) L-Ascorbic acid (x10), b_2) L-ascorbic acid (x40); c_1) CH (x10), c_2) CH (x40); d_1) $NiSO_4$ (x10), d_2) $NiSO_4$ (x40). CH, chronic hypoxia.

Figure 5.6.7a and b shows histopathology of the lung tissues in all experimental groups. Control and L-ascorbic acid (**Figure 5.6.7a)** shows normal lung parenchyma consisting of bronchi, bronchioles and thin-walled alveoli separated by intervening interstitial connective tissue containing small pulmonary capillaries. Histopathology of the lung in chronic hypoxia (CH), $NiSO_4$ (**Figure 5.6.7a**) and CH + $NiSO_4$ (**Figure 5.6.7b**) exposed rats demonstrate hyalinised respiratory bronchioles and the alveolar spaces were dilated and filled with eosinophilic oedema fluid. The interstitial septa are thickened by oedema, congestion and haemorrhage and mixed inflammatory leucocytic infiltration. In groups supplemented with L-ascorbic acid (L-ascorbic acid + CH and L-ascorbic acid + $NiSO_4$) (**Figure 5.6.7b**) has shown some improvements in the pathological alterations caused by CH and $NiSO_4$.

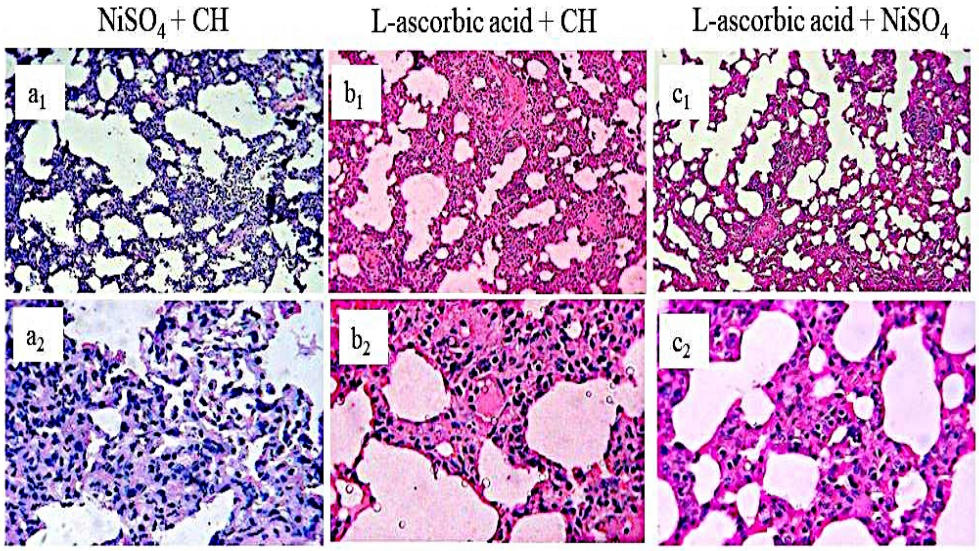


Figure 5.6.7b: Photo micrograph of lung tissue stained with Haematoxylin & Eosin stain from a_1) $NiSO_4$ + CH (x10), a_2) $NiSO_4$ + CH (x40); b_1) L-ascorbic acid + CH (x10), b_2) L-ascorbic acid + CH (x40); c_1) L-ascorbic acid + $NiSO_4$(x10), c_2) L-ascorbic acid + $NiSO_4$ (x40).CH, chronic hypoxia

CHAPTER 6

DISCUSSION

6.1 GRAVIMETRY

Results clearly indicate that chronic hypoxia (CH), $NiSO_4$ and CH + $NiSO_4$ has adverse effects on % body weight gain of rats. Many theories and pathways have been proposed about the control and maintaenance of the body composition. But the present results of our study are supported by electrophysiological parameters. Nickel causes severe gastritis and malabsorption which ultimately leads to less calorie consumption and decreases the body weight. It has been reported that nickel exposed rats reduces somatic growth, longitudinal bone growth and bone strength during the pubertal period (Das Gupta et al., 2007). According to Bray *et al* (2000), increased sympathetic activity reduces food intake and our study also showed increased sympathetic activity in CH, $NiSO_4$ and CH + $NiSO_4$ group (Bray, 2000). But L-ascorbic acid supplementation in CH and $NiSO_4$ exposed rats has shown statistically significant body weight gain when compared to CH and $NiSO_4$ treated rats respectively. Messina *et al* hasreported positive correlation between energy expenditure and sympathetic dominance (Messina et al., 2013). In earlier studies it has been reported that L-ascorbic acid helps in the restoration of growth rate in experimental ratsexposed to nickel (Das and Büchner, 2007).

6.2 ELECTROPHYSIOLOGY

Electrophysiological parameters were recorded to know the influence of L-ascorbic acid on cardiovascular hemmodynamics of experimental rats exposed to chronic hypoxia and $NiSO_4$.

In the present study, we have observed an increase of mean arterial pressure (MAP) in the rats of CH, $NiSO_4$ and CH + $NiSO_4$ groups. There may be multiple factors adding to an increase of MAP in these groups. From the present study we found some influencing factors promoting to higher MAP, crucial being increased sympathetic drive as reflected by HRV analysis (vide chapter: 5.2; Table:5.2.4). Increased MAP may alsobe due to the high generation of reactive oxygen species (ROS) or disturbed oxidant-antioxidant balance and cyclooxygenase 2 (COX2) dependent endothelium contracting factors (EDCFs) by endothelial cells (Julian, 2007; Lippmann et al., 2006; Vallejo et al., 2006; Wani et al., 2018). Nitric oxidea potent vasodilator plays a key role in the maintenance of arterial blood pressure. It also acts as a counter-regulatory factor opposing the vasoconstrictor effects of endothelin, angiotensin II and renal sympathetic nerve activity. We observed increased MAP values despite of elevated NO concentrations in CH and $NiSO_4$ groups, this may be due to decreased bioavailability of NO causing endothelial dysfunction (vide chapter: 5.5; Table: 5.5) (Calbet, 2003). NO, in addition, has a role in pressure natriuresis (Barton, 2003). Golovko*et al* (2003) reported that nickel ion induces vaso constriction in the isolated canine coronary artery by enhancing $CaCl_2$ influx into vascular smooth muscle cells (Golovko et al., 2003; Koller et al., 1982). These increased concentrations of intra cellular smooth muscle Ca^{2+} could lead to the increased arterial tone which in turn leading to an increase of blood pressure (Forstermann and Sessa, 2012a). But, simultaneous supplementation of L-ascorbic acid (L-ascorbic acid + CH and L-ascorbic acid + $NiSO_4$) significantly improved the altered blood pressure parameters in rats. L-ascorbic acid is found to be a potent antioxidant which improves

endothelial functions by reducing the oxidative stress, suppressing endothelial cell apoptosis by cytokines and tumour necrosis factor-α (TNF-α) and angiotensin II (Das et al., 2010).

Heart rate variability (HRV) analysis in all groups of rats considered as a clinically approved indicator of cardiac autonomic balance (Chuang et al., 2013). Our observations clearly indicated a sympatho-vagal imbalance in CH, $NiSO_4$ and CH + $NiSO_4$ group of rats by sympathetic overdrive with concomitant under drive of para sympathetic actions. The increase of heart rate in CH, $NiSO_4$ and CH + $NiSO_4$ group rats in the present study further indicates a possible increase in sympathetic stimulation (Julian, 2007; Lippmann et al., 2006; Vallejo et al., 2006). These autonomic malfunctions in CH group may be by stimulating the carotid bodies and triggering the chemoreceptor reflex, in $NiSO_4$ group it may be due to nickel-initiated inflammatory response, increased oxidative stress and decreased availability of antioxidants (Kasprzak, 2003). Altered sympatho-vagal balance due to CH or $NiSO_4$ administration was significantly improved by the supplementation of antioxidant L-ascorbicacid, suggesting that concurrent response of oxidative stress involved in the modulation of cardiac autonomic function (Lippmann et al., 2006).

6.3 HEMOGRAM

It has been known that red blood cells plays an importnat role in O_2 transport in our biological system. Sveral physiochemical systems play an important role in maintaing the oxygen homeostasis. We observed significant increase of RBC count in chronic hypoxia exposed rats but RBC count was significantly decreased in $NiSO_4$ and CH + $NiSO_4$ group animals. Oxygen sensing factors like hypoxia-inducible factor (HIF) plays an eminent role in hypoxia adaptation pathway by enhanced production of erythropoietin (Epo) which inturn enhances the RBC production to combat the low oxygen tissue environment (Haase, 2013; Rankin et al., 2007; Wang and Semenza, 1993). Recently various researchershad shown theimportnat role of HIF-2 in controllingEpoexpression in the biological systems. Various findings shown a positive correlation between HIF-2, Epo and severity of hypoxia (Haase, 2013).

Nickel has long been known to alter the hematological system by inhibiting the activities of several enzymes involved in heme biosynthesis (Lange et al., 1999; Park et al., 2001). Anemia induced by nickel is primarily the result of both inhibition of heme synthesis and shortening of erythrocyte life span, but nickel also can induce inapprpriate production of the hormone erythropoietin leading to inadequate maturation of red cell progenitors, which can contribute to the less RBC count (Secchi et al., 1974). The decrease of RBC count may be attributed to the destructive influence of nickel on the cell membranes of erythrocytes through binding of the toxicants with immunoglobulins or through disturbance of the activity of erythrocyte enzymes, especially those responsible for reduction of glutathione and thiol group of proteins (Sun et al., 2002).In our study decrease in RBC count may be due to non regenerativeanemia arise from nickel induced direct injury of hematopoietic stem cells resulting in decreased erythrocytes. Toxic insult by nickel also resulted into a marked decline in tissue thiols and ascorbic acid concentrations (Luca et al., 2007). Oral supplementation of vitamin C (100 mg/kg for 3 days) completely restored blood delta aminolevulinic acid

dehydratase, uroporphyrinogen I synthase and a few drug metabolizing enzymes (Wang et al., 2011). L-ascorbic acid facilitates the rate of production of blood cells by stimulating bone marrow. It was also reported that L-ascorbic acid promotes the production of RBC in bone marrow and contributes to hemoglobin synthesis and even prevents blood clotting (Jeong et al., 2011). Unlike the other studies we have not found any significant changes in WBC counts and hemoglobin concentrations among experimental groups (Tkeshelashvili et al., 1989).

6.4 BIOCHEMISTRY

6.4.1 Lipid Profile

In this study, chronic hypoxia (CH) and heavy metals like nickel-induced increase of serum LDL-cholesterol, total cholesterol and triglycerides could be due to changes in the gene expression of hepatic enzymes like HMG- CoA reductase (hydroxy- methyl glutaryl-CoA reductase). This in turn depresses LDL-receptor gene expression. Defects in the LDL-receptor interfere with cholesterol uptake from the blood stream, which in turn causes excess cholesterol synthesis in the liver and high levels of serum total cholesterol and LDL-cholesterol (Kantola et al., 1998). Heavy-metal-induced change in the gene expression of HMG-CoA reductase in rats has already been reported(Amrita Gupta et al., 2008; Kojima et al., 2004). In the present study, when compared with nickel treated group, the significant improvement in lipid profiles in rats treated concomitantly with l-ascorbic acid and nickel sulfate agrees with reports that ascorbic acid can help decrease the levels of total and LDL-cholesterol and triglycerides (Tofler et al., 2000). L-ascorbic acid induced increase in LDL-receptors and facilitation of lipoprotein-cholesterol clearance has also been reported (Tofler et al., 2000). It has also been observed that, ascorbic acid can counteract nickel induced changes in HMG-CoA reductase activities and thereby improve the serum lipid profile (Harwood et al., 1986). It is referred that chronic hypoxia (CH) and divalent cation like Ni can cause intracellular depletion of vitamin C (ascorbic acid), which is a potent antioxidant as well as helps in recycling of inactive vitamin E to its active form during the scavenging process (Aulinskas et al., 1983; Bulger and Helton, 1998).

6.4.2 Serum Oxidative Stress:

Chronic hypoxia and heavy metal like nickel are known atherogenic factors which may exert its effects by increasing the ROS production which in turn increases lipid peroxidation by the increase of oxygen free radicals and over utilization of membrane antioxidants (Detmar et al., 1997). Increased oxidative stress in the physiological system is

considered to be a key factor underlying cardiovascular dysfunction, hence increase of serum, heart and lung tissue MDA with the decrease of L-ascorbic acid and α-tocopherol in serum and decrease of L-ascorbic acid in heart and lung tissues in hypoxia or nickel exposed rats ascertain cardio and pulmono toxicities due to nickel or hypoxia induced oxidative stress (Niki, 1987). Reactive oxygen species (ROS) are implicated as important pathologic mediators in many disorders. Increased generation of ROS and enhanced lipid peroxidation are considered responsible for the toxicity of wide range of compounds (Das et al., 2001). Lipid peroxidation constitutes a complex chain reaction of free radicals, which leads to a degradation of polyunsaturated fatty acid in cell membrane (Halliwell and Gutteridge, 1986). Ascorbic acid can be oxidized by most reactive oxygen and nitrogen species thought to play roles in tissue injury associated with various diseases. These species include superoxide, hydroxyl, peroxyl and nitroxide radicals, as well as non-radicals reactive species such as singlet oxygen, peroxynitrite and hypochlorate. By virtue of their scavenging activity, ascorbate inhibits lipid peroxidation, oxidative DNA damage and oxidative protein damage. It is also reported that L-ascorbic acid enters mitochondria of a cell in its oxidized form via GLUT-1 and protects mitochondria from oxidative injury. Since mitochondria contribute significantly to intracellular ROS, protection of the mitochondrial genome and membrane may be beneficial (KC et al., 2005). As nickel decreases the cellular transportation of ascorbic acid and deplete intracellular ascorbic acid resulting in intracellular hypoxia and metabolic dysfunction (Salnikow and Kasprzak, 2005). Supplementation of L-ascorbic acid in chronic hypoxia or nickel treated rats was found to be beneficial to counteract hypoxia or nickel induced cardio toxicities or pulmono toxicities due to oxidative stress.

6.4.3 Tissue oxidative stress:

The mechanism of nickel-induced oxidative stress involves an imbalance between generation and removal of ROS (reactive oxygen species) in tissues and cellular components

causing damage to membranes, DNA and proteins. The presence of double bonds in the fatty acid on cell membrane weakens the C–H bonds on the carbon atom adjacent to the double bonds and makes H removal easier. Therefore, fatty acids containing zero to two double bonds are more resistant to oxidative stress than polyunsaturated fatty acids with more than two double bonds (Land, 1990). The intrinsic mechanism underlying nicke-induced oxidative damage to membranes is associated with changes in its fatty acid composition(Knowles and Donaldson, 1990). The fatty acid chain length and unsaturation are the determinant for membrane susceptibility to peroxidation, and nickel induced arachidonic acid elongation might be responsible for the enhanced lipid peroxidation of the membrane. Thus, nickel affects membrane related processes such as the activity of membrane enzymes, endo and exocytosis, transport of solutes across the bilayer, and signal transduction processes by causing lateral phase separation (Hattiwale et al., 2013).

There has been considerable debate concerning the relationship between vitamin C nutritional status and nickel-induced toxic effects. Early reports suggest vitamin C as a possible chelator of nickel, with similar potency to that of EDTA (Das and Buchner, 2007). Current evidence suggests that the primary sensor of hypoxia for the development of pulmonary vasoconstriction is the PASMC (Primary Pulmonary Artery Smooth Muscle Cells) mitochondria, which increases the production of ROS at low pressures of O_2, probably in the complex III of the electron transport chain. It is possible that there are secondary sensing mechanisms that contribute to this effect, which will increase the production of ROS during hypoxia such as sarcolemmal NADPH oxidase from pulmonary vasculature. Researchers have demonstrated an increase in the mitochondrial ROS generation in various tissues in response to hypoxia, including PASMC. The probability that lower concentrations of oxygen in lungs are sufficient to produce similar effects to those observed in other tissues at higher concentrations is true (Das and Saha, 2015).

6.5 MOLECULAR MARKERS

6.5 Molecular Markers

Oxygen sensing molecular markers were assessed by estimating serum VEGF, NOS3 (eNOS), and nitric oxide concentrations. Hypoxia triggers transcription of more than a hundred genes via hypoxia inducible factor (HIF) particularly HIF-1α which regulates both VEGF and NOS3 (Ke and Costa, 2006). In many studies, it has been reported that nickel or any other heavy metals create cellular hypoxia by binding to the heme portion of oxygen sensing molecules (Detmar et al., 1997). Along with VEGF, HIF also activates various angiogenic factors like placental growth factor (PGF), platelet derived growth factor B (PDGFB), angiopoietins (Liu et al., 1995; Semenza, 2003). Similarly we found increased VEGF production in chronic hypoxia (CH) as well as $NiSO_4$ exposed rats which indicates exposure to nickel induces hypoxia like stress in the cells.

VEGF causes angiogenesis and increases NO production by stimulating the NOS3 gene to maintain adequate blood flow in the tissues (Takahashi and Shibuya, 2005). In the present study, we observed an increase in serum, cardiac tissue NO concentrations in chronic hypoxia or nickel exposed rats may be due to the over expression of inducible nitric oxide synthase (i-NOS/NOS 2) (Aktan, 2004). Despite of increased NO concentration we have recorded high mean arterial pressure values in CH, $NiSO_4$ and in CH + $NiSO_4$ groups. The increased MAP values might be because of increased nitrosative stress or nickel induced alteration of vascular smooth muscle pathophysiology. Increased levels of nitric oxide can react with the superoxide anion (O_2^-) and form peroxynitrite anion ($ONOO^-$) (Surh et al., 2009). Peroxynitrite is a potent oxidant and may trigger lipid peroxidation, inhibit mitochondrial electron transport and oxidize thiol compounds (Das Gupta et al., 2007). i-NOS gene expression reported being increased in conditions like cellular hypoxia, oxidative stress and pro-inflammatory conditions (Pautz et al., 2010). Hypoxia or nickel mediated activation of the transcription factors NF-κB and STAT-1α reported being an essential step

for the i-NOS induction in most of the cells (Forstermann and Sessa, 2012). Supplementation of L-ascorbicacid may decrease the NO production by blocking the NF-κB pathway in hypoxia or nickel exposed rats.

The present study indicated that hypoxia or nickel induces the increase of VEGF, potent oxygen sensing growth factor upregulates in cellular hypoxia, vascular damage, triggers hypoxia cell signalling and angiogenesis pathways to protect tissue from further damage (Das et al., 2019). It has been reported that nickel depletes the intra cellular ascorbate levels and leads to the stabilization of HIF -1 alpha and 2-alpha and induce cellular hypoxic stress (Salnikow and Kasprzak, 2005). Supplementation of L-ascorbic acid in hypoxia or nickel exposed rats decreased VEGF concentration and oxygen free radicals lead to decrease cellular damage and protect heme containing oxygen sensing biomolecules in physiological systems including the cardiovascular or pulmonary systems.

6.6 CARDIOVASCULAR & PULMONARY REMODELING

6.6 Cardiovascular Remodelling

6.6.1 Cardiosomatic Index

We observed thatanimals exposed to CH, $NiSO_4$ and CH + $NiSO_4$ shown increased cardiac mass. This increased cardiac mass could be due to cardiac hypertrophy in response to increased mean arterial pressure (supported by histopathology reports and MAP values) or may be direct effect of deprived oxygen causing cardiac hypertrophy. Fan *et al* in their experiment on mice demonstrated that hypoxia upregulates protein synthesis in cardiac tissue contributing to cardiac hypertrophy (Fan et al., 2011). But simultaneous supplementation of L-ascorbic acid in $NiSO_4$ and L-ascorbic acid + CH administered groups showed statistically insignificant reduction in cardiac mass. So further study may be required to know the beneficial effect of long-term supplementation or pharmacological intravenous administration of L-ascorbic acid in nickel or CH induced decrease of % body weight gain and cardiac hypertrophic changes.

6.6.2 Histopathological Examination of the Ventricles and Intramyocardial Coronary Artery:

Histopathological studies of cardiac tissues along with coronary artery and aorta show cardiovascular remodelling due to chronic hypoxia or nickel exposures. The results also indicate a possible association between hypoxia or nickel and vascular architecture by the development of hypertrophy and hyperplasia in vessels, expansion of the endothelial and adventitial layers along with increased arterial wall thickness (Doggrell, 1998; Maillet et al., 2013). Low oxygen microenvironment or nickel induced oxidative, nitrosative stress and concomitant decrease of antioxidant defensive agents are responsible for changes in cardiovascular pathophysiology and affect structural and functional homeostasis. But supplementation of L-ascorbic acid mediated improvements of both heart and aortic tissue

architectures in nickel treated rats may be because of decreased oxidative, nitrosative stress and increased antioxidant agents (Das et al., 2001).

In the present study we found that CH and $NiSO_4$ exposures leads to cardiovascular remodelling and leads to malfunctioning of cardiovascular system. High normalised wall index (NWI) values of coronary artery in CH and $NiSO_4$ exposed rats clearly depicts the changes in arterial architecture by low oxygen environment.It has been reported that NWI can be useful tool to assess early athersclerotic changes in the small blood vessels (Saam et al., 2009). Increased NWI was due to decreased arterial lumen size and increase in arterial wall thickness in hypoxic or metal induced pathologic assaults.It has been reported that hypoxia and differential PO_2 grades, decreased oxygen diffusions within the arterial wall lead to increase wall thickness (Crawford and Blankenhorn, 1991). Further many studies have reported that increased ROS production and decreased antioxidants in the vascular tissues leads to lipid peroxidation and atherogenic changes in small arteries. Zhao et al (2013) reported that increased vascular smooth muscle cell (VSMC) proliferation, upregulation of fibrinogenic growth factors, endothelin I and increased collagen deposition and cross linking leads to increased NWI.In a study it has been shown that vitamin C inhibit collar induced intimal thickening in experimental rabbits by decreasing the expression of MMP2 (Arun et al., 2015). Patil et al (2019) showed the protective effect of amla extract on high fat diet induced atherosclerotic changes in the valscular system. Similarly, our study also shown improvemnts in NWI values in L-ascorbic acid supplemented groups (L-ascorbic acid + CH and L-ascorbic acid + $NiSO_4$).

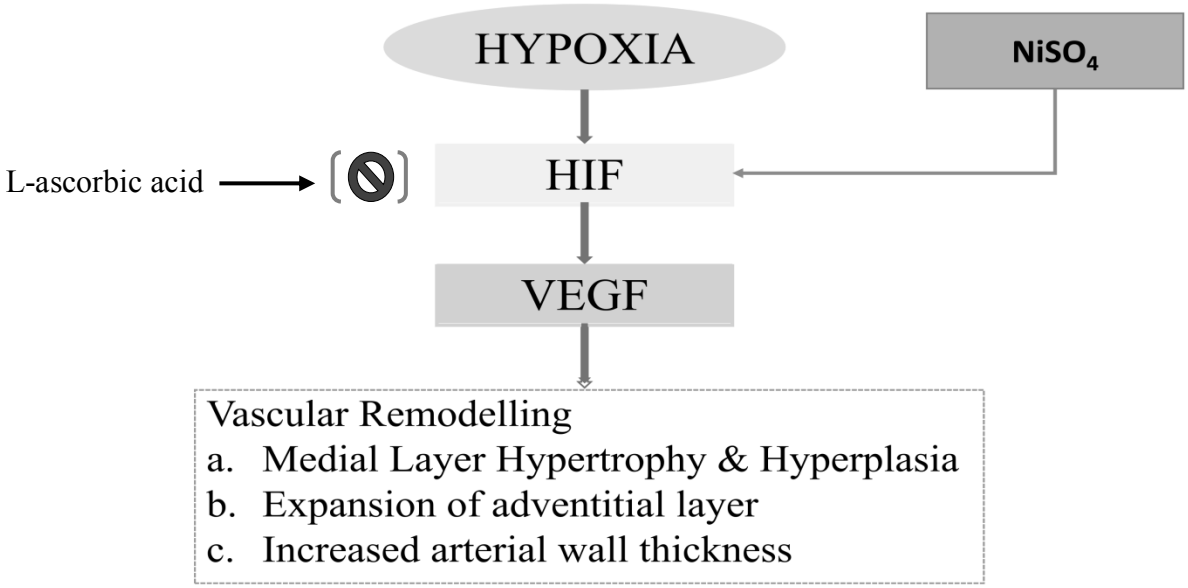


Figure 6.6.2 Possible mechanism of hypoxia or nickel induced Cardiovascular remodeling

6.6.3 Pulmonary Remodeling:

Lungs play an important role in gaseous exchange (O_2 and CO_2) between the biological system and the external environment. In accordance with our results other research findings also shown increased pulmonosomatic index in rats and mice exposed to hypoxia (Hunter et al., 1974). In lung tissue homogenate of chronic hypoxia (CH), $NiSO_4$ and CH + $NiSO_4$ groups shown oxidant-antioxidant imbalance by increased MDA, NO and decreased ascorbic acid concentration. Further, histopathological observations clearly shows the lung damage by CH and $NiSO_4$ exposures. Lungs of CH, $NiSO_4$ (Figure 5.6.7a) and CH + $NiSO_4$ (Figure 5.6.7b) exposed rats shown hyalinisation of respiratory bronchioles, eosinophilic oedema fluid filled alveolar spaces. We have also observed mixed inflammatory responses, like leucocytic infiltration, oedema, congestion and haemorrhage in interstetial septa. It has been shown that L-ascorbic acid protects lungs from oxidative and nitrosative stress and

improves the histopathological alterations caused by nickel (Hattiwale et al., 2013). In groups supplemented with L-ascorbic acid (L-ascorbic acid + CH and L-ascorbic acid + $NiSO_4$) (Figure 5.6.7b) has shown some improvements in the pathological alterations caused by CH and $NiSO_4$.

CHAPTER 7

SUMMARY and CONCLUSION

7.1 SUMMARY

All multi cellular life is based on the utilization of O_2 for the generation of high energy compounds, and its consumption depends on the mass and metabolic activity of organisms. Decreased oxygen availability or its utilization by the cells causes hypoxia; it may be due to decreased PO_2 in the atmosphere, lung disorders, abnormal hemoglobin, anemia, ischemia, carbon monoxide poisoning, cyanide poisoning and heavy metal poisoning etc.Conservation of oxygen homeostasis is important for cell growth and survival. Amongst environmental heavy metal pollutants, a divalent cation nickel (Ni) is most widely spread environmental contaminant because of its various industrial applications like electroplating of metals, stainless steel and battery manufacturing, as a catalyst, electrical and electronic industries, metallurgy and metal alloys preparations.

In the present research work, we studied the influence of antioxidant vitamin L-ascorbic acid on the mechanisms of cardiovascular cell signaling pathways by chronic hypoxia and heavy metal nickel highlighting on the involvement of the hypoxia signalling pathway by metal-induced generation of reactive oxygen species and oxidative stress generation in cardiovascular remodeling and autonomic functions of heart.

In the present study we included forty-two adult male Albino Wistar rats (Rattus Norvegicus) and randomly allocated into seven groups and named groups as control; L-ascorbic acid (50 mg/ 100 g b.wt, oral); chronic hypoxia (CH) (10% O_2, 90% N_2); $NiSO_4$ (2.0 mg/ 100 g b.wt, i.p, every alternate day); CH + $NiSO_4$; L-ascorbic acid + CH and L-ascorbic acid + $NiSO_4$ and respective interventions were given for 21 days.

Before and after the intervention period weights of all the experimental animals were recorded and % body weight gain was calculated. Electrophysiological parameters like ECG, blood pressure and pneumogram of animals were recorded in anaesthetized/conscious animals after the intervention period. Cardiovascular autonomic function was assessed by

HRV analysis. Oxidative stress and antioxidant defence were assessed by estimating MDA, ascorbic acid, α-tocopherol in the serum and MDA in heart, and lung tissue homogenate by spectrophotometric methods. Oxygen sensing molecular markers like VEGF, NOS3 and NO were estimated in the serum. Cardiovascular remodeling was studied by assessing cardiosomatic index, and histopathological examination of H and E stained sections of the ventricles, intramyocardial coronary artery, and elastic artery. Further, to know the vascular remodeling normalized wall index (NWI) of coronary artery was calculated. In addition to these histopathological examination of the lung was also done.

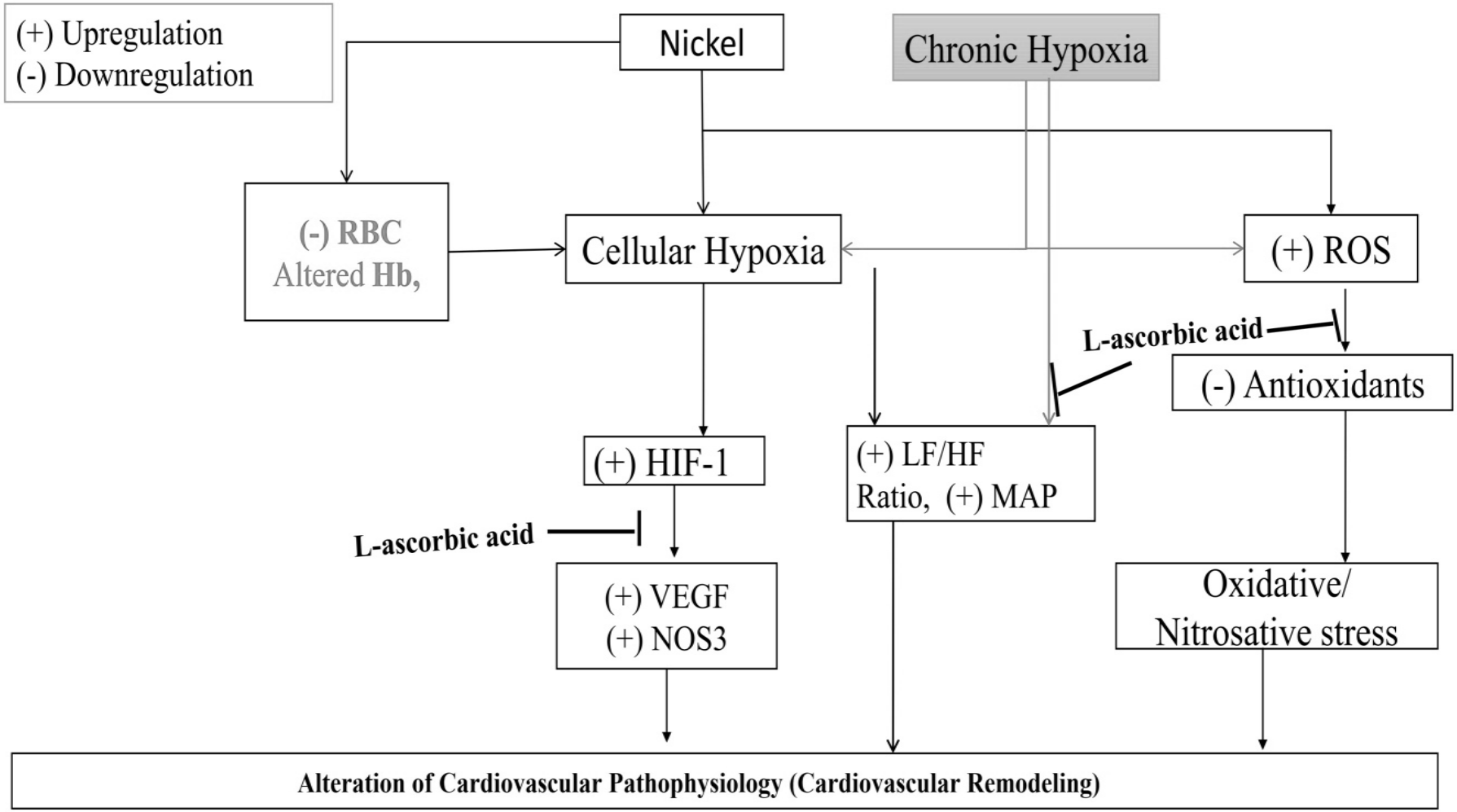
Results suggest that chronic hypoxia (CH), $NiSO_4$ and CH + $NiSO_4$ impairs overall gravimetry of experimental animals, cardiac autonomic functions, vascular functions, induces oxidative and nitrosative stress, upregulates oxygen sensing molecular markers like VEGF and NOS3 proteins. Further, chronic hypoxia and heavy metal nickel toxicity have adverse impact on histopathology of lung, cardiac and aortic tissues.Additionally, increased NWI values supports the cardiovascular remodelling. All these results indicate a possible link between nickel toxicity and hypoxia in sympathetic over activity, oxidative stress, leading to pathological cardiovascular remodeling.

Antioxidant vitamin L-ascorbic acid supplementation was able to 1) Reduce sympathetic overactivity 2) reduce MAP 3) decrease oxidative stress 4) decreases nitrosative stress and 5) ameliorate cardiovascular remodelling resulting from chronic hypoxia and $NiSO_4$ exposure. These effects of L-ascorbic acid could be attributed to its antioxidant property. Thus, the results of the present study suggest a possible use of antioxidant vitamin L-ascorbic acid supplementation as an add-on therapy against hypoxia or nickel toxicity-related pathophysiology.

7.2 CONCLUSION:

Present study shows that CH or nickel induce hypoxia cell signaling mechanisms through high production of reactive oxygen species and deprivation of antioxidant vitamins like L-ascorbic acid and alpha tocopherol, sympathetic over activity, cardiovascular, and pulmonary remodelling and hypertension. Results also indicate that heavy metal nickel induce cardiovascular pathophysiology is sensitive to oxygen hence alteration of cardiovascular anatomy and physiological function in the experimental animal by nickel or chronic hypoxia derive similar outcome. The results obtained in this study may have clinical value in humans and the effect of L-ascorbic acid on CH or nickel induced cardiovascular toxicities deserves further exploration.

7.3 GRAPHICAL ABSTRACT



Limitations and Future Perspectives of the Study

- Nickel induced gene mutations and DNA sequence studies needs to be done to know the types of mutations caused by nickel toxicity.
- Insilico, in-vitro and in-vivo epigenetic and proteomic studies also needed to know the deeper aspects of common hypoxia signalling pathways induced by chronic hypoxia or nickel exposure.

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ANNEXURES

ANNEXURE I

PLAGIARISM VERIFICATION CERTIFICATE

BLDE(DEEMEDTOBEUNIVERSITY)

PLAGIARISM VERIFICATION CERTIFICATE

1. **Name of the Student:** R Chandramouli Reddy **RegNo:** 15PHD003

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ANNEXURE III

PRESENTATIONS AND AWARDS

Presentations

1. "Alteration of Cardiovascular Pathophysiology of Nickel treated Rats with L-Ascorbic acid supplementation" at Association of Clinical Biochemist of India - South Zone Conference, from December 6th – 8th 2018, organized by Department of Biochemistry, Kasturba Medical College, Manipal, Karnataka, India.
   ----- Received **Best Poster Award.**

2. "Heart Rate Variability and Baroreceptor Sensitivity on Experimental Chronic Sustained Hypoxia Exposed Rats" at ASSOPICON-2016 during 15th -17th September, 2016, organized by Department of Physiology, Shri B. M. Patil Medical College, Hospital & Resaerch Centre, Vijayapur, Karnataka, India.

3. "Alteration of Cardiovascular Pathophysiology of Nickel treated Rats Supplemented with L-Ascorbic acid" on Research Day, 6th June 2018, organized by BLDE (Deemed to be University), Vijayapur, Karnataka.
   --------Received **'Consolation Prize'**

ANNEXURE IV

PUBLICATIONS

1. Das S, **Reddy RC**, Chadchan KS, Patil AJ, Biradar MS, Das KK. Nickel and Oxidative stress: Cell Signaling Mechanisms and Protective Role of Vitamin C. *Endocrine, Metabolic & Immune Disorders - Drug Targets*. 2019. *19*. https://doi.org/10.2174/1871530319666191205122249. [Epub ahead of print]**. (IF-1.104).**
2. **Reddy RC**, Devarnavadagi B, Yendigeri SM, Bagali S, Kulkarni RV, Das KK. Effect of L-Ascorbic acid on nickel-induced alteration of cardiovascular pathophysiology in Wistar rats. Biol Trace Elem Res. 2019. doi: 10.1007/s12011-019-01829-w. [Epub ahead of print]. **(IF-2.43).**
3. **Reddy RC**, Devaranavadagi B, Das KK. Nickel Induced Alteration of Pathophysiology of Lungs in Experimental Rats. *Indian Journal of Public Health Research & Development*. 2019. *10*(8), 145. https://doi.org/10.5958/0976-5506.2019.01867.9. (Scopus)
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Book Chapter

1. Das KK, Honnutagi R, Mullur L, Reddy RC, Das S, Majid, Dhundasi S, Biradar MS. Heavy Metals and Low-Oxygen Microenvironment—Its Impact on Liver Metabolism and Dietary Supplementation. *Dietary Interventions in Liver Disease,* **2019**, 315-332.

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REVIEW ARTICLE

Nickel and Oxidative Stress: Cell Signaling Mechanisms and Protective Role of Vitamin C

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Abstract: ***Background:*** Nickel activates the signaling pathways through the oxygen sensing mechanism and the signaling cascades that control hypoxia-inducible transcriptional gene expressions through oxidative stress. This review emphasizes on the recent updates of nickel toxicities on oxidant and antioxidant balance, molecular interaction of nickel and its signal transduction through low oxygen microenvironment in the *in-vivo* physiological system.

Discussion: **Nickel and oxidative stress:** Nickel alters intracellular chemical microenvironment by increasing ionized calcium concentration, lipid peroxidation, cyclooxygenase, constitutive nitric oxide synthase, leukotriene B4, prostaglandin E2, interleukins, tumor necrosis factor-α, caspases, complement activation, heat shock protein 70 kDa and hypoxia-inducible factor-1α. The oxidative stress induced by nickel is responsible for the progression of metastasis. It has been observed that nickel exposure induces the generation of reactive oxygen species which leads to the increased expression of p53, NF-kβ, AP-1, and MAPK. Ascorbic acid (vitamin C) prevents lipid peroxidation, oxidation of low-density lipoproteins and advanced oxidation protein products. The mechanism involves that vitamin C is capable of reducing ferric iron to ferrous iron in the duodenum, thus the availability of divalent ferrous ion increases which competes with nickel (a divalent cation itself) and reduces its intestinal absorption and nickel toxicities.

Conclusion: Reports suggested the capability of ascorbic acid as a regulatory factor to influence gene expression, apoptosis and other cellular functions of the living system exposed to heavy metals, including nickel.

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1. INTRODUCTION

Nickel is a naturally occurring element and its concentration mainly depends on the geographical location and anthropogenic input [1]. Nickel, the 28th element in the periodic table, was first purified by Swedish chemist Axel Cronstedt in the year 1751 [2]. Nickel (Ni) is a silvery-white lustrous metal with a slight golden tinge. It belongs to the group of transition metals in the periodic table, which is hard and ductile. Because of its electromagnetic properties and chemico-thermal stabilities, nickel ferrite nanoparticles are widely used in many medical applications like magnetic resonance imaging (MRI), target-based drug delivery and hyperthermia [3].

About 9% Ni produced is used in Ni plating for the corrosion resistance and is widely used in making coins. Nearly about 2 million tonnes of nickel is produced every year around the world [4]. Nickel as a compound finds a lot of applications such as a catalyst for hydrogenation, cathodes for batteries, pigments manufacturing and metal surface treatments. Nickel is an essential trace element for many microbes, plants and animals that have enzymes like glyoxalase I, acireductonedioxygenase etc. which require nickel for their active functioning. The most common form of nickel is Ni^{2+}but compounds with Ni^{0}, Ni^{+} and Ni^{3+} are also well known. However, the rare oxidation states Ni^{2-}, Ni^{-} and Ni^{4-} have also been produced and studied [5].

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It should be noted that the nickel exposure pathway is not only the environmental medium or any route of exposure but also all the elements that link a contaminant source and a receptor population. It is also important to note that the measure of exposure is proportional to variables such as intensity, and frequency and duration of contact with the contaminant is crucial. Ni salts are considered as an important industrial hazard [4].

The nickel toxicity has become a great interest because of its widespread environmental occurrence and the incidence of accidental poisoning in industrial workers where nickel is used as a major raw material [6]. The toxicity of nickel depends on the route of exposure and solubility of nickel compounds. Pulmonary absorption is the major route for nickel toxicity than the gastrointestinal and dermal absorption. Diet is the most significant nickel exposure pathway for some organisms [7]. The absorption of nickel takes place due to soluble Ni^{2+} ions which are either in the form of particulate or as a coordination complex [8]. In detail, Das *et al.* reported the different routes of human exposures, and the pharmacokinetics of nickel in the previous reviews and book chapters [9, 10].

International Agency for Research on Cancer (IARC) informed that there are sufficient reports and research evidence on humans and animals for the carcinogenicity of nickel. According to the US Agency for Toxic Substances and Disease Registry (ATSDR, 2005), approximately 10-20% of the general population is sensitive to nickel [11]. The normal reference values for serum nickel for healthy adults are 0.2μg/L, and in urine, it is 1-3 μg/L [11].

Maintaining a healthy biological system mainly depends on the oxidant and antioxidant balance. At normal physiological conditions, there will be a continuous generation of reactive oxygen species (ROS) and will be counteracted by endogenous antioxidants like superoxide dismutase (SOD), glutathione peroxidase, catalase (CAT) etc. or by exogenous (vitamin C, vitamin E etc.) antioxidant systems [12]. Many *in vivo* and *in vitro* studies demonstrated that environmental heavy metal pollutants like nickel, chromium, vanadium, arsenic, cobalt etc. show their toxicity by stimulating the high production of ROS, decreasing the antioxidants availability, inflammatory pathway and apoptosis pathway [13, 14].

The purpose of the current review is to provide deep insights into nickel mediated oxidative stress mechanisms, altered cell signaling pathways, DNA damage and the protective actions of ascorbic acid on nickel-induced toxicity.

2. NICKEL TOXICITY AND IT'S PUBLIC HEALTH CONSEQUENCES

A major amount of nickel enters our body via food and water intake, but inhalation exposure is most common in occupational workers. It has been reported that acute inhalation exposure of nickel in humans shows symptoms like headache, nausea, respiratory problems, and death [15, 16]. Warner *et al*., in 1979 reported that there is no clinical evidence of developmental and reproductive toxicity in the women working in nickel refinery, but Chashschin *et al.,* reported reproductive and developmental effects of nickel in occupationally exposed populations (0.13-0.2 mg nickel/m^3) [17, 18]. Goyer *et al.,* reported that acute inhalation of nickel carbonyl leads to headache, nausea, vomiting, chest pain, hyperpnea, cyanosis, respiratory failure, and ultimately death if the exposure is severe [19]. In the year 1994, Nicklin and Nielsen categorized asthmatic attack response caused by nickel inhalation as i) a rapid onset attack (antibody-mediated Type I hypersensitivity) with bronchospasm, ii) a late response reaction at 6-12 hours after exposure (antigen-antibody immune complex-mediated inflammatory reaction), and iii) a mixed or combined response [20]. A study reported the increased blood reticulocytes in workers who drank water from a water fountain contaminated with nickel sulfate, nickel chloride, and boric acid (estimated dose of 7.1–35.7 mg Ni/kg) [11]. Huang *et al.,* conducted a survey of 50 peritoneal dialysis (PD) patients and 50 normal patients for urinary nickel concentration and analyzed the possible association of urinary nickel concentrations with clinical outcomes and inflammatory biomarkers. Based on their study, it was found that nearly 50% of the patients undergoing PD had higher levels of urinary nickel and also these patients had increased serum levels of high sensitivity C-reactive protein [21]. In another research, Jouybari *et al.,* studied the role of toxic elements as biomarkers for breast cancer (BC), which showed a significant difference in the cadmium (Cd) and Ni statuses between healthy and BC patients, clearly indicating a direct and positive association between Cd and Ni concentrations and BC risk [22]. Also, increased urinary total protein levels, β2-macroglobulin, retinal binding protein, and N-acetyl-β-D-glucosaminidase were reported in electroplating workers who consumed nickel contaminated water [23].

3. NICKEL TOXICITY

3.1. Nickel Generates ROS

In authors' laboratory, by experimenting on animals, it has been reported that nickel induces oxidative stress accompanied by the overproduction of reactive oxygen species, lipid peroxides (malondialdehyde) and decreasing concentrations of endogenous antioxidants like SOD, CAT, glutathione (GSH), glutathione peroxidase, glutathione reductase and exogenous antioxidants like l-ascorbic acid and alpha-tocopherol [24-27]. The molecular mechanism of nickel toxicity and carcinogenicity may be due to the overproduction of ROS [28]. Chen *et al.* carried out an experiment to assess the effect of nickel chloride on isolated human lymphocytes and showed that nickel toxicity led to cell membrane oxidative damage by a significant increase in the production of ROS in lymphocytes [29]. Das *et al.,* showed that subchronic exposure of experimental rats to nickel sulphate ($NiSO_4$) led to increased production of ROS, lipid peroxidation and decreased antioxidants [27]. Topal *et al.,* assessed the effect of nickel on nuclear factor kappa-light chain-enhancer of activated B cells (NF-kβ) activity, antioxidative response and histopathological effects on the liver, gill and

kidney tissues of Rainbow trout fish exposed to different concentration of nickel chloride ($NiCl_2$) (1 mg/L and 2 mg/L) for 21 days. They found a significant increase in the lipid peroxidation by promoting peroxidative damage and a resultant increase in antioxidant enzymes SOD and CAT activity [30]. Nickel toxicity may disturb the biochemical and physiological functions of fish by causing changes in NF-kβ activity and oxidative and histopathological damage in the tissues of rainbow trout [30].

3.2. Nickel Influences Cytokines Signaling

Nickel and other heavy metals are absorbed in the intestine, transported by metal transporter protein 1 and then enter into the portal circulation. By the circulatory system, nickel is distributed to all the tissues and organs of the body [31]. It has been reported that the accumulation of heavy metals and increased oxidative stress in hepatocytes stimulate the production kupffer cells and lead to cell necrosis. Many studies have reported that heavy metal poisoning induces an inflammatory pathway via tumor necrosis factor α (TNF)-α signaling pathway [31-33]. It has been shown that Ni^{2+} induces the expression of inflammatory genes such as IL-8 in human primary monocytes [34]. In an experiment conducted to study the expression of interleukin (IL)-8 in human monocytes THP-1 cell, it has been confirmed that $NiCl_2$ induces the expression of IL-8. The use of PX-478 and TAK-242 clearly indicated the expression of IL-8 which is mainly dependent on hypoxia-inducible factor 1 (HIF-1)-α activation [34].

TNF-α is a cell-signaling protein that is involved in systemic inflammation. The primary role of TNF-α is in the regulation of immune cells. Dys-regulation of TNF is associated with many human diseases which include Alzheimer's disease [35], cancer [32], major depression [36] and psoriasis [37]. Ding *et al.,* demonstrated that nickel exposure induces the production of inflammatory cytokine TNF-α and the trans-activation of nuclear factor of activated T cells, NF-kβ and activator protein 1 (AP)-1 in human bronchial epithelial cells (BEAS-2B). Further studies have shown that TNF-α was induced by the exposure of nickel in BEAS-2B cells specifically through extracellular signal-regulated kinases/AP-1 dependent pathway [38].

4. NICKEL AND CELL SIGNALING

4.1. Nickel Induces Expression of COX

Cyclooxygenases (COX) are also called as prostaglandin-endoperoxide synthases (PTGSs). There are two isoforms of cyclooxygenases present in mammals: COX-1 (PTGS1), which is typically expressed in all types of cells at low levels and maintains homeostasis; and COX-2 (PTGS2), is an early expressed gene in response to the wide variety of cell challenges and stress inducers [39]. Both the isoforms catalyze the synthesis of prostaglandins, thromboxanes, and levuloglandins [40]. These COX enzymes are inhibited by aspirin (acetyl salycyclic) and other non-steroidal anti-inflammatory drugs [40]. For decades, COX inhibitors have been in use as anti-inflammatory and anti-pyretic agents and also in the treatment of neurodegenerative disorders and cancer [40]. Ding *et al.,* 2006 reported that nickel exposure up-regulates the expression of COX-2 by the ROS/NF-κB/IKK β/p65-dependent pathway and plays a crucial role in antagonizing nickel-induced cell apoptosis in human bronchial epithelial Beas-2B cells [41]. Many types of medical alloys contain nickel. The elution of Ni ions from these metals causes toxicity and inflation. In a study carried out by Sato *et al.,* it was found that Ni ions eluted from subcutaneous implanted Ni wire increased the expression of COX-2 and mPGES-1 mRNA [42]. The induction of COX-2 and mPEGS-1 mRNA is due to the concentration of Ni ions in the region, but not due to the physiological stimulation of the implemented wire. It has been proved that Ni induced expression of COX-2 is observed not only in mice cells but also in the human cell lines [42]. It has been demonstrated that c-Jun/AP1 downstream pathways of JNK1 play a crucial role in nickel-induced COX-2 expression and carcinogenesis [43].

4.2. Nickel Influences Oxygen Sensing Cell Signaling

There are multiple explanations for the nickel-induced "oxygen sensor" activation. Earlier research findings showed that intravenous injection of $NiSO_4$ in rats leads to the increased erythrocytosis and development of local tumors [44-46]. Goldberg *et al.,* in 1988 showed enhanced expression of erythropoietin (EPO) mRNA in human hepatoma cells (Hep3B or HepG2) by exposing cells to hypoxic conditions, Ni (II) or Co (II) [47]. Physiologically, up-regulation of EPO mRNA expression is seen when there is diminished oxygen supply to the kidneys. Semenza *et al.,* showed that EPO production and transcription mainly occurs in the control of hypoxia-inducible factor 1 (HIF-1) [48]. Nickel-induced "oxygen sensor" hypothesis infers that less redox-active Ni (II) may impede with high redox-active iron (Fe) (II) metabolism. Fe (II) is an important cofactor for enzymes like dioxygenases and hydroxylases. Hydroxylation of HIF by prolyl hydroxylases (PHD)1-3 is a crucial step for the degradation of HIF which is requires O_2 and ascorbic acid [49]. It has been reported that transition heavy metals like nickel, cobalt etc., induce cellular hypoxia by diminishing heme synthesis which further leads to a decrease in intracellular oxygen tension and inhibits PHD_2 [50]. HIF-1α is considered as the transcriptional regulator which regulates the cellular and developmental response to hypoxia [51]. HIF-1α is a subunit of the heterodimeric transcription factor, Hypoxia-inducible factor 1 that is encoded by the HIF1A gene [48, 49]. The deregulation or overexpression of HIF-1α either by hypoxia or genetic alterations has been associated with cancer and other pathophysiologies of vascularization and angiogenesis, energy metabolism, cell survival and tumor invasion. Under normal oxygen conditions, the HIF 1A gene is expressed in low levels, but in hypoxic conditions, HIF 1A transcription is significantly up-regulated [52, 53]. Some research works have revealed that exposure to nickel causes accumulation of HIF-1α in several cells and trigger HIF-1α to regulate hypoxia-mimic responses which induce overexpression of microRNA-210 (miR-210) which is most sensi-

tive hypoxic mRNA and is ideal for the regulation of hypoxia [53-55]. Viemann *et al.* reported that the contact allergen nickel induced the production of IL-6 by activating HIF-1α in addition to NF-κβ [56].

4.3. Nickel and Calcium Channels

Wani *et al.,* showed that nickel induced aortic hyper contraction by the overproduction of ROS in endothelial cells, increased the release of endothelial hyper contractile prostanoids through COX-2 pathway, and increased the influx of Ca^{2+} through T-type Ca^{2+} channels to smooth muscle cells [57]. They also concluded that acute exposure to nickel increased vascular resistance, which consequently led to the commencement and continuation of hypertension on rats [57].

An opening of calcium channels and Ca^{2+} ions influx into the cells plays an important role in the regulation of blood glucose levels by controlling the secretion of insulin by pancreatic beta cells [58]. It has been shown that although $NiSO_4$ blocks the calcium channel and prevents the release of insulin in the bloodstream which leads to increase in the blood glucose level, but it has been observed that nickel induces low oxygen-sensitive expression of vascular endothelial growth factor (VEGF) protein that mainly depends on intracellular calcium store release, not on extracellular calcium influx. Consistent with these reports, it has been found that intracellular calcium is essential for VEGF induction by nickel compounds irrespective of calcium channel functions [59-61].

5. ANTIOXIDANT VITAMIN C

Vitamin C, also known as ascorbic acid and L-ascorbic acid is a vitamin found in various foods and also available as a dietary supplement. Vitamin C is found to be the most effective antioxidant in humans. Vitamin C functions physiologically as an antioxidant in the aqueous fluid and tissue compartments. As an antioxidant, vitamin l-ascorbic acid has its own significance for years, but now ascorbic acid is also acknowledged to play an important role in hydroxylation reactions of proteins that are involved in many important cell-signaling pathways and in controlling the interactions and functions of many cellular proteins [49]. A co-factor α-ketoglutarate (2OG) dependent non-heme iron-containing dioxygenase enzymes like lysyl, prolyl and asparginyl hydroxylases, and DNA repair enzymes, human ABH2 and ABH3 proteins play an important role in hydroxylation reactions [5, 6]. Iron and 2OG are two important factors required for the dioxygenase enzymes activity and ascorbic acid plays a crucial role in maintaining the iron in Fe (II) active form [62, 63]. It has been shown that vitamin C helps in the generation of other antioxidants within the body, including alpha-tocopherol i.e., Vitamin E [64]. It is specifically required in the activity of various human enzymes involved in the synthesis of collagen, hormones and amino acids [46, 47].

Studies are being conducted to examine the ameliorating effect of vitamin C in preventing or delaying the development of cancers, cardiovascular diseases and other diseases in which oxidative stress plays a crucial role. Frei *et al.,* demonstrated the role of ascorbate in the protection of plasma lipids against peroxidative damage caused by peroxyl radicals [65]. Their experimental results suggested that ascorbate is the most effective antioxidant in human blood plasma which is of major importance in the protection against diseases and degenerative processes caused by oxidative stress [65].

6. NICKEL-INDUCED OXIDATIVE STRESS AND PROTECTIVE ACTION OF VITAMIN C

Many researchers have shown that for anti-oxidant therapy, vitamin C is an ideal compound because it is an endogenous agent and can be given orally and parenterally with similar efficiency [66]. Das *et al.,* conducted a study to find out the effect of ascorbic acid supplementation on nickel-induced lipid peroxidation in the liver of Wistar rats and found that ascorbic acid supplementation showed a remarkable improvement of lipid peroxide, glutathione, SOD, CAT and GSH-Px status in the liver in comparison with rats treated with nickel alone [27]. This shows that ascorbic acid possesses relative protection against nickel hepatotoxicity [27]. In another experiment conducted to study the role of ascorbic acid on nickel-induced hepatic nucleic acid concentration in rats, the effect of oral treatment of ascorbic acid (50 mg/100 g.b.wt.) on $NiSO_4$ induced (2.0 mg/100 g.b.wt.) alteration of nucleic acids. Moreover, the total protein concentration in the liver of rats was also studied. Nucleic acids and total protein concentration significantly decreased in the nickel treated rats when compared to untreated rats. Furthermore, on simultaneous supplementation of ascorbic acid with $NiSO_4$, a remarkable improvement of nucleic acids and total protein concentrations in the liver was observed. These results indicate that nickel influences the expression of genetic information by reducing hepatic DNA, RNA and protein concentration in animals and also the supplementation of ascorbic acid is beneficial against nickel-induced toxicity [67]. Functions of L-ascorbic acid as a protective antioxidant against nickel-induced toxicity aretabulated below (Table **1**).

CONCLUSION

It may be postulated from a series of experiments carried out by the corresponding author of this review and other researchers that nickel causes serious cellular damages by increased ROS production, deprivation of antioxidants availability and by triggering inflammatory pathways (Fig. **1**) [74].

As stated in this review, nickel induces cellular hypoxia which leads to expression and stabilization of HIF-1α followed by the generation of ROS. Overexpression of HIF-1α alters the cell signaling mechanism by changing oxygen sensing gene expressions, which lead to cellular damage. It was further reported that dietary supplements such as vitamin C is beneficial in suppressing the metal or hypoxia-induced oxygen sensing gene expressions [31].

Table 1. Antioxidant vitamin C against nickel-induced oxidative stress.

S. No	Tissue/organ/System Affected	Antioxidant Used	Result	Citation
1	Pulmonary nitrosative stress	l-ascorbic acid	Pulmonary nitrosative stress decreased through NOS3.	Hattiwale *et al*., 2013 [68]
2	Brain tissue	l-ascorbic acid	Lipid peroxide, nitric oxide levels decreased and antioxidant enzymes status restored in serum and brain tissue samples.	Das *et al.,* 2010[69]
3	Regulation of blood glucose	l-ascorbic acid	Blood glucose homeostasis improved.	Tikare *et al.,* 2008 [60]
4	Testicular lipid peroxide and antioxidants	l-ascorbic acid	Testicular lipid peroxides decreased, GSH and antioxidant enzyme activities restored.	Gupta *et al*., 2007 [70]
5	Erythrocyte MDA and antioxidants	l-ascorbic acid	Improved the status of Erythrocyte MDA and all the endogenous erythrocyte antioxidant defence system restored.	Das *et al.,* 2007 [71]
6	Lung antioxidant defence system	l-ascorbic acid	Lung tissue lipid peroxides decreased and antioxidant enzyme concentrations restored.	Gupta *et al.,* 2006 [72]
7	Serum dyslipidemia and pathological changes of Hepatic Tissue	l-ascorbic acid	Dyslipidemia corrected and histopathological alterations corrected.	Das *et al.,* 2006 [73]
8	Hepatic lipid peroxidation	l-ascorbic acid	Hepatic tissue lipid peroxides decreased, GSH and antioxidant enzyme activities improved.	Das *et al.,* 2001 [27]

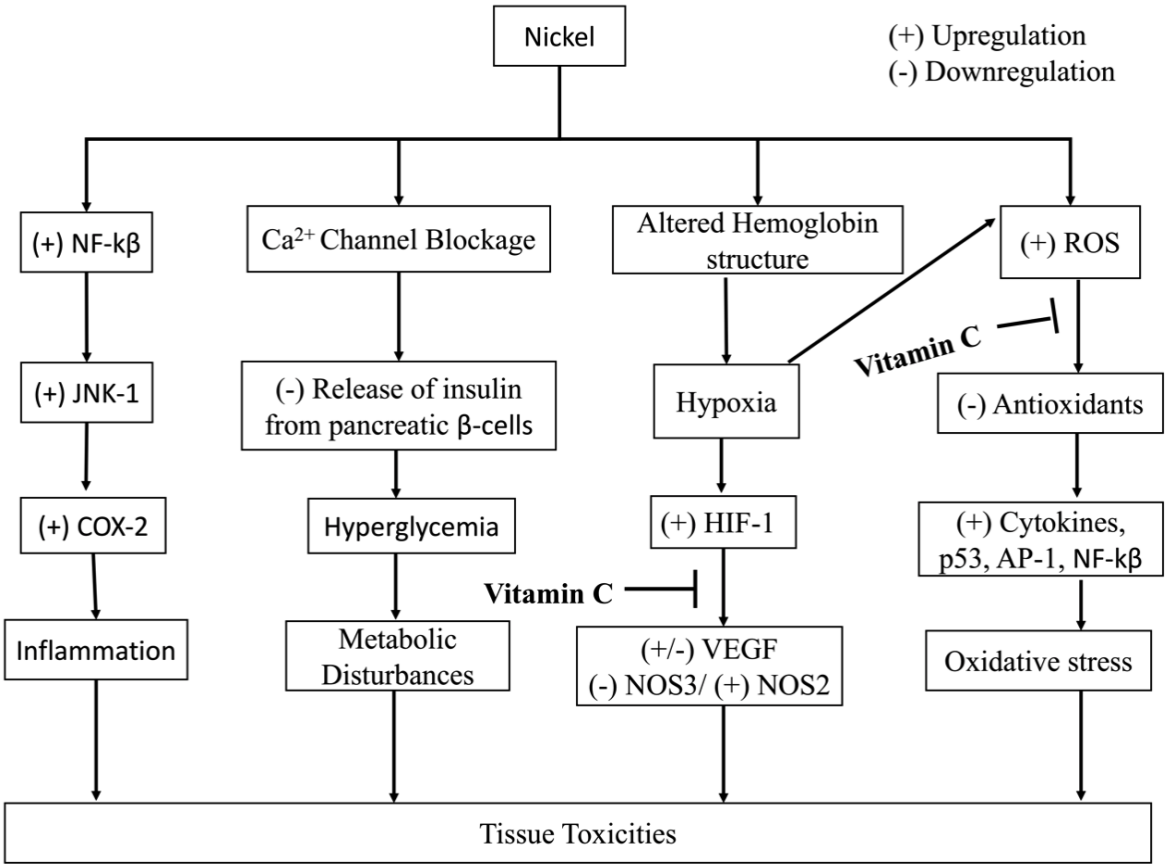

Fig. (1). Nickel Toxicity-Cell Signaling Mechanism and Vitamin C Supplementation. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

However, more studies are required for a better understanding of the molecular aspects of nickel toxicities in humans. Precise clinical trials are needed for the physiological, pathological and molecular impact of nickel on the occupationally exposed population to generate more viable therapeutic options in treating nickel toxicity-related diseases.

LIST OF ABBREVIATIONS

AP-1	=	Activator Protein 1
ASTDR	=	Agency for Toxic Substances and Disease Registry
BC	=	Breast Cancer
BEAS	=	Bronchial Epithelial Cells
CAT	=	Catalase
Cd	=	Cadmium
COX	=	Cyclooxygenase
EPO	=	Erythropoietin
Fe	=	Iron
GSH	=	Glutathione
HIF	=	Hypoxia Inducible Factor
IARC	=	International Agency for Research on Cancer
IL	=	Interleukin
MDA	=	Malondialdehyde
miRNA	=	Micro RNA
mPGES	=	Microsomal Prostaglandin E Synthase
MTP	=	Metal Transporter Protein
NF-κβ	=	Nuclear Factor Kappa-Light Chain-Enhancer of Activated B Cells
Ni	=	Nickel
$NiCl_2$	=	Nickel Chloride
$NiSO_4$	=	Nickel Sulphate
NOS	=	Nitric Oxide Synthase
PD	=	Peritoneal Dialysis
PHD	=	Prolyl Hydroxylase
PTGSs	=	Prostaglandin Endoperoxide Synthases
ROS	=	Reactive Oxygen Species
SOD	=	Superoxide Dismutase
TNF	=	Tumor Necrosis Factor:
VEGF	=	Vascular Endothelial Growth Factor

CONSENT FOR PUBLICATION

Not Applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Effect of L-Ascorbic Acid on Nickel-Induced Alteration of Cardiovascular Pathophysiology in Wistar Rats

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Abstract

Nickel, a widely used heavy metal is suspected as a cardiotoxic element. The aim of the present study was to assess the possible protective role of l-ascorbic acid on nickel-induced alterations of cardiovascular pathophysiology in male albino rats. Twenty-four albino rats (b.wt. 170–250 g) were randomized into four groups: control; l-ascorbic acid (50 mg/100 g b.wt., orally); $NiSO_4$ (2.0 mg/100 g b.wt., i.p.); $NiSO_4$ with l-ascorbic acid. Cardiovascular electrophysiology, serum and cardiac tissue malondialdehyde (MDA), nitric oxide (NO), ascorbic acid, serum α-tocopherol and serum vascular endothelial growth factor (VEGF) were evaluated. Histopathology of cardiac and aortic tissues was also assessed. $NiSO_4$-treated rats showed a significant increase in heart rate, LF/HF ratio and blood pressure (SBP, DBP and MAP). A significant increase of serum MDA, NO and VEGF in $NiSO_4$ treatment with a concomitant decrease of serum ascorbic acid and α-tocopherol as compared to their respective controls were also observed. Simultaneous supplementation of l-ascorbic acid with $NiSO_4$ significantly decreased LF/HF ratio, BP and oxidative stress parameters, whereas ascorbic acid and α-tocopherol concentration was found to be increased. Histopathology of cardiac and aortic tissues showed nickel-induced focal myocardial hypertrophy and degeneration in cardiac tissue with focal aneurism in aortic tissues. Supplementation with l-ascorbic showed a protective action in both cardiac and aortic tissues. Results indicated the possible beneficial effect of l-ascorbic acid on nickel-induced alteration of the cardiovascular pathophysiology in experimental rats.

Keywords Nickel sulfate · Oxidative stress · Electrophysiology · VEGF · Cardiac tissue · L-ascorbic acid

Introduction

Recent studies are showing a strong link between environmental pollutants and various cardiovascular diseases (CVD) [1–5]. Amid environmental heavy metal pollutants, nickel, a trace element with having a divalent cation, has enormous industrial applications. Because of its properties, it has been most widely used in electroplating of metals, battery manufacturing, as a catalyst, electrical and electronic industries, metallurgy and metal alloys preparations [6]. Nickel particles are widely distributed in the soil, air and water; its concentrations are excessive in industrial areas [7]. Nickel is a silver-white transition metal that belongs to the group VIIIb of the periodic table. Nickel concentrations in ambient air is minimal (approximately 6–20 ng/m^3) but higher concentrations (up to 150 ng/m^3) are found in the air polluted by anthropogenic sources [6]. Among the various nickel compounds, nickel sulfate contributes predominant nickel pollutant of ambient air. Nickel facilitates absorption of iron (Fe) in Fe^{3+} form in gastrointestinal tract (GIT) [8]. Nickel is considered as essential trace element for several animal species, but in humans deficiency manifestations have not been clearly mentioned [6]. In biological systems, absorption, distribution and clearance of nickel compounds mainly depend on their

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solubility in water and the route of exposure [6]. The absorption and toxic manifestations of nickel compounds depend on the solubility and order of absorption as follows: nickel carbonyl > soluble nickel compounds (chloride, nitrate, sulfate) > insoluble nickel compounds (oxides, sulphides) [9].

The degree of severity of nickel toxicities is manifested by its route and quantum of exposure [10]. It has been found that toxic manifestations of nickel usually occur through (1) increased ROS production and deprivation of the antioxidant defence system, and (2) alterations in the autonomic nervous system [1]. Nickel-induced increase of oxidative and nitrosative stress is well established and possible altered role of NOS2 (inducible nitric oxide synthase), NOS3 (endothelial nitric oxide synthase) and oxygen sensing cell signalling pathways is also found to be the potential factors to induce nickel toxicities [1]. Studies revealed that divalent cation (Ni^{2+}) influences cardiovascular functions and causes aortic hyper contraction, but exact physiological mechanisms are yet to be known [11].

Very few studies have been conducted to know the toxic effects of divalent cations like nickel on cardiovascular health. Moreover, the effect of water-soluble antioxidant vitamins like l-ascorbic acid on nickel-induced cardiovascular abnormalities were found to be least investigated. Water-soluble chain breaking antioxidant vitamin C scavenges oxygen radicals in aqueous phase, but lipophilic antioxidants like alpha tocopherol (vitamin E) scavenge oxygen radicals within the membrane [12]. Vitamin C also acts as a co-antioxidant and facilitates regeneration of alpha tocopherol from its ascorbyl radical [12]. So, it is well known that some antioxidant vitamins like l-ascorbic acid (vitamin C) or α-tocopherol (vitamin E) may re-establish oxidant/antioxidant balance hence may provide additional defence against metal-induced cell injuries through oxidative stress [13, 14]. In many studies, it has been shown that vitamin C protects against cell death caused by various obnoxious stimuli and a major proportion of this protection has been associated with its antioxidant ability.

Hence, the present study was undertaken to explore the possible protective role of l-ascorbic acid on nickel-induced alterations of cardiovascular pathophysiology in experimental rats.

Materials and Methods

Experimental Design

Twenty-four healthy adult male albino Wistar rats (*Rattus novergicus*) of 8–10 weeks old with the weight of 170–250 g were procured from BLDE (Deemed to be University), Shri B.M. Patil Medical College, Hospital and Research Centre animal house. Experimental rats were acclimatized to standard laboratory conditions for 7 days before starting the experimentation. All standard laboratory conditions like temperature with 22 ± 2 °C and 12 h light-dark cycle were maintained throughout all days of the experiment. All the protocols were scrutinized and approved by the Institutional Animal Ethical Committee (Ref No: BLDE/BPC/641/2016-2017 dated 22 October 2016). Experimental animals were pair fed with normal laboratory diet and water provided ad libitum throughout the study period. All the experimental protocols were performed according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, Government of India [15]. All the experimental rats were divided into four groups and given respective interventions as shown in Table 1. Throughout the adaptation period and the experiment, the rats were accommodated in triplets in standard polycarbonate cages (24 in. × 12 in. × 8 in.) with stainless steel mesh on the top.

Gravimetry

Each animal body weight of all groups was measured on day 1 of intervention and the day of sacrifice by using Sartorious electronic balance (Model: Practum 1102-10IN) and percentage body weight gain was calculated. After sacrificing the experimental rats, heart tissue was dissected out and weighed immediately by using electronic balance (Sathyam digital scale, Model: H2F-A + 300). Organo-somatic index of heart was calculated by organ weight in gram per 100 g of animal body weight.

Electrophysiology

After 21 days of intervention, rats were kept overnight fasting and acclimatized to a small animal restrainer for assessment of non-invasive blood pressure (NIBP). Once rats acclimatized to restrainer, systolic and diastolic blood pressures were recorded by NIBP monitor with tail cuff sensor (Biopac NIBP200A model) connected to a PC. After that, rats were anaesthetised by injecting ketamine (60 mg/kg b.wt.; i.p.) and xylazine (6 mg/kg b.wt., i.p.) [18]. ECG was recorded by using an MP45 Biopac instrument with a PC-based BSL 4.1 (Biopac Student Lab 4.1) software. For ECG, bipolar electrodes were attached to the upper and lower limbs of the rats; heart rate variability (HRV) was analysed by using Kubios HRV software, version 2.0, developed by Department of Physics, University of Kuopio, Finland. Analysis of HRV was performed by using 5 min ECG R-R interval data. The following HRV parameters were measured: low frequency (LF) power (sympathetic activity), high frequency (HF) power (parasympathetic activity) and LF/HF ratio.

Animal Sacrifice and Sample Collection

After 21 days of the intervention, rats were kept overnight fasting, anaesthetized, recorded electrophysiological

Table 1 Experimental groups and the intervention

Experimental groups	Dosage for 21 days
Control	Placebo; oral gavage daily
L-ascorbic acid	50 mg/100 g b.wt.; oral gavage daily [16]
$NiSO_4$	2.0 mg/100 g b.wt.; i.p.; alternate day, 10 doses [17]
$NiSO_4$ + l-ascorbic acid	$NiSO_4$ 2.0 mg/100 g b.wt.; i.p. alternate day, 10 doses + l-ascorbic acid 50 mg/100 g b.wt.; given by oral gavage daily

b.wt. body weight, *i.p.* intraperitoneal

parameters as said above and opened thoracic cage carefully, blood was collected by cardiac puncture and stored in a plain tube with clot activator. The samples were kept at room temperature for 45 min and centrifuged at 600×*g* for 15 min. Serum was separated, protected from light and stored at − 20 °C for further biochemical analysis. After blood collection, rats were sacrificed as per CPCSEA guidelines between 09.00 AM and 11.00 AM.

Oxidative Stress Markers and Antioxidant Vitamins

The concentration of lipid peroxidation product malondialdehyde (MDA) in serum and cardiac tissue homogenate samples was estimated by thiobarbituric acid (TBA) method, where MDA reacts with TBA in hot acidic conditions gives red coloured complex and this colour intensity was directly proportional to the concentration of MDA [19]. Nitrosative stress marker nitric oxide (NO) in its stable form nitrate in serum and cardiac tissue homogenate reduced to nitrite by cadmium reduction and forms coloured complex with N-napthylene diamine. The colour absorbance was measured by UV-Visible spectrophotometer (Shimadzu, Model: UV1800) [20, 21]. Ascorbic acid was estimated in serum and heart tissue homogenate by the method of Roe and Koether [22]. Serum alpha tocopherol concentrations were estimated by the method of Jargar et al. [23]. In the protein-free serum samples, alpha tocopherol was extracted into the xylene layer which reduces ferric to ferrous ions and forms red coloured complex with 2,2′-bipyridyl reagent. The intensity of red colour developed was red at 492 nm against blank by using microplate reader (Meril EIAQuant, Meril Diagnostics Pvt. Ltd., India) [23].

Molecular Markers

Serum VEGF was estimated by a commercially available ELISA kit (Biospespvt. Ltd., China) using a microplate reader (Model: Merilyzer EIAQUANT, Meril Diagnostics Pvt. Ltd).

Histopathology

After the blood collection, animals were sacrificed and cardiac tissue and thoracic aorta were dissected and washed in cold saline to remove the excess blood and then tissues were stored in 10% neutral-buffered formalin for histopathological evaluations. Paraffin blocks were made with fixed tissues and made sections of 3–5 μm thickness, deparaffinized, rehydrated and stained with haematoxylin and eosin (H&E) [24]. The stained tissue sections were observed under a photomicroscope and photographed (Olympus BH-2 with Samsung digital colour camera, Model No. SDC-242).

Statistical Analysis

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 t-test, by using Windows-based SPSS software version 15.0. The p value < 0.05 is considered statistically significant.

Results

Gravimetry

Figure 1 shows that $NiSO_4$-treated rats gained the least % body weight during 21 days of intervention period when compared to all other groups. Both control and l-ascorbic acid-treated rats showed greater % body weight gain among all groups. Rats simultaneously treated with l-ascorbic acid and $NiSO_4$ showed little improvement in body weight gain when compared to animals treated with $NiSO_4$ alone but it is not statically significant.

Organo-Somatic Index

Figure 2 shows a significantly higher cardiac organo-somatic index of $NiSO_4$ alone-treated rats when compared to control and l-ascorbic acid groups. Rats simultaneously treated with l-ascorbic acid and $NiSO_4$ did not show a statistically significant decrease when compared to $NiSO_4$ alone-treated rats.

Electrophysiology

Heart Rate Variability and Heart Rate

Table 2 shows the LF, HF, LF/HF ratio and heart rate of all groups of experimental rats. Results showed a significant increase of LF power, a decrease of HF power and an increase of LF/HF ratio and heart rate (HR) in $NiSO_4$ alone-treated rats when compared to all other groups. Rats simultaneously treated with $NiSO_4$ and l-ascorbic acid show a significant decrease

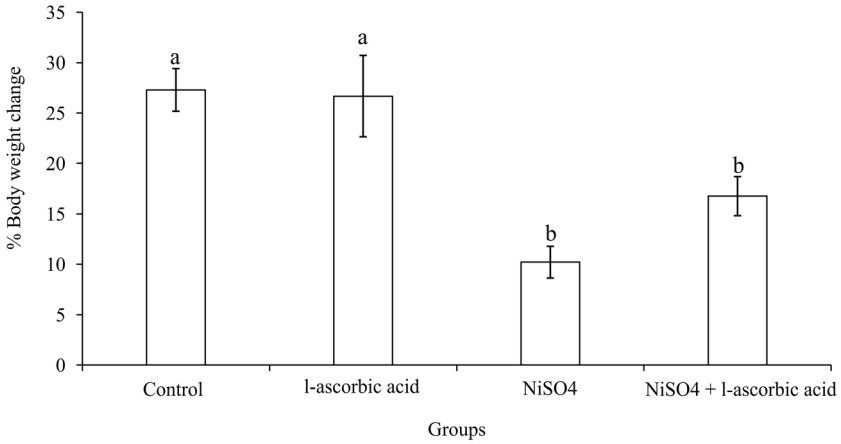

Fig. 1 Comparison of % body weight gain in control, l-ascorbic acid, $NiSO_4$ and $NiSO_4$ + l-ascorbic acid. $n = 6$ rats in each group. Values with different superscripts (a, b) are significantly different from each other ($p < 0.05$)

of LF, an increase of HF and a decrease of LF/HF ratio and HR as compared to nickel alone-treated rats.

Blood Pressure

Table 3 shows significantly increased SBP, DBP and MAP in $NiSO_4$ alone-treated rats whereas simultaneous supplementation of l-ascorbic acid with $NiSO_4$ shows a significant decrease in blood pressure parameters.

Oxidative Stress Markers and Antioxidant Vitamins

Table 4 shows a significant increase in serum and cardiac tissue MDA concentrations in $NiSO_4$ alone-treated rats in comparison to control and l-ascorbic acid groups, whereas rats supplemented with l-ascorbic acid and $NiSO_4$ showed a significant decrease in MDA concentration. Serum and cardiac tissue nitric oxide concentrations in $NiSO_4$ group showed a significant increase when compared to control and l-ascorbic acid groups. Supplementation of l-ascorbic acid with $NiSO_4$ significantly decreased heart tissue NO concentration whereas serum NO concentration remained unchanged as compared to $NiSO_4$ alone-treated group.

Serum and cardiac tissue ascorbic acid concentrations are found to be significantly decreased in $NiSO_4$-treated rats when compared to control and l-ascorbic acid groups. $NiSO_4$ + l-ascorbic acid-treated group of rats showed a significant increase in serum ascorbic acid concentrations but no significant increase was found in case of cardiac tissue l-ascorbic acid concentration as compared to $NiSO_4$ alone-treated rats. $NiSO_4$ alone-treated rats showed a significant decrease in serum α-tocopherol concentration when compared to control and l-ascorbic acid groups, but the group simultaneously treated with $NiSO_4$ and l-ascorbic acid did not show any significant changes as compared to $NiSO_4$ alone-treated group.

Molecular Marker

Figure 3 shows a significant increase of serum VEGF concentration in $NiSO_4$ alone-treated rats when compared to control and l-ascorbic acid-treated rats, whereas rats supplemented with l-ascorbic acid with $NiSO_4$ showed significantly decreased serum VEGF concentration when compared to $NiSO_4$ alone-treated rats.

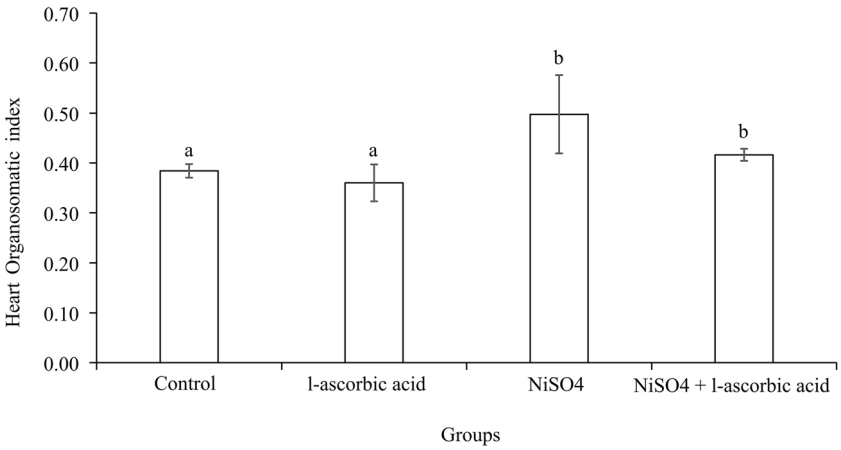

Fig. 2 Comparison of the organosomatic index of cardiac tissue in control, l-ascorbic acid, $NiSO_4$ and $NiSO_4$ + l-ascorbic acid. $n = 6$ rats in each group. Values with different superscripts (a, b) are significantly different from each other ($p < 0.05$)

Table 2 Effect of l-ascorbic acid supplementation on $NiSO_4$-induced alterations of heart rate variability (HRV) parameters like LF, HF, LF/HF ratio and heart rate

Groups	LF (Power n.u)	HF (Power n.u)	LF/HF ratio	Heart rate (BPM)
Control	49.87 ± 1.60^a	49.37 ± 1.52^a	1.01 ± 0.06^a	255.70 ± 6.97^a
L-ascorbic acid	49.09 ± 1.10^a	49.04 ± 1.22^a	0.99 ± 0.02^a	325.07 ± 8.92^a
$NiSO_4$	59.54 ± 1.73^b	41.89 ± 1.24^b	1.42 ± 0.01^b	364.03 ± 20.26^b
$NiSO_4$ + l-ascorbic acid	53.40 ± 3.08^a	46.33 ± 3.17^a	1.15 ± 0.14^a	270.82 ± 26.94^a
p value	0.001	0.006	0.001	0.037

Horizontal values are mean ± SD of six observations in each group: control, l-ascorbic acid, $NiSO_4$ and $NiSO_4$ + l-ascorbic acid. Values with different superscripts (a, b) are significantly different from each other ($p < 0.05$)

LF low frequency, *HF* high frequency, *BPM* beats per minute

Histopathology of Cardiac Tissue and Aorta

Histopathology of Cardiac Tissue

Figure 4 shows sections of the cardiac tissues of all groups. H&E-stained tissue sections of control and l-ascorbic acid-treated rats studied under the microscope show normal histological features like myocardium composed of branching and anastomosing, striated muscle fibres arranged in parallel fashion, separated by capillaries, myocardial fibres are connected to each other by intercalated discs and coronary arteries appear normal. $NiSO_4$ alone-treated tissue sections show focal myocardial hypertrophy, degeneration, capillary congestion and moderate arteriosclerosis of coronary arteries. Rats simultaneously treated with l-ascorbic acid and $NiSO_4$ show some histological improvements in architecture by showing myocardium composed of branching and anastomosing striated muscle fibres arranged in parallel fashion, separated by capillaries; there is no evidence of arteriosclerosis and coronary arterial congestion.

Histopathology of Aorta

Figure 5 showing aortic tissue sections of all groups. H&E-stained thoracic aortic tissue sections of control and l-ascorbic acid-treated rats studied under the microscope show large-sized artery normal histological structures and no evidence of arteriosclerosis, atherosclerosis, arteritis, calcification, aneurysm and dysplasia. But $NiSO_4$ alone-treated rats' aortic tissue sections show mild thickening of tunica intima, medial sclerosis with hyperplastic smooth muscle cells and focal aneurysm. Tissues sections of rats simultaneously treated with $NiSO_4$ and l-ascorbic acid show near normal architecture with only mild thickening of tunica media.

Discussion

Results indicate that $NiSO_4$ has an adverse effect on % body weight gain of rats. Many theories and pathways have been proposed about the control and maintenance of the body composition. But the present results of our study are supported by electrophysiological parameters. In accordance with the experimental findings of Bray et al. (2000), increased sympathetic activity reduces food intake and our study also showed increased sympathetic activity in $NiSO_4$-treated rats [25]. But l-ascorbic acid supplementation in $NiSO_4$-treated rats has shown statistically insignificant body weight gain when compared to $NiSO_4$ alone-treated rats. After 21 days of intervention with $NiSO_4$ alone, an increased cardiac mass showed when compared to control and l-ascorbic acid groups (Fig. 2). This increased cardiac mass could be due to cardiac hypertrophy in response to increased mean arterial pressure (supported by histopathology reports and MAP values) [26,

Table 3 Effect of l-ascorbic acid supplementation on $NiSO_4$-induced alterations of Blood Pressure Values

Groups	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)
Control	124.55 ± 3.23^a	102.22 ± 3.93^a	110.80 ± 4.41^a
L-ascorbic acid	118.28 ± 6.62^a	97.20 ± 5.66^a	120.36 ± 0.51^a
$NiSO_4$	166.11 ± 7.71^b	136.80 ± 12.67^b	146.56 ± 10.67^b
$NiSO_4$ + l-ascorbic acid	133.78 ± 15.44^a	106.01 ± 12.38^a	115.23 ± 12.66^a
p value	0.001	0.004	0.004

Horizontal values are mean ± SD of six observations in each group: control, l-ascorbic acid, $NiSO_4$ and $NiSO_4$ + l-ascorbic acid. Values with different superscripts (a, b) are significantly differ from each other ($p < 0.05$)

SBP systolic blood pressure, *DBP* diastolic blood pressure, *MAP* mean arterial pressure

Table 4 Effect of l-ascorbic acid supplementation on $NiSO_4$-induced alterations of serum and cardiac tissue stress markers and antioxidant vitamins

Parameters	Control	L-ascorbic acid	$NiSO_4$	$NiSO_4$ + l-ascorbic acid	*p* value
Serum MDA (μM/L)	1.63 ± 0.29^a	1.27 ± 0.13^a	2.4 ± 0.39^b	1.38 ± 0.17^a	0.003
Cardiac tissue MDA (μM/g of tissue)	3.48 ± 0.87^a	2.94 ± 1.07^a	13.72 ± 0.67^b	6.99 ± 1.00^c	0.000
Serum NO (μM/L)	6.11 ± 0.19^a	7.78 ± 1.44^a	12.22 ± 2.26^b	10.01 ± 1.49^b	0.000
Cardiac tissue NO (μM/g of tissue)	26.0 ± 5.86^a	23.61 ± 3.64^a	82.25 ± 8.27^b	52.60 ± 8.19^c	0.000
Serum ascorbic acid (mg/dL)	0.81 ± 0.03^a	1.28 ± 0.14^b	0.61 ± 0.02^c	1.28 ± 0.13^b	0.000
Cardiac tissue ascorbic acid (mg/g of tissue)	4.76 ± 1.25^a	7.69 ± 1.93^a	3.23 ± 1.64^b	5.12 ± 1.43^b	0.050
Serum α-tocopherol (μg/mL)	2.86 ± 0.38^a	3.46 ± 0.85^a	1.32 ± 0.30^b	1.39 ± 0.06^b	0.001

Horizontal values are mean ± SD of six observations in each group: control, l-ascorbic acid, $NiSO_4$ and $NiSO_4$ + l-ascorbic acid. In each column, values with different superscripts (a, b, c) are significantly different from each other ($p < 0.05$)

MDA malondialdehyde, *NO* nitric oxide

27]. But simultaneous supplementation of l-ascorbic acid in $NiSO_4$ administered group showed statistically insignificant reduction in cardiac mass. So further study may be required to know the beneficial effect of long-term supplementation with l-ascorbic acid in nickel-induced decrease of % body weight gain and cardiac hypertrophic changes.

The present experiment on HRV in nickel-treated rats considered as a clinically approved indicator of cardiac autonomic balance [28]. The results clearly indicated a vago-sympathetic imbalance in nickel-treated rats by sympathetic overdrive with concomitant under drive of parasympathetic actions. The increase of heart rate in $NiSO_4$ treatment in the present study further indicates a possible increase in sympathetic stimulation. These autonomic malfunctions may also be due to nickel-initiated inflammatory response, increased oxidative stress and decreased availability of antioxidants [29]. Altered sympatho-vagal balance due to $NiSO_4$ administration was significantly improved by the supplementation of antioxidant l-ascorbic acid, suggesting that concurrent response of oxidative stress involved in the modulation of cardiac autonomic function [1].

In this study, we have also observed an increase of SBP, DBP and MAP in the rats treated with $NiSO_4$. This may also be due to the generation of reactive oxygen species (ROS) and cyclooxygenase 2 (COX2)-dependent endothelium contracting factors (EDCFs) by endothelial cells [1, 4, 11, 27]. Golovko et al. (2003) reported that nickel ion induces vasoconstriction in the isolated canine coronary artery by enhancing $CaCl_2$ influx into vascular smooth muscle cells [30, 31]. These increased concentrations of intracellular smooth muscle Ca^{2+} could lead to the increased arterial tone which in turn leading to an increase of blood pressure [32]. However, simultaneous supplementation of l-ascorbic acid with $NiSO_4$ significantly improved the altered blood pressure parameters in rats, as l-ascorbic acid is found to be a potent antioxidant which improves endothelial functions by reducing the oxidative stress, suppressing endothelial cell apoptosis by cytokines and tumour necrosis factor-α (TNF-α) and angiotensin II [33].

Increased oxidative stress in the physiological system is considered to be a key factor underlying cardiovascular dysfunction, hence increase of serum and heart tissue MDA with the decrease of l-ascorbic acid and alpha tocopherol in serum and decrease of l-ascorbic acid in heart tissues in nickel treated rats ascertain cardiotoxicities due to nickel-induced oxidative stress [12]. Nickel is a known atherogenic substance which may exert its effects by increasing the ROS production which in turn increases lipid peroxidation by the increase of oxygen

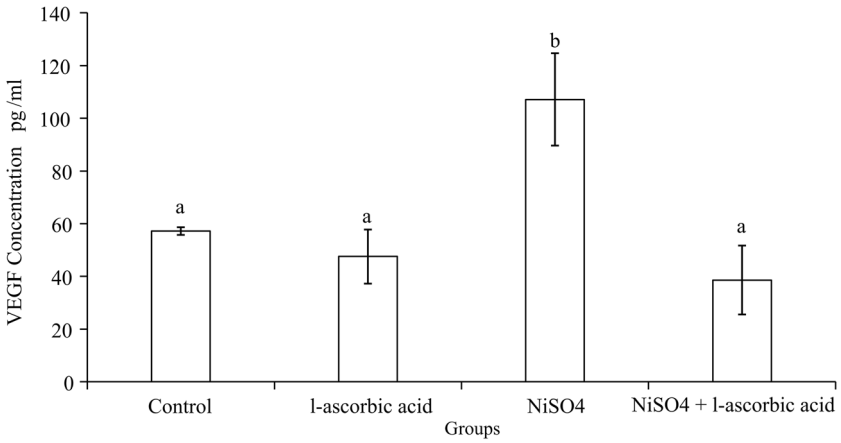

Fig. 3 Comparison of VEGF concentration in control, l-ascorbic acid, $NiSO_4$ and $NiSO_4$ + l-ascorbic acid. $n = 6$ rats in each group. Values with different superscripts (a, b) are significantly different from each other ($p < 0.05$)

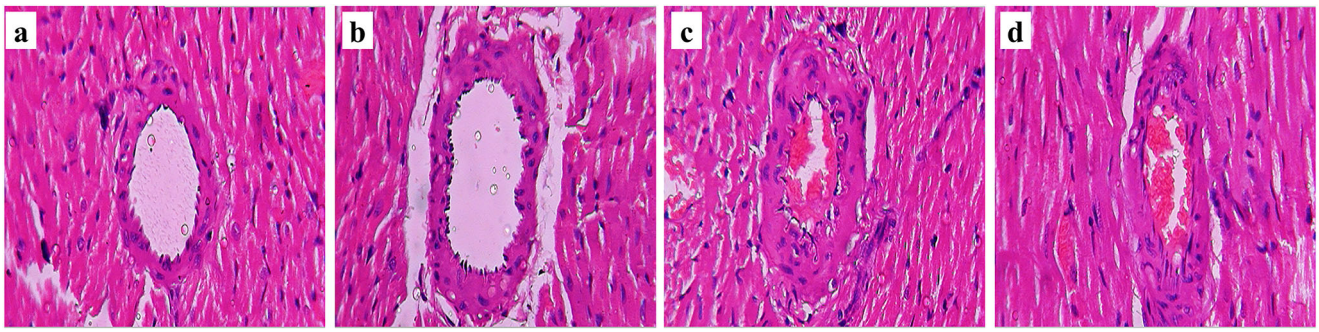

Fig. 4 Haematoxylin and eosin-stained photo-micrographs of cardiac tissue with coronary artery from **a** control (×40); **b** l-ascorbic acid (×40); **c** $NiSO_4$ (×40); **d** $NiSO_4$ + l-ascorbic acid (×40)

free radicals and overutilization of membrane antioxidants [34]. Supplementation of l-ascorbic acid in nickel-treated rats was found to be beneficial to counteract nickel-induced cardiotoxicities due to oxidative stress.

In the present study, we observed an increase in serum and cardiac tissue NO concentrations in nickel-treated rats may be due to the overexpression of inducible nitric oxide synthase (i-NOS) [34]. Increased levels of nitric oxide can react with the superoxide anion (O^{-}_{2}) and form peroxynitrite anion (ONOO–). Peroxynitrite is a potent oxidant and may trigger lipid peroxidation, inhibit mitochondrial electron transport and oxidize thiol compounds [35]. i-NOS gene expression reported being increased in conditions like cellular hypoxia, oxidative stress and pro-inflammatory conditions [36]. Nickel mediated activation of the transcription factors NF-κB and STAT-1α reported being an essential step for the i-NOS induction in most of the cells [32]. Supplementation of l-ascorbic acid may decrease the NO production by blocking the NF-κB pathway in nickel-treated rats.

In many studies, it has been observed that nickel or any other heavy metals create cellular hypoxia by binding to the heme portion of oxygen sensing molecules [34]. The present study indicated that nickel-induced increase of VEGF, potent oxygen sensing growth factor causes cellular hypoxia, vascular damage, triggers hypoxia cell signalling and angiogenesis pathways to protect tissue from further damage [37]. Supplementation of l-ascorbic acid in nickel-treated rats decreased oxygen free radicals which in turn decrease cellular damage and protect heme-containing oxygen sensing biomolecules in physiological systems including the cardiovascular system.

Histopathological studies of cardiac tissues along with coronary artery and aorta show cardiovascular remodelling due to nickel toxicity. The results also indicate a possible association between nickel and vascular architecture by the development of hypertrophy and hyperplasia in vessels, expansion of the endothelial and adventitial layers along with increased arterial wall thickness [36]. Nickel-induced oxidative, nitrosative stress and concomitant decrease of antioxidant defensive agents are responsible for changes in cardiovascular pathophysiology and affect structural and functional homeostasis. But supplementation of l-ascorbic acid mediated improvements of both heart and aortic tissue architectures in nickel-treated rats may be because of decreased oxidative, nitrosative stress and increased antioxidant agents [14].

Conclusion

Results suggest that heavy metal like nickel impairs cardiac autonomic functions, vascular functions, oxidant-antioxidant balance and oxygen sensing cell signalling mechanism (VEGF). Further, nickel sulfate was also found to have an adverse impact on cardiac and aortic tissue histopathology.

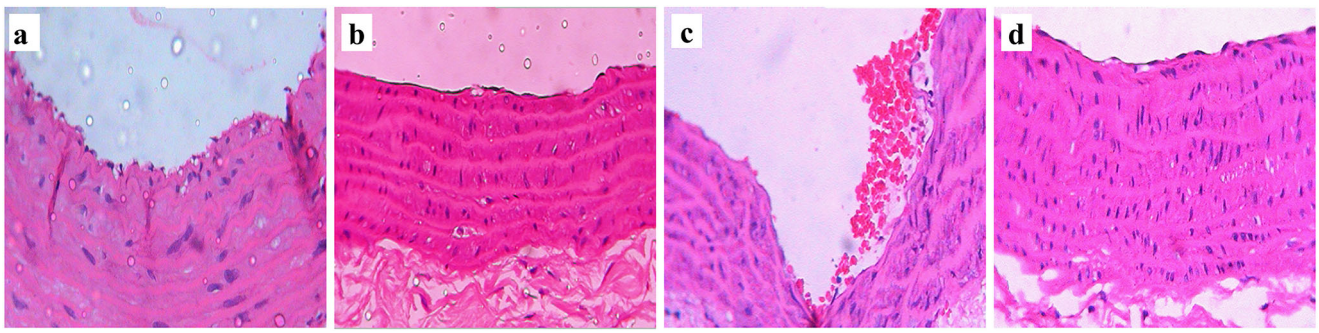

Fig. 5 Haematoxylin and eosin-stained photo-micrographs of the thoracic aorta from **a** control (×40); **b** l-ascorbic acid (×40); **c** NiSO4 (×40); **d** $NiSO_4$ + l-ascorbic acid (×40)

Supplementation of l-ascorbic acid, a potent antioxidant, was found to be beneficial against nickel-induced cardiovascular toxicities. The results obtained in this study may have clinical value in humans and the effect of l-ascorbic acid on nickel-induced cardiovascular toxicities deserves further exploration.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval Institutional Animal Ethical Committee (IAEC) approval was taken from BLDE Association's Shri Sanganabasava Mahaswamiji College of Pharmacy & Research Centre, BLDE (Deemed to be University), Vijayapur, Karnataka, India (Ref No: BLDE/BPC/641/2016-2017 dated 22 October 2016) as per institutional CPCSEA (Committee for the Purpose of Control and Supervision of |Experiments on Animals), Ministry of Environment, Forests and Climate Change, Government of India Reg. No. 1076/PO/ERs/S/07 CPCSEA dated 20 August 2014.

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Nickel Induced Alteration of Pathophysiology of Lungs in Experimental Rats

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Abstract

Background: Nickel and its compounds are known lung carcinogens, hypoxia mimicking effect of divalent nickel (Ni (II)) plays a crucial role in transformation of cells and the progression of tumours. In this study we aimed to evaluate the possible pathophysiological alterations of lung tissues of rats exposed to nickel sulphate.

Materials & Method: For this study 12 male albino rats were randomly divided into two groups: control (placebo), $NiSO_4$ and given respective interventions for 21 days. % body weight gain, pulmonary-somatic index was calculated. In lung tissue homogenates of all groups of animals, concentration of lipid peroxidation product malondialdehyde (MDA), nitric oxide (NO) and antioxidant l-ascorbic acid was estimated spectrophotometrically and lung tissue histopathological observations were also made.

Results: Our results shows significantly decreased % body weight gain, elevated lung tissue MDA, NO and concomitant decrease of l-ascorbic acid concentration in $NiSO_4$ treated groups when compared to control group. Histopathological observations of lung tissue sections of $NiSO_4$ treated group showed eosinophilic oedema fluid filled alveoli, cystic macrophages, thickened interstitial septa, leucocytic infiltration, haemorrhage and acute bronchiolitis.

Conclusion: Our results demonstrate that heavy metal nickel exposure leads to increased pulmonary oxidative and nitrosative stress, decreased concentrations of antioxidants like l-ascorbic acid and histopathological alterations in lungs. So we can conclude that people exposed to heavy metals like nickel may be more prone for lung disorders irrespective of the route of exposure.

Keywords: *nickel, lungs, histopathology, malondialdehyde, nitric oxide, l-ascorbic acid, lipid peroxidation*

Introduction

Industrial usage of heavy metals like nickel (II), cobalt and chromium (VI) is increasing, so industrial workers are more prone to exposure related health consequences[1]. Occupational exposure of nickel mainly occurs in the people working in industries like stainless steel manufacturing, electroplating, mining and metallurgy[1]. It has been reported that experimental animals administered with nickel sulfate leads to increased concentrations of lipid peroxide products, decreased activities of antioxidants like glutathione, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px)[2]. In many studies it has been reported that nickel toxicity leads to oxidative stress and the depletion of ascorbic acid levels in the cells might be one of the reasons[3]. Further it has been observed that lungs are the primary target organs

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in case of nickel exposure. It has also reported that consumption of nickel through water and food develop pneumo-toxicity[4]. Hence the present study has been undertaken to evaluate the effect of nickel sulphate on pathophysiological changes in lung tissue.

Materials and Method

Selection of Animals and Treatment: 12 male rats of albino Wistar strain (*Rattus norvegicus*) weighing 170 to 200 gm were acclimatized 12 hours light-dark cycle, 32 $\pm$ 2^{o} C temperature for 10 days in the Central Animal House facility of BLDE (Deemed to be University), India. Animals were fed with standard pellet diet and water *ad libitum*.

Rats were randomly distributed into 2 groups having 6 animals in each group. Animals of control group were treated with placebo and $NiSO_4$ group rats were treated with nickel sulphate in double distilled water at a dose of 2.0 mg/100 gm b.wt intraperitoneally for 21 days[5].

% Body Weight Gain

Body weight of all groups of animals was recorded by using Sortorious digital scale before and after the intervention period. % body weight change was calculated by using the following formula[6].

$$\%\ Body\ weight\ gain = \frac{Final\ body\ weight\ (gm) - Initial\ body\ weight(gm)}{Initial\ body\ weight(gm)} \times 100$$

Animal sacrifice and Tissue collection:

After the 21 days of intervention period all groups of animals were anesthetized and sacrificed by cervical dislocation. Animals were dissected and lungs were taken out carefully, half lung stored at -20^{o}C in phosphate buffer for biochemical parameters and half stored in buffered 10% formalin for histopathological procedures.

Pulmonary- somatic index

After dissection both the lungs were washed in cold saline to remove excess blood and weight was taken by using digital scale. Pulmonary somatic index was calculated by using the following formula[7].

$$Pulmonary\ somatic\ index = \frac{Lungs\ weight(gm)}{Final\ body\ weight(gm)} \times 100$$

Lung tissue lipid peroxide estimation

Lipid peroxidation product malondialdehyde (MDA) was estimated in lung tissue homogenate by Buege and Aust (1978) method[2]. In brief 10% of lung tissue homogenate was prepared in 0.1 M phosphate buffer by using a tissue homogenizer (REMI Motors Pvt. Ltd, Mumbai, India), homogenate was centrifuged and supernatant was used for MDA estimation. In acidic conditions MDA reacts with Thiobarbituric acid (TBA) and gives pink colour and the absorbance was measured at 535 nm by using UV-Visible spectrophotometer (Schimadzu UV 1800, Japan).

Lung nitric oxide and l-ascorbic acid estimation

Lungs were dissected out, washed with chilled saline immediately and stored in tissue container at -20 °C until used. Lung tissue homogenate was prepared by using 0.9 % saline (500 mg of lung/ 5 ml 0.9 % saline); 1 ml homogenate was mixed with 2 ml of ethanol (1:2 v/v) and cold centrifuged (4 °C) at 1500xg for 15 min and used for tissue nitric oxide estimation[9]. 1ml of supernatant was collected and mixed with 2 ml of 10 % TCA and centrifuged at 4°C at 1500xg for 15 min for the quantitation of tissue l-ascorbic acid[10].

Lung Histopathology Procedure

Lung tissues were dissected and washed in cold saline to remove the excess blood and then tissues were stored in 10% neutral buffered formalin for histopathological evaluations. Paraffin blocks were made with fixed tissues and made sections of 3-5 µm thickness, deparaffinized, rehydrated and stained with haematoxylin and eosin (H&E)[11]. The stained tissue sections were observed under a photomicroscope and photographed (Olympus BH-2 with Samsung digital colour camera, Model No. SDC-242).

Statistical Methods

Values will be presented as Mean $\pm$ SD. *P* value $\leq$ 0.05 will be considered as statistically significant. Comparison of values between two groups was done by unpaired '*t*' test by using SPSS (Version.16.0) software.

Results

Our results showed significant decrease in % body weight gain in $NiSO_4$ treated rats when compared to control rats (**Table 1)**. We had also observed no statistically significant difference in pulmonary-somatic index between control and $NiSO_4$ rats.

Table 1: Statistical comparison of % body weight gain, pulmonary-somatic index and concentrations of

MDA, NOx, l-ascorbic acid in lung tissues of control and $NiSO_4$ groups (n=6 in each group).

Parameters	**Control**	**$NiSO_4$**	***p* value**
% Body weight gain	27.85 ± 1.48	8.15 ± 2.60	0.00*
Pulmonary-somatic index	0.91 ± 0.15	0.81 ± 0.23	0.425
Lung MDA (µM/gm of tissue)	27.83 ± 3.33	56.47 ± 6.81	0.00*
Lung NOx (µM/gm of tissue)	15.89 ± 3.15	31.41 ± 4.86	0.00*
Lung l-ascorbic acid (mg/gm of tissue)	14.10 ± 3.87	4.95 ± 1.25	0.001*

Values are expressed as Mean ± SD. Unpaired 't' test was done to compare the values between two groups. *p* value (>0.05) not significant, *p** (<0.001) highly significant; MDA,malondialdehyde; NOx,nitric oxide.

We have observed significant increase of lung tissue MDA and NO concentration in $NiSO_4$ treated rats when compared to control group. We have also observed a significant decrease in antioxidant vitamin l-ascorbic acid concentration in $NiSO_4$ treated rats when compared to control (**Table 1)**.

Histopathology

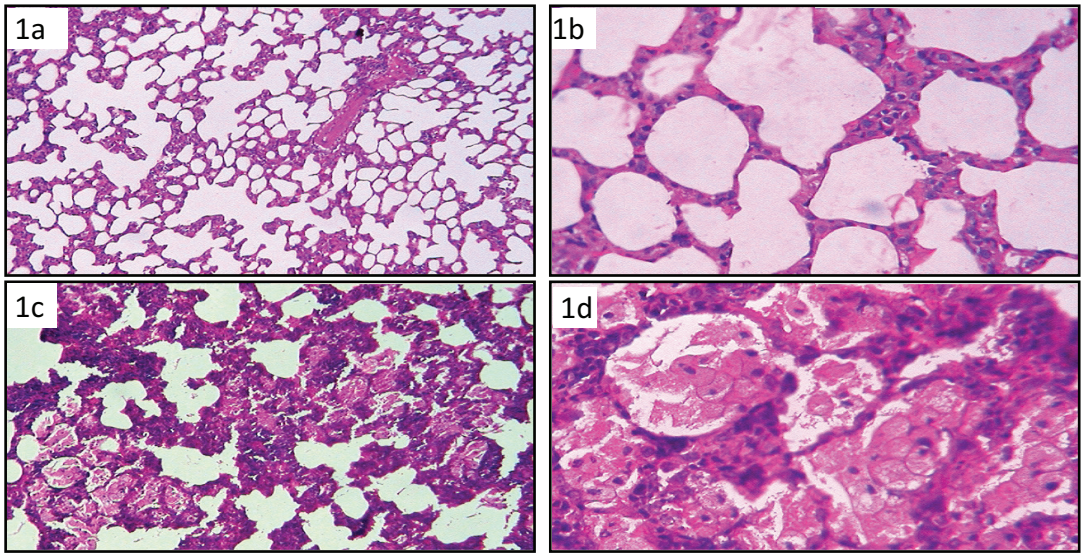

Fig.1: Haematoxylin and eosin stained sections of lung tissues of all groups of rats , (1a) control (10x), (1b) control (40x), (1c) NiSO4 (10x), (1d) NiSO4 (40x).

H&E stained lung tissue sections of control group rats (Fig. 1a & 1b) showed normal lung histological architecture like lung parenchyma consisting of bronchi, bronchioles and thin walled alveoli separated by intervening interstitial connective tissue containing small pulmonary capillaries.

H&E stained lung sections (Fig. 1c & 1d) of NiSO4 treated group showed dilated alveolar spaces filled with eosinophilic oedema fluid and cystic macrophages. It also showed thickened interstitial septa by oedema, congestion and haemorrhages and leucocytic infiltration and acute bronchiolitis.

Discussion

Pulmonary somatic index represents the degree of damage to the pulmonary system. Decreased lung somatic index in NiSO4 treated group may be due to decreased protein synthesis, inflammation, fibrosis, haemorrhage etc6.

NiSO4 is known to be inducer of reactive oxygen species (ROS), and we have observed increased concentrations of MDA in lung tissues. All biological

membranes contain unsaturated fatty acids in their structure, hence peroxidation of membrane lipids leads the membrane damage and cell necrosis8,12. MDA is the product resulted from peroxidation of poly unsaturated fatty acids (PUFA)8. Formation of lipid endoperoxides in unsaturated fatty acids containing at least 3 methylene interrupted double bonds lead to the formation of malondialdehyde as a breakdown product8. Increased MDA levels in lung tissue indicating increased oxidative stress and cell damage in the present study.

Nickel sulphate induces increased lung nitric oxide concentration which may be due to increased activities of inducible nitric oxide synthase (i-NOS) in lung tissues6. Increased NOx level in nickel treated rats probably reacted with superoxide and produced peroxynitrite to develop nitrosative stress in the lungs12. Peroxynitrite interns alter the protein structure and affects its function in target tissues12. These findings in the present study were supported by the results of histopathological studies.

Intra cellular ascorbic acid concentration is a marker of oxidant-anti oxidant balance and it has been found that nickel induces intracellular ascorbic acid depletion leads to altered cell signaling mechanism resulted in carcinogenesis 13. Our study also showed decreased concentration of ascorbic acid in lung tissues, these decreased antioxidant levels may lead to oxidant-antioxidant imbalance, oxidative and nitrosative stress which finally leads to cell necrosis and even cell death.

The histopathological observations on lung tissues in nickel treated rats clearly indicate pathological alterations in lung tissue by inducing integrity of alveolar tissues. The observations from the present study clearly indicate nickel induced oxidative stress and oxidant-antioxidant imbalance, generate lung tissue inflammatory responses 14. Possibly nickel induced alveolar damages may lead to develop cellular hypoxia and may alter pathophysiology of lung tissues 15.

Conclusion

Nickel sulphate is found to be a pulmonary toxic heavy metal which leads to develop oxidative and nitrosative stress in lung tissues. Histopathology of lungs in nickel treatment supports these observation.

Conflict of Interest: Authors declare that there is no conflict of interest.

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Ethical approval: Institutional Animal Ethics Committee (IAEC) approval was taken from BLDE Association's Shri Sanganabasava Mahaswamiji College of Pharmacy & Research Centre, BLDE (Deemed to be University), Vijayapur, Karnataka, India (Ref: BLDE/BPC/641/2016-2017 dated 21.10.2016) and all the experiments on animals were carried out by following CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Government of India.

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Review

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Primary concept of nickel toxicity – an overview

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Abstract: Toxic metals, including excessive levels of essential metals tend to change biological structures and systems into either reversible or irreversible conformations, leading to the derangement of organ functions or ultimate death. Nickel, a known heavy metal is found at very low levels in the environment. Nickel is available in all soil types and meteorites and also erupts from volcanic emissions. In the environment, nickel is principally bound with oxygen or sulfur and forms oxides or sulfides in earth crust. The vast industrial use of nickel during its production, recycling and disposal has led to widespread environmental pollution. Nickel is discharged into the atmosphere either by nickel mining or by various industrial processes, such as power plants or incinerators, rubber and plastic industries, nickel-cadmium battery industries and electroplating industries. The extensive use of nickel in various industries or its occupational exposure is definitely a matter of serious impact on human health. Heavy metals like nickel can produce free radicals from diatomic molecule through the double step process and generate superoxide anion. Further, these superoxide anions come together with protons and facilitate dismutation to form hydrogen peroxide, which is the most important reason behind the nickel-induced pathophysiological changes in living systems. In this review, we address the acute, subchronic and chronic nickel toxicities in both human and experimental animals. We have also discussed nickel-induced genotoxicity, carcinogenicity, immunotoxicity and toxicity in various other metabolically active tissues. This review specifically highlighted nickel-induced oxidative stress and possible cell signaling mechanisms as well.

Keywords: carcinogenicity; genotoxicity; heavy metals; immunotoxicity; nickel; oxidative stress.

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Introduction

Heavy metals are chemical elements that have a specific gravity that is at least five times that of water. They are innate ingredients of the earth's outer layer and are found in varying concentrations in all ecosystems. The heavy metals constitute key portions of the periodic table and include metals from groups IIA (most of the alkaline earth metals) to VIA (chalcogens like selenium, polonium, tellurium etc.) of the periodic table. Among the environmental heavy metal pollutants, nickel is considered as an industrial and occupational health risk, as many nickel compounds are accessible in the human environment [1]. Swedish chemist Axel Cronstedt in 1951 was the first person to obtain purified nickel, the 28th element in periodic table. Earlier, copper miners mistook nickel ore for copper ore and described it as *kupfernickel* or "the devil's copper". It appears as a silvery white metal, which is found to be in multiple states of oxidation, commencing from –1 to +4 [2]. It has also been observed that the +2 oxidation or divalent state nickel is the main widespread analogue of nickel in biological systems. Most nickel subsists as a firm form of hydroxides at pH >6.7 whereas all the nickel complexes are found to be relatively soluble at pH <6.5 [3].

Metal toxicity

The beneficial and adverse effects of metals are well known to many branches of life sciences, but their influences in physiological chemistry have been studied in-depth only in recent decades. Metals play an integral role by conjugating at the dynamic sites of enzymes and contributing directly in the catalytic process, thus stabilizing the macromolecular structures of proteins and nucleic acids and affecting the structural and functional integration.

The possible recognition of the essential biological roles of metals in no way obviates the primary objective of ecological and toxicological investigation, that is, to eliminate the hazards created by metals. Thus, it is important to understand the actions of metals in the physiological and toxicological aspects [4]. Metals induce a two-fold, elevated, biphasic dose response curve, which allows a gross division into two general regions (Figure 1)

(i) Potentially, each of the element has a biological meaning which can be evaluated properly only against a milieu of deficit state.
(ii) Potentially, every element is toxic when presented to an organism in high enough concentration or threshold level.

Nickel toxicities (human and experimental)

The hazards from heavy metals, such as nickel, are absolutely man made and the selected groups who are occupationally exposed to it are the main victims of toxicities. The toxic effects are restricted to a relatively slender group of individuals who are exposed to toxic metals in their workplace [5]. During last few decades, trace metal toxicity-related health problems outshined the mere understanding of occupational health issues of professionally exposed individuals. The broad scope of environmental changes in the air, water and soil, through industrialization, urbanization, transportation and the overuse of chemicals in agriculture-related industries has threatened the physical well-being of individuals through nutrition and has caused grave concern in terms of exposure to certain trace metals [6–8].

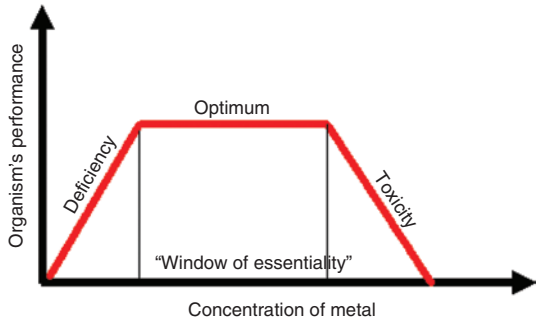

Figure 1: The characteristic of metals as environmental pollutants.

Environmental nickel levels depend especially on natural sources, pollution from nickel-manufacturing industries and airborne particles from combustion of fossil fuels. Absorption from atmospheric nickel pollution is of least concern. Interestingly, vegetables usually contain more nickel than do other food items; high levels of nickel have been found in legumes, spinach, lettuce and nuts. Certain products, such as baking powder and cocoa powder, have also been found to contain excessive amounts of nickel, perhaps related to nickel leaching during the manufacturing process. Soft drinking-water and acid beverages may dissolve nickel from pipes and containers. Leaching or corrosion processes may contribute significantly to the oral nickel intake, occasionally up to 1 mg/day [9].

The environmental sources of lower levels of nickel include tobacco, dental or orthopedic implants, stainless steel kitchen utensils and inexpensive jewelry [10]. Tobacco smoking is another source of non-occupational exposures to nickel. It has been observed that each cigarette contains 1.1–3.1 µg of nickel and that about 10%–20% of the nickel inhaled is present in the gaseous phase. According to some studies, nickel in tobacco smoke may be present in the form of nickel carbonyl, a form which is extremely hazardous to human health. Pipe tobacco, cigarettes and other types of tobacco products do not greatly differ from one another in terms of nickel content [11, 12].

The route of nickel exposure is mainly responsible for the severity of the impact on system biology, immunology, neurology, reproduction, development and carcinogenicities, either through acute (01 day), subchronic (10–100 days) and chronic (>100 days) exposure periods. One of the most common pathways to nickel toxicity is an allergic skin reaction sensitive population. A report indicated that nickel is a potential immunomodulatory and immunotoxic agent aside from its action as an allergen in humans [13, 14]. The International Agency for Research on Cancer (IARC) [15] and the U.S. Department of Health and Human Services classified nickel compounds as human carcinogens on the basis of various studies in human and experimental animals [16].

Acute toxicity (01 day)

Humans

Acute toxicity in humans resulting from absorption through the gastrointestinal tract or by inhalation through

lungs was primarily reported by Sunderman [17]. Nickel carbonyl inhalation causes two kinds of acute toxic effects: instant and delayed. The symptoms of acute toxicities include nausea, vomiting, vertigo, irritation, etc. These symptoms last for a few hours to a couple of days. Instant symptoms are followed by delayed symptoms like stiffness of the chest, constant cough, dyspnea, cyanosis, tachycardia, palpitations, sweating, visual disturbances and weakness etc. [18]. Death due to cardiac arrest has been reported in a 2 ½ year old girl, who consumed nickel sulfate accidentally [19]. Deaths due to respiratory distress syndrome (ARDS) among spray painting workers exposed to nickel have already been documented [20]. Sunderman et al. reported shortness of breath and giddiness among electroplating workers who accidentally drank nickel chloride-polluted water (1.63 g/L) [21].

Experimental animals

One observation in a single- dose nickel chloride injection in male rats showed elevated circulating prolactin levels after 1 day and elevated levels for 4 consecutive days [22]. Acute nickel toxicity also caused renal damages and frank hematuria [23]. Water-soluble nickel compounds are more toxic than the less soluble compounds. The less soluble nickel compounds like nickel oxide and subsulfide have been found to have LD50 greater than 3600 mg Ni/kg.b.wt. in rats, whereas soluble nickel compounds, i.e. nickel sulfate and acetate, exhibited an LD50 range of 39–141 mg Ni/kg.b.wt. in rats and mice [24].

Subchronic toxicity (10–100 days)

Humans

A study on 6-week exposure to nickel fumes (0.07–1.1 mg nickel/m^3) to welders caused a breathing rate increase and visual dysfunctions with tiredness [25]. In the case of women who were occupationally exposed to soluble nickel compounds (0.75 mg Ni/m3 average concentration), they showed elevated urinary protein, β2-microglobulin, retinal binding protein and N-acetyl-β-D-glucosaminidase [26]. Such changes of biomarkers reflect tubular dysfunction. Interestingly, another study on workers exposed to nickel sulfate did not observe any proteinurea [27].

Experimental animals

A study on rats showed remarkable reductions in body weight and signs of liver and kidney failures due to exposure to oral nickel intake in a 3-week study [28]. This has also been observed that significant dose-dependent hyperglycemia, decrease in serum urea and significant increase in urine urea in male rats treated with $NiCl_2$ in different doses (0.38, 0.75 or 1.5 mg/kg/day, 28 days) [29]. A decrease in blood hemoglobin and Packed Cell Volume after nickel exposure has also been reported [30]. Nickel-treated rats also showed toxic symptoms like ataxia, hypothermia, salivation and diarrhea [13]. A study on rats treated with 5, 35 or 100 mg nickel/kg/day for 2 months showed complete mortality among high-dose group B [31]. A dose of 35 mg/kg of nickel sulfate showed high WBC and platelets counts with lower blood glucose levels in rats [32]. Several weeks' exposure to dietary nickel acetate, a degenerative change in kidney tubular systems has also been reported [33]. The Inhalation Toxicology Research Institute's 13-week inhalation study on rodents exposed to various nickel compounds revealed inflammation and fibrosis of the lungs as well as alveolar macrophage hyperplasia corresponding to the water solubility of the nickel compounds, with nickel sulfate as the most toxic effect [34].

Chronic toxicity (>100 day)

Humans

Occupational exposure to nickel dust or nickel vapors resulting from welding nickel alloys is the most common chronic exposure routes in humans. Chronic inhalation and exposure to nickel dusts and aerosols contribute to all the types of respiratory disorders, including asthma, bronchitis, etc. [35]. Another study reported that nickel refinery workers were displaying higher incidences of pulmonary and nasal cancer [36, 37]. A study on women working in a nickel refinery did not suggest any type of growth or reproductive hazards [38]. However, incidental occupational nickel exposure (0.13–0.2 mg nickel/m^3) in men has been found to be hazardous to growth and reproductive health [39].

The main cause of concern when handling nickel, its alloys or its salts, is its ability to produce allergic dermatitis. Such reactions can occur through soil, water or direct contact with metal that contains nickel and even metallic jewelry or coins. Due to its omnipresence and occurrence in daily-use items, nickel is the most common reason of immediate and delayed hypersensitivity in occupationally exposed and non-exposed population [40]. Chronic nickel also induced increase loss of nitrogen, urinary glucose output as well as loss of urinary phosphates, calcium and zinc ions. Chronic exposure resulting in reduced nicotinamide induces a

disruption in oxidative phosphorylation [41]. Thus far, no intermediate-duration human inhalation exposure studies have been identified; rather, some chronic exposure studies have examined the potential of nickel and nickel compounds to induce respiratory effects in workers. Most of these studies are cohort mortality studies that have been unable to find significant increases in the number of deaths from nonmalignant respiratory system diseases [42].

Experimental animals

Prolonged exposure to nickel oxide (42 mg nickel/m^3) developed emphysema and other proliferative and inflammatory changes in rats [43]. Rats that consumed nickel sulfate (100 mg/L)-contaminated water has resulted in serious loss of kidney weights with significant albuminurea [26]. Further, it has been observed that rats fed with nickel for 2 long years showed severe reduction of body weight [26]. The available chronic-duration database was considered inadequate for minimum risk level (MRL) derivation given that intermediate-duration studies found an overall significant decrease in survival of the offspring of rats exposed to ≥1.3 mg Ni/kg/day [44].

Some specific aspects of nickel toxicities

Genotoxicity

An increase in the incidence of chromosomal abnormalities but with no chromosome distortion was reported among nickel refinery workers, which were found to be similar with another report on workers exposed to manganese, nickel and iron [45, 46]. Most of the *in vivo* studies revealed that nickel and its compounds are not mutagenic, although some oral and intra peritoneal studies have reported the presence of micronuclei in the bone marrow in nickel exposed rodents [47, 48]. Nickel subsulfide exposure to both nickel-sensitized and non-sensitized individuals showed genotoxicity like the alteration of DNA configuration, resulting in cross linking and strand break in the human lymphocyte [49–52]. A very high degree of mutagenicity at the guanine phosphorybosyl transferase gene with low soluble nickel compound exposure in the Chinese hamster G12 cell line has been reported [53]. Nickel causes the mutation of the p53 gene, which is an important tumor suppressor gene and transcription factor, in kidney epithelial cells [54]. Nickel also inhibits DNA repairing by possibly binding to DNA-repair enzymes and generates free radical result in irreversible protein degradation [48, 55].

Carcinogenicity

Nickel exposure to various workers in nickel industries demonstrated carcinogenic effects. Possibility, multiple carcinogenic factors that are also found along with nickel may be the reasons for such a phenomenon. Various studies have reported that divalent nickel is a potent carcinogen that can induce malignancy in both humans and rodents. Human exposure of nickel through industries like refinery, mining and smelting, stainless steel industries, and battery manufacturing facilities causes cancer, although it is difficult to identify the speciation of nickel compounds. The International Committee on Nickel Carcinogenesis is currently working on identifying the specific nickel carcinogen [56].

Animal studies have shown the carcinogenic potential of various nickel compounds like nickel subsulphide, nickel chloride, nickel oxide, and nickel sulfate, etc. [57]. A study on rodents showed lung tumors, including adenomas, adenocarcinomas, squamous cell carcinomas, and fibrosarcoma with an exposure to nickel oxide (7 mg Ni/m^3; 6 h/day; 5 days/week) [58]. However, the inhalation of 6.3 mg Ni/m^3 as nickel oxide for 1 month did not show any significant increase in lung cancer in rats [59], although rats exposed to nickel oxide of about 1–2 mg Ni/m^3 showed alveolar/bronchiolar carcinoma or adenoma [60]. Results suggest that the genetic factors, including epigenetic factors and oxidative stress, are the probable causes of nickel-induced carcinoma. It has also been reported that some of the nickel compounds induce cell proliferation, which may induce mild DNA abrasions into extreme mutations [3].

Immunological effects

Nickel generates multiple reactions in the human immune system in a diverse fashion [40]. Experimental works have proven that nickel is an immunomodulatory and immunotoxic agent. It has been reported that nickel contact caused allergic dermatitis and immunologic urticarial; hence, nickel can be marked as both immune sensitive as well as an allergen [14, 61–63]. However, it remains unclear how a small nickel particle generates allergic manifestation. When metal oxidizes, it develops a substance named hapten, which can elicit an immune response by binding with tissue protein like large molecules [13]. Nickel exposure to workers has been found to have a significant impact on the increase of IgG, IgA and IgM with the concomitant decrease in IgE levels [64, 65]. Further significant elevations of other serum proteins of cell-mediated immunity,

including α1-antitrypsin, α2-macroglobulin and ceruloplasmin, have also been observed [61]. Nickel can also significantly reduce the circulating antibody response of immunized rats treated with a viral antigen [61, 66, 67].

Endocrine effects

Nickel causes severe adverse effects on the hypothalamic-pituitary-gonadal axis, which is further aggravated in protein restricted dietary condition [68]. It has been reported that the inhalation of nickel causes no impact on endocrine profiles in humans but seriously impairs the functions of most of the vital endocrine glands of rats or mice [60, 69, 70]. Rats exposed to about 0.73–2 mg Ni/m^3 as nickel oxide demonstrated adrenal medullary hyperplasia with benign pheochromocytoma [24, 69, 70]. Nickel chloride given orally at doses of ≥20 mg Ni/kg/day for up to 30 weeks showed an increase of pituitary glands only in male rats [71–73]. Female rats treated with nickel chloride (31 mg Ni/kg/day, orally) showed a decrease of prolactin level [68, 74]. Histopathological observations in rats (187.5 mg Ni/kg/day) and dogs (62.5 mg Ni/kg/day) did not show any adverse effects on most of the endocrine glands [44]. An increase of blood glucose level in rats has been found after a 21-day treatment of nickel sulfate (2.0 mg/100 g b.wt.; i.p.) [75].

Neurogenic effects

Neurologic effects, including giddiness, weariness and headache, have been observed in shift employees who consumed nickel-contaminated water [21]. One study on humans found that a person who ingested a single dose of nickel ($NiSO_4$; 0.05 mg Ni/Kg, b.wt.) developed homonymous hemianopsia (intraocular effect) for 2 h. [76]. A microscopic examination on rats and mice showed no remarkable changes in whole brain pathophysiology after exposure to several nickel compounds though some atrophy of the olfactory epithelium [77]. Force feeding with nickel chloride for 3 months in rats resulted in severe neurological disorders, including sluggishness, abnormal breathing, impaired body temperature regulations and ataxia [32, 78].

Cardiovascular effects

No increases in the number of deaths from cardiovascular diseases have been reported in workers exposed to nickel [79]. Nickel chloride treatment (8.6 mg Ni/kg/day for 91 days) in rats showed a reduction in organ weights, including the heart [32]. Interestingly, increased heart weight in rats exposed to 75 mg Ni/kg/day as nickel sulfate for 2 years Hs been reported, although no histopathological changes on cardiac tissues have been observed [44, 80]. Inhalation of Ni in a low dosage (1.2 mg/m^3) caused delayed bradycardia, hypothermia and arrhythmogenesis [81]. In another study, a long-term average ambient air level of Ni (1.9 ng/m^3) in the United States resulted in a significant progression of cardiovascular mortality in humans [82]. A study on nickel exposure (100 mg/L $NiSO_4$) showed significant increased lipoperoxide and total lipid concentrations in cardiac tissue. The mechanism through which nickel acts to increase cardiovascular risk factors remains unknown, although impaired antioxidants metabolism and oxidative stress may be considered as possibilities [83]. Another study showed no cardiovascular effects in rats or mice exposed to inhalation of 0.44, 1.83 or 7.9 mg Ni/m^3 as nickel sulfate, nickel subsulfide or nickel oxide, respectively, 6 h/day, 5 days/week for 13 weeks [69, 70]. Hence, it can be postulated that a low dose of nickel through inhalation does not show any significant cardiovascular abnormalities; however, a moderate to higher dose may induce pathophysiological changes relevant to atherogenic events, including increased oxidative stress, inflammatory response, and coagulation activity [84].

Gastrointestinal effects

Workers who consumed water during one work shift from a water cascade contaminated with nickel showed symptoms related to gastrointestinal (GI) disorders [21]. The symptoms included nausea, abdominal cramps, diarrhea and vomiting. In the case of rats treated with nickel chloride (25 mg Ni/kg/day; 3 months), the animals showed severe gastritis, including diarrhea [30]. However, such GI disorders were not found in rats treated with dietary nickel sulfate (28.8 mg Ni/kg/day; 3 weeks) [80] or nickel sulfate (187.5 mg Ni/kg/day for 2 years) as well [44, 85, 86].

Musculoskeletal effects

Similarly, workers accidentally consumed nickel in drinking water reported muscle pain [21]. However, any such skeletal muscle histological abnormality was not found in experimental nickel exposed rats (187.5 mg Ni/kg/day) [44].

Dermal effects

Nickel exposure to skin causes contact dermatitis in the general population. Several investigations on single or multiple oral doses of nickel sulfate showed the increase of severity of dermatitis in nickel-sensitive individuals [14, 85–93]. The study further revealed body erythema, hand eczema and a flare-up at the patch test site after coming into contact with nickel sulfate. An oral challenge dose of nickel sulfate (0.014 mg/kg) showed signs and symptoms of dermatitis on subjects who had gone for patch testing 1 month before the test [94].

Metabolic effects

An increase in serum glucose concentrations has been found in male rats exposed to nickel oxide (0.385 and 0.784 mg Ni/m3; 28 days) [29]. Interestingly, a decrease in serum glucose concentration has been observed in the case of female rats exposed to nickel oxide (0.8 and 1.6 mg Ni/m^3; 28 days) [29]. Results clearly suggested a gender sensitive metabolism in nickel-exposed rats. It has been further revealed that a single-dose injection of nickel chloride (4.5 mg Ni/Kg b.wt.) significantly increased serum glucose concentration in rabbits along with histopathogical changes in pancreatic cells [29]. Drinking nickel chloride for 28 days resulted in an increase of serum glucose concentrations in rats [94, 95]. Another study on rats showed a decrease of blood glucose levels after being treated with nickel (8.6 mg Ni/kg/day for 91 days) by force feeding [32]. However, it may be noted that in both studies, a significant reduction in body weight (20% and higher) has also been observed at the same dose effect levels. Hence, an ambiguity regarding altered metabolism due to the primary or secondary effects of nickel remained remains [24, 96].

Ni(II) Induces oxidative stress

Divalent nickel enhances lipid peroxidation at all DNA bases by either *in vitro* or *in vivo* systems [52, 97, 98]. Nickel-induced oxidative stress is rather weak; however, depleting glutathione and oxidatively activating various transcription factors cannot be ignored as possible indications of oxidative stress [98–101]. Even though divalent nickel itself is not a good free radical generator from oxygen or hydrogen peroxide or lipid hydroperoxides, the entire reactionary mechanisms with all those oxygen derivatives can be controlled by the process of chelation with some ligands like histidine or cysteine [102–104]. It has been observed that Ni(II) incubated with cysteine in the presence of an oxygen environment generates hydroxyl radicals, which then react with cysteine and produce a carbon-centered alkyl radical and, subsequently, free radicals from lipid hydroxyperoxides in presence of oligopeptides [105, 106].

Hence it may be noted that Ni(II) toxicity lies on free radical generation from Ni(II) – thiol complexes or singlet oxygen or lipid hydroxyperoxides in a complex manner. It is possible that the nickel-induced accumulation of iron may be directly responsible for the formation of and the reactive oxygen species (ROS) subsequent enhancement of lipid peroxidation via redox pathways [107]. Nickel induces oxidative stress with generation of ROS may stimulate cell signaling pathways by developing an intracellular low-oxygen microenvironment. This, in turn, activates the hypoxia-inducible factor-1α (HIF-1α) transcription factor and regulates all the hypoxia gene expressions. The pathway may turn into either adaptive response against nickel induced cellular hypoxia or apoptosis. Further, it was also observed that heavy metals like nickel(II) through ROS may mimic cellular hypoxia but may not always activate HIF-1 dependent genes [96]. The possible reason behind the nickel-induced activation of HIF-1α transcription factor is that Ni(II) replaces Fe(II) in the oxygen carrier and produces a hybrid form of nonfunctional hemoglobin. This phenomenon develops into permanent intracellular hypoxia, which then activates HIF-1α [108].

Human studies

The oxidative effects of nickel on human lymphocytes *in vitro* manifested increased levels of intracellular ROS, lipid peroxidation and hydroxyl radicals after acute exposure to inorganic nickel, which supported the concept of nickel chloride induced oxidative stress [106]. In the human bronchial epithelial cell line BEAS-2B, however, nickel was only mildly active in inducing an oxidative stress response compared with other metal species measured as ROS [109]. Arranging various metal species in order of increasing toxicity yields the following: Ni(II) < Cr (VI) < Cd (II) [106, 110]. Several extensive studies on cell lines and blood lymphocytes clearly indicate nickel-induced oxidative stress in humans [111, 112]. Nickel carbonate hydroxide-induced genotoxicities and lymphocytic destructions are mediated through oxidative stress involving H_2O_2, singlet oxygen or

the hydroxyl radical [112]. The pretreatment with endogenous antioxidant enzymes like catalase (CAT) and superoxide dismutase (SOD) on human lymphocytes have been proven effective to reduce such nickel-induced oxidative stress [109, 113].

After controlling for confounders, plasma lipid peroxidation levels significantly increased and erythrocyte antioxidants significantly decreased in a group of nickel-plating workers [114].

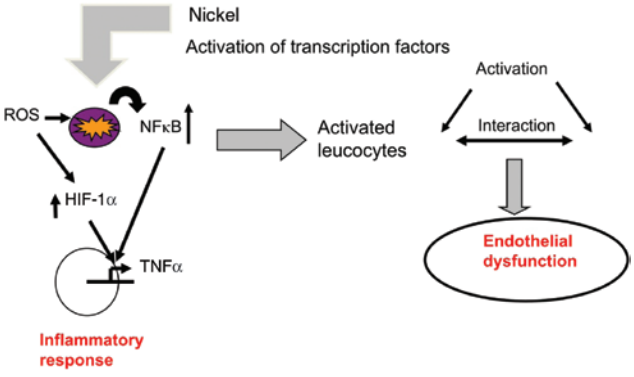

Figure 2: The nickel-induced inflammatory pathways.
ROS, reactive oxygen species; NF-κB, nuclear factor κB; HIF-1α, hypoxia inducible factor 1α; TNFα, tumor necrosis factor-α.

Experimental animal studies

The intraperitoneal administration of nickel chloride results in increased hepatic, renal and pulmonary lipid peroxidation, as indicated by malondialdehyde (MDA) in fresh tissue homogenates [115–117]. Using a mouse model, a previous study reported that the intraperitoneal administration of nickel chloride enhances hepatic lipid peroxidation and depletes glutathione [118]. In a mouse study, multiple intraperitoneal doses of the compound elicited a moderate increase in lipid peroxidation in whole testis homogenates and higher dose-related increases in both mitochondrial and microsomal fractions [119]. The extent of the nickel-induced lipid peroxidation showed an inverse relationship with some of the endogenous cellular antioxidant defense systems, except SOD, CAT and glutathione-S-transferase. Moreover, the exposure of rat lymphocytes to nickel subsulfide increases the formation of ROS in a concentration-dependent manner [113].

Oxidative stress and cell signaling by nickel

Inter and intra cellular communication with response to extracellular stimuli through biological mechanisms is

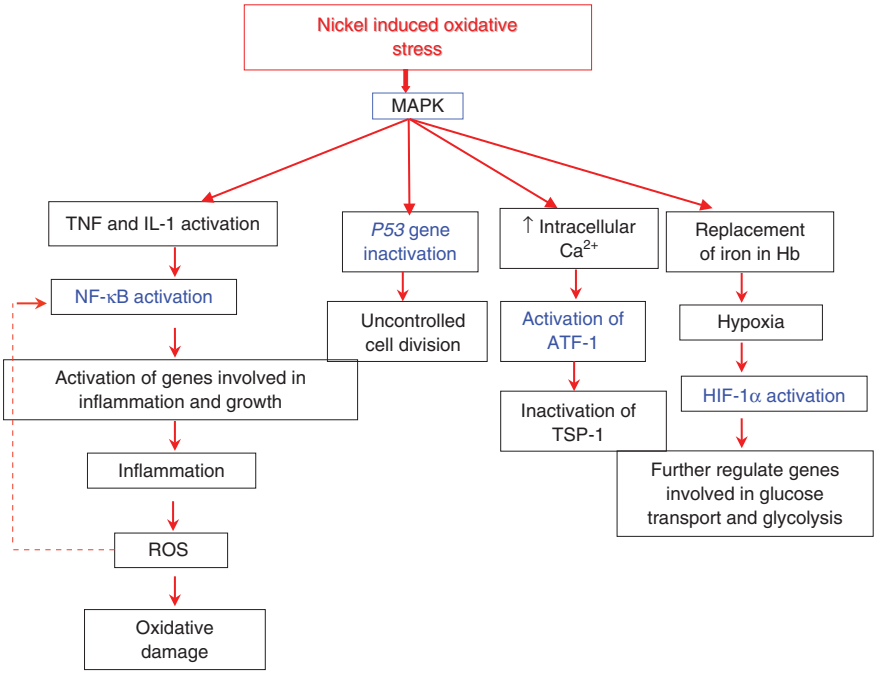

Figure 3: The nickel-induced oxidative stress.
MAPK, mitogen-activated protein kinase; TNFα, tumor necrosis factor α; IL-1, interleukin 1; NF-κB, nuclear factor κB; HIF-1α, hypoxia inducible factor 1α; ARF-1, Cyclic AMP-dependent transcription factor; TSP-1, Thrombospondin 1; ROS, reactive oxygen species.

called “cell signaling” or “signal transduction”. These cell signaling pathways follow transcription mechanisms that are responsible for specific gene expressions via proteins named as transcription factors. These transcription factors bind with specific DNA sequences and further activate RNA polymerase II. The cell signal transduction pathways modulate various physiological functions, including gene expression, muscle contraction, nerve impulse propagation or inflammation. Interestingly ROS, which are found to damage cells and are harmful for physiological functions are found to be intracellular signaling regulators [120]. A study revealed that ROS influence several gene expressions through signal transduction pathways [121]. Given that ROS are oxidants and behave as secondary messengers, they control redox as per their concentration and are capable of inducing either cell proliferation or cell death [33, 122, 123]. Figure 2 shows the nickel-induced inflammatory pathways that are extended to even endothelial function regulations.

This cell signaling mechanism also includes cytosolic calcium concentration, which also regulates both inflammatory and endothelial functions, protein phosphorylation and the activation of nuclear factor κB (NF-κB) and the AP-1 proteins [124]. Nickel induces mitogen-activated protein kinase (MAPK) upregulations, which in turn, activate TNF and the IL-1 pathways to further activate NF-κB. ROS and metal ions primarily inhibit phosphor-serine/threonine-, phosphotyrosine- and phospholipid-phosphatases by interacting with sulphydryl groups on their cystein residues, thus further generating disulphide bonds after oxidation [125].

These structural changes alter protein conformation, which leads to the upregulation of several signaling cascades, most important of which are the growth factor kinase-, src/Abl kinase-, MAPK- and PI3- kinase-dependent signaling pathways**.** Figure 3 presents the overall oxidative stress pathways, which make multiple cascades to activate redox-regulated transcription factors (AP-1, NF-κB, p53, HIF-1, NFAT).

Conclusions

Based on the literature, including the research carried out in the authors’ laboratory, we can say that nickel is a potentially toxic heavy metal that affects multiple organs of living systems. Moreover, the toxicities of nickel manifested based on the manner of exposure, dose and duration. Further, nickel-mediated toxicity in organisms may occur through oxidative stress pathways.

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Subchronic hypoxia pretreatment on brain pathophysiology in unilateral common carotid artery occluded albino rats

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Abstract:

OBJECTIVE: This study was aimed to assess the effect of unilateral common carotid artery occlusion on brain pathophysiology in rats pretreated with subchronic hypoxia.

MATERIALS AND METHODS: Rats (200 ± 20 g) were randomized into three groups: Group 1 served as sham, Group 2 were normoxic (21% O_2 and 79% N_2), and Group 3 were hypoxia preconditioned (10% O_2 and 90% N_2) for 21 days before left common carotid artery occlusion (LCCAO). The LCCAO was done for 75 min followed by reperfusion for 12 h. Neurological scores were recorded. Serum malondialdehyde (MDA) and nitric oxide (NO) levels at pre- and 12 h post-LCCAO were measured. Brain histopathological assessments were also done.

RESULTS: Higher neurological deficits scores in Group 2 as compared to Group 3 rats were noticed. Serum MDA and NO levels at 12 h post-LCCAO in Group 2 rats showed significant elevation as compared to preocclusion levels. Group 3 rats did not show such elevations. On histopathology of left and right cerebral hemispheres of Group 1 (sham) did not show any specific changes. In Group 2 rats, the right cerebral hemisphere (nonoccluded) showed no areas of ischemia-induced brain changes, but in the left side (occlusive), there were features of ischemic brain damage including cerebral edema. In the case of Group 3 rats, there were less ischemic damages in the left occluded side as compared to the left side of the Group 2 rats.

CONCLUSION: This study clearly demonstrates that subchronic hypoxia pretreatment can reduce ischemic brain injury by unilateral common carotid artery occlusion in rats.

Keywords:

Brain histopathology, cerebral edema, left common carotid artery occlusion, neurological scores, pretreated hypoxia

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Introduction

The significance of cerebrovascular ischemia in medical practices made medical scientists to take up preparation of various experimental ischemic models on rodents.[1] It is reported that after occlusion of extracranial or common carotid artery the functional competence of the intracranial collateral circulation through internal carotid artery and basilar artery protects further ischemic brain damages except some preexisting vascular irregularities or exposure of hypoxia.[2] It is also evidenced that the duration to tolerate cerebral ischemia depends on the severity of ischemia, duration of reperfusion, and many other conditions including metabolic reserves. It is further stated that different areas of brain differentially tolerate ischemia.[3] Studies reported that rats engaged in exercise for 2 weeks before

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transient forebrain experimental ischemia show marked improvement in neuronal damages.[4] Hypoxia leads to altered intracellular chemical microenvironment by increasing calcium concentration, lipooxygenase, lipid peroxidation, and cyclooxygenase.[5] Cells exposed to hypoxia follow adaptive changes for its survival. One such response is to activate hypoxia-inducible factors 1α. Hypoxia microenvironment stimulates cell signaling mechanism to stabilize many proteins such as signal transducer and activator of transcription (STAT3) and protein kinase B (Akt) to adapt against ischemic assaults.[6]

The study was aimed to evaluate the consequences of the left common carotid artery occlusion for 75 min and subsequent reperfusion for 12 h on brain histopathology in normoxic (21% O_2) and subchronic hypoxia pretreated (10% O_2 for 21 days) rats.

Materials and Methods

Animals

Adult male Wistar-strain albino rats (*Rattus norvegicus;* 180–200 g, b.wt.) from central animal house of BLDE Deemed to be University Vijayapur, Karnataka, were obtained. Rats were divided into the three groups (n = 6 in each group) under 12 h light: 12 h dark cycle to avoid diurnal rhythm. Metabolic wire cage (60 cm × 30 cm × 20 cm) is used to keep maximum 3 rats in each one. Standard pellets (Hindustan Lever, Mumbai, India) were used as normal diet, and drinking water were provided *ad libitum* during the entire experimental protocol.

Experimental groups

Table 1 shows the study design of experimental groups of rats which are divided into three groups with six rats in each group.

Subchronic hypoxia exposure

The hypoxia was set up with the inflow of a combination of air (oxygen 10% and nitrogen 90%) that was regulated by an oxygen analyzer (model 175518A, Gold Edition, Vacuum Med, Sanchung, Taipei). CO_2 was absorbed by soda lime (27 granules), and excess humidity was removed by a desiccators. The room temperature was maintained at 24°C–26°C. Twice in a week for 1 h, the hypoxia chamber was opened to clean the cages and provide food and water. The exposure to low oxygen in all the rats of Group 3 was continued for 21 days.[7]

Table 1: Experimental groups of rats (n=6 in each group)

Groups	Experimental protocols
Group 1	Sham operation (surgical incision at carotid triangle, no occlusion and immediate allow for instant reperfusion)
Group 2	Normoxia, 21% oxygen (75 min LCCAO and subsequent reperfusion for 12 h)
Group 3	Hypoxia (10% oxygen) pretreatment for 21 days (subchronic) before 75 min LCCAO and reperfusion for 12 h

LCCAO=Left common carotid artery occlusion

Experimental protocol

Group 1 served as sham, Group 2 was normoxic, and Group 3 served as hypoxic. Electrocardiography (ECG) and pneumogram were recorded using Biopac (MP 45) before, during the surgical procedure and after 12 h of reperfusion. Data were evaluated and analyzed with SPSS version 16.0 (SPSS Inc., Chicago, USA).

Group 1 (sham) was taken as a control group; Group 2 (normoxic) was exposed with normal atmospheric oxygen under normal pressure, and Group 3 (hypoxic) was exposed to subchronic sustained hypoxia by keeping the rats in 10% oxygen and 90% nitrogen for 21 days as per the standard protocol before left CCA occlusion. The occlusion (genuine and sham) of the left common carotid artery at carotid triangle level was performed in all the rats of Group 1, Group 2, and Group 3 under proper anesthesia (Ketamine, 60 mg/kg b.wt and Xylazine, 6 mg/kg b.wt.).[8] Sham operations in Group 1 rats were performed by similar left common carotid artery occlusion (LCCAO) technique except occlusion and allowing instant reperfusion.[9] Once the left carotid artery at the carotid triangle was exposed, it was occluded for 75 min in all the rats of Group 2 and Group 3 [Figure 1]. After 75 min, carotid occlusion was slowly released, surgical incision was closed with catgut, and reperfusion was allowed for 12 h till sacrifice.[10] Utmost postoperative care was taken for each rat. At the end of 12 h, all three groups of rats were euthanized by deep anesthesia. Before the sacrifice, blood samples were collected from retro-orbital plexus. Blood samples were also collected in a similar way before LCCAO from

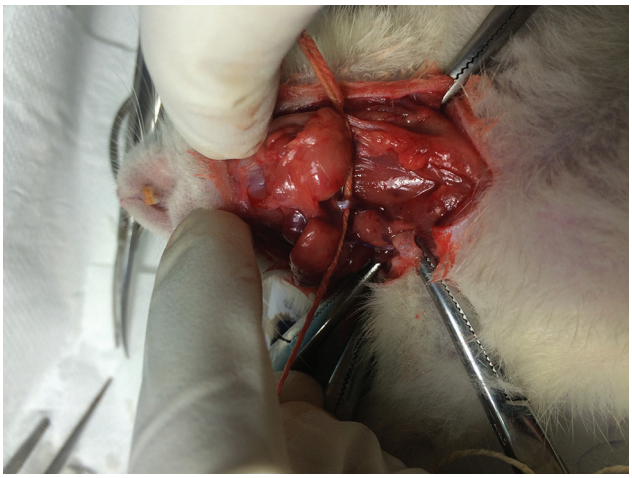

Figure 1: Ligation of the left common carotid artery with silicon thread

all the rats of Group 1, Group 2, and Group 3. The brain was carefully dissected out and immersion fixed in 10% formalin for 2 days. Serum malondialdehyde (MDA) levels as a marker of oxidative stress were assessed in all the three groups of rats before the occlusion and after 12 h of reperfusion by Kei Satoh method.[11] Similarly, serum nitric oxide (NO), a nitrosative stress marker, and also a regulator of oxidative stress were estimated using Griess reagents spectrophotometrically.[12]

Neurological scores

Neurologic examinations were performed on all the rats in Group 1, Group 2, and Group 3 in just before the sacrifice. Neurological scores were evaluated as per standard protocol.[13]

Histopathological procedure

Cerebral hemispheres were sliced into coronal slices of 2-mm thickness. The brain stem was cut vertical to its long axis. The cerebellum also cut into two slices perpendicularly to the folia of the dorsal angle of each hemisphere. Bilateral blocks of the brain were embedded in paraffin wax and sections were cut on a rotary microtome from 2.0 μ to 3.0 μ and stained with routine hematoxylin and eosin (H and E) stains. The brain sections of all animals were examined under a conventional light microscopy (Olympus CH20i) with Samsung Digital Color Camera, Model No. SDC-242, N. J 07094, U. S. A.

Statistical analysis

Results were obtained as mean ± standard deviation values for each group. To determine the significance of intergroup differences, one-way analysis (ANOVA) followed by "*post hoc t*-test" were done.

Ethics

Animal experiments were conducted as per the ethical norms approved by CPCSEA, the Ministry of Social Justice and Empowerment, Government of India (Reg. No. 1076/PO/ERs/S/07 CPCSEA dated Aug 20.,2014), and Institutional Animal Ethical Committee, BLDE University, Vijayapur (BLDE/BPC/641/2016-17 dated Aug 22, 2016).

Results and Discussion

Physiological and biochemical parameters

The ECG pattern (heart rate [HR]), pneumogram (respiratory rate [RR]), and blood pressure (systolic blood pressure [BP], diastolic BP, and mean arterial pressure) were also found within normal ranges during the entire experimental protocol in all the animals of all the groups till sacrifice [Table 2]. Results suggested that no vital parameters such as HR, BP, and RR were affected in all the three groups during the entire experimental procedure. After 12 h of reperfusion and just before sacrifice, we observed cognitive and neurological impairments in Group 2 and Group 3 rats [Table 3]. Results showed the maximum neurological deficit scores in Group 2 (normoxia) rats (35/60; 58%) followed by Group 3 (subchronic hypoxia pretreatment) rats (26/60; 43%). Oxidative and nitrosative stress levels were measured by assessing serum MDA, and NO in Group 2 (normoxic) rats following 12 h of reperfusion showed a significant increase compared to preocclusion levels. In the case of subchronic hypoxia pretreated rats (Group 3), no such significant alteration of MDA and NO levels (pre vs post) were observed [Figures 2 and 3]. Higher MDA and NO levels in Group 3 rats before surgery (pre-LCCAO) may be due to hypoxia-induced lipid peroxidation, and subsequently, the formation of peroxynitrites due to exposure of subchronic hypoxia but such preconditioning actually facilitate greater adaptation from cerebral ischemic stress (post-LCCAO) induced lipid peroxidation. The increase of lipid peroxidation or rise of reactive oxygen species and reactive nitrogen species may probably induce neuronal injury from apoptosis and further contribute to ischemic brain injuries. Our results indicate a greater protection from oxidative stress-induced inflammatory response due to LCCAO in rats preconditioned with hypoxia.[14]

Table 2: Cardiovascular and respiratory parameters of all the group of rats

Group	Status	HR (bpm)	SBP (mmHg)	DBP (mmHg)	RR (cycles/min)
Group 1	Before occlusion	273.45±45.34	100.23±13.23	75.50±15.50	14.34±5.35
	During occlusion	289.45±40.50	96.78±20.50	70.56±10.50	12.50±7.60
	Re-perfusion after 12 h	280.56±47.00	99.00±19.50	73.50±19.00	12.70±5.70
Group 2	Before occlusion	270.25±35.45	104.23±20.45	73.45±11.54	15.50±5.00
	During occlusion	290.45±54.50	105.56±21.34	78.00±18.50	15.50±5.20
	Re-perfusion after 12 h	285.00±67.00	98.90±18.00	70.68±12.50	13.75±7.50
Group 3	Before occlusion	290.25±76.50	100.45±15.34	69.50±12.45	14.45±5.50
	During occlusion	291.45±6.50	103.34±20.00	73.92±18.34	15.50±6.00
	Re-perfusion after 12 h	285.00±67.00	98.50±20.00	72.23±11.02	15.70±7.00
P		0.095	0.120	0.085	0.110

Values are mean±SD of each group (*n*=6). Group 1=Sham operated, Group 2=Normoxic, Group 3=Subchronic hypoxia pretreated, HR=Heart rate, SBP=Systolic blood pressure, DBP=Diastolic blood pressure, RR=Respiratory rate, SD=Standard deviation

Table 3: Neurological deficit scores after 12 h reperfusion and before sacrifice

Neurological signs	Score (0-5 scale) for each rat	Total scores (n=6)		
		Group 1	Group 2	Group 3
Failure to extend left forepaw fully	1	0	6	4
Circling to the left	2	0	12	12
Falling to the left	3	0	9	6
Did not walk spontaneously	4	0	8	4
Grand total score	10 × 6=60	0	35 (58%)	26 (43%)
Mean±SD		0	5.833±3.430	4.333±3.141*

Mean±SD values of Group 2 and Group 3 are statistically significant from each other (*P<0.05). Group 1=Sham operated, Group 2=Normoxia, Group 3=Hypoxia pretreatment for 21 days, SD=Standard deviation

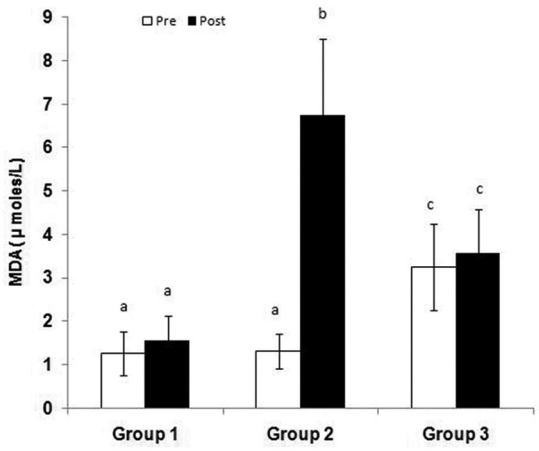

Figure 2: Serum malondialdehyde levels in Group 1 (sham), Group 2 (normoxia), and Group 3 (subchronic hypoxia pretreated) rats before (pre) left common carotid artery occlusion and 12 h after reperfusion (post). n = 6 rats in each group. Values with different superscripts (a and b) are significantly differ from each other (P < 0.05)

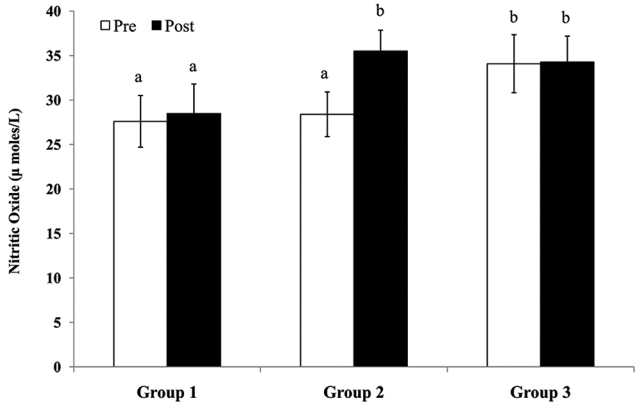

Figure 3: Serum nitric oxide levels in Group 1 (sham), Group 2 (normoxia), and Group 3 (subchronic hypoxia pretreated) rats before (pre) left common carotid artery occlusion and 12 h after reperfusion (post). n = 6 rats in each group. Values with different superscripts (a and b) are significantly differ from each other (P < 0.05)

Histopathological observations in cerebral cortex

Sections of all the three groups under H and E stain were studied serially on the left and right cerebral hemispheres of the brain (n = 6), from the frontoparietal cortex and dorsolateral portion of the neostriatum. Figure 4a to l shows H- and E-stained sections of the left and right cerebral hemispheres of the brain. The sections from sham animals showed normal cerebral cortex consisting of gray matter made of cell bodies of neurons and glial cells along with their processes blood vessels and white matter made up of bundles of axons. No visible pathology was noted in the group. The blood vessels appeared normal on both sides of the hemispheres [Figure 4c and d]. Group 2 (normoxic with left-CCA occlusion) reveals histopathological changes in gray matter of left cerebral cortex with a decrease in number of pyramidal cells and stellate (granular) cells which appear to be small, multipolar, and vacuolated to eosinophilic cytoplasm and pyknotic nuclei [Figure 4e]. There were foci of wedge-shaped areas of cerebral infarcts with diffuse interstitial edema of the cerebrum [Figure 4g]. The junction between gray and white matter was blurred. Right cerebral hemisphere of the brain (nonoccluded side) showed normal brain parenchyma [Figure 4f and h]. Group 3 (subchronic hypoxia pretreated rats with left-CCA occlusion subsequently sacrifice after 12 h) shows in the gray matter of cerebral cortex, a decrease in number of pyramidal cells, small multipolar stellate (granular) neurons, and vacuolated eosinophilic cytoplasm with pyknotic nuclei having features of early cellular damage (Red cells) in the left cerebral hemisphere [Figure 4i]. There were also foci of interstitial edema of the left cerebrum but without the features of cerebral ischemia which were observed in case of Group 2 experimental animals [Figure 4k]. Besides these findings, blurring of the junction between gray and white matter was also noticed in Group 3 rats (subchronic hypoxia pretreated). Right cerebral hemisphere of brain (nonoccluded side) of Group 3 rats showed normal brain parenchyma. Dilated blood vessels, lined with prominent blood vessels cells on the right side of the hemispheres, were also observed in Group 3 rats [Figure 4j and l].

Histopathological observations in subcortex

There were no changes in the subcortical structures of sham-operated rats [Figure 5a]. In case of Group 2 (normoxic), most of the caudate nuclei, internal capsule, globus pallidus, substantia nigra, and putamen of basal ganglia showed small lacunar infarcts [Figure 5b],

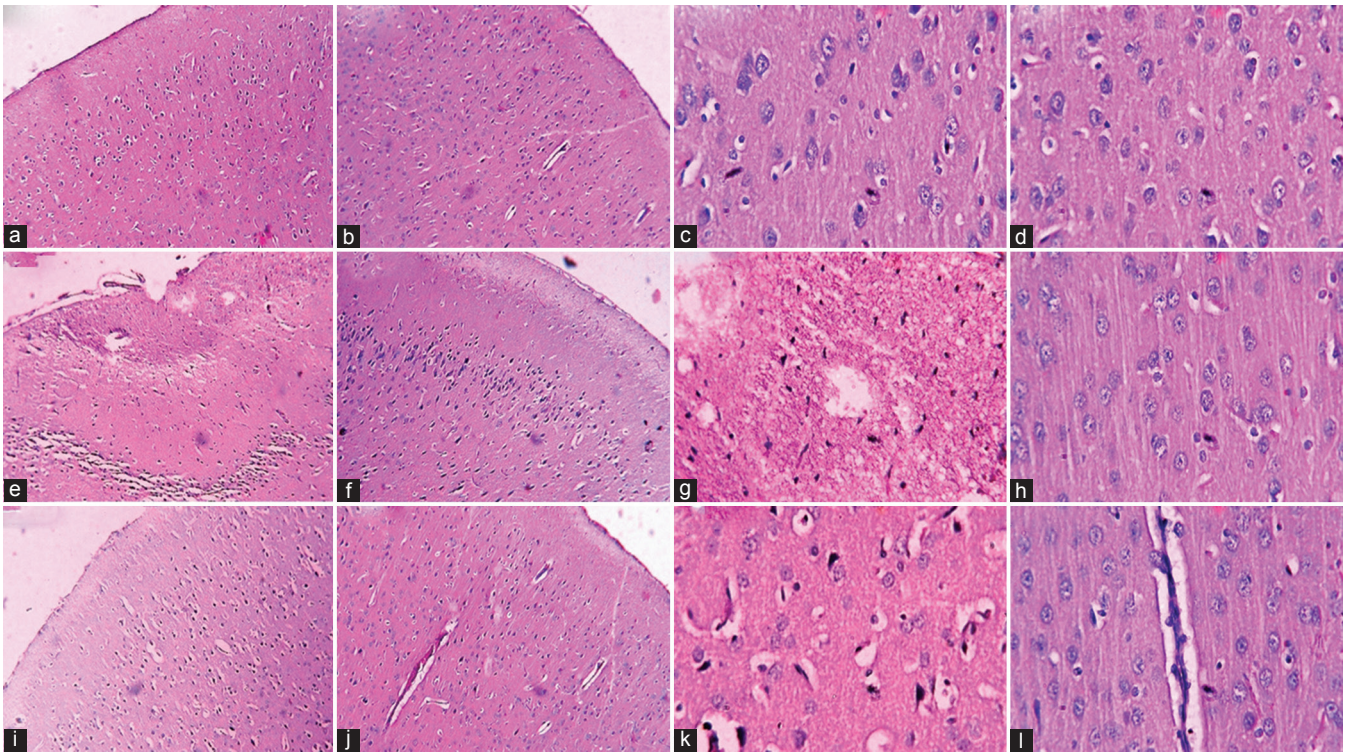

Figure 4: Hematoxylin and Eosin stain showing (a) sham's left cerebral cortex (×10), (b) sham's right (×10), (c) sham's left cerebral cortex (×40), (d) sham's right (×40), (e) normoxic left (occluded) cerebral cortex (×10), (f) normoxic right (nonoccluded) (×10), (g) normoxic left (occluded) cerebral cortex (×40), (h) normoxic right (nonoccluded) (×40), (i) hypoxia pretreated left (occluded) cerebral cortex (×10), (j) hypoxia pretreated right (non occluded) (×10), (k) hypoxia pretreated left (occluded) cerebral cortex (×40), and (l) hypoxia pretreated right (nonoccluded) (×40)

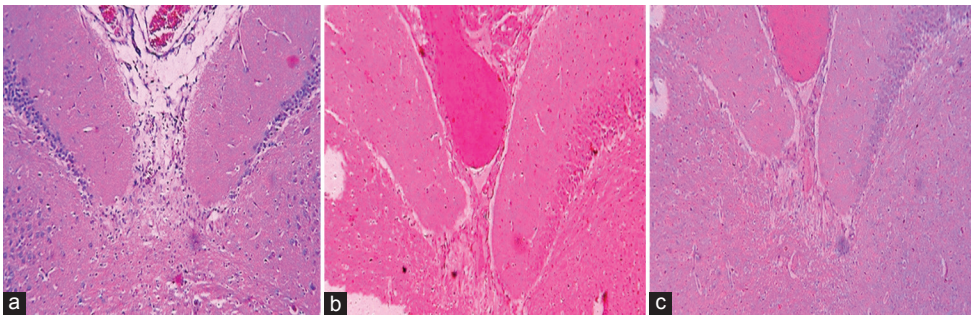

Figure 5: Photomicrograph stained with hematoxylin and eosin stain showing subcortical structures mainly the caudate nuclei, internal capsule, globus pallidus, substantia nigra, and putamen of basal ganglia (×10) of (a); sham's left and right side, (b); normoxic left (occluded side) and right (nonoccluded) side, and (c); subchronic hypoxia pretreated left (occluded side) and right (nonoccluded) side

whereas Group 3 (subchronic hypoxia pretreated) rats such small lacunar infarcts were found relatively less as compared to normoxic rats. This group showed pallor of staining and vacuolization of the white matter [Figure 5c].

The partial/incomplete infarct section, defined as the area with lack of staining (pallor or failure to perfuse) of the cortex, brain edema, cell body degeneration, and suggestive of early focal neuronal damage (red neurons), was determined from serially cut sections from the frontoparietal cortex and dorsolateral portion of the neostriatum of both left (ischemia-induced) and right brain hemispheres of all the sham, normoxic, and subchronic hypoxia pretreated rats (n = 6). In normoxic rats, there were no areas of ischemia-induced brain changes in the right side of the (nonoccluded) cerebral hemisphere and left side (left occluded side), there were features of diffuse global ischemic brain damage involving mainly frontal, frontoparietal regions of the cortex, and basal ganglia (striatum), particularly in the lateral segment of the caudate.[15] The striking feature in both sides of the brain in subchronic hypoxia pretreated rat was the dilated blood vessels lined by prominent endothelial cells signifying a compensatory mechanism in response to subchronic hypoxia. The reduced brain damage in subchronic hypoxic pretreated rats was well correlated morphologically with vascular adaptive

changes whereas the changes of occipital cortex and medial striatum were concerned only uncommonly and variable elsewhere. In contrast to the left side of the subchronic hypoxia pretreated rats with less ischemic damages, the left side of the normoxic rats showed more extensive damages.[16]

Our study demonstrated a reduction in brain edema associated with reduced infarct volume in animals preconditioned (21 days) to subchronic hypoxia by comparing the infarct size difference in brain between the left (ischemic site) and right (nonischemic) hemispheres [Figure 4e-k]. Hypoxia preconditioned for 21 days before cerebral ischemia possibly reduced the ischemic alterations and decreased ischemic brain damage after focal ischemia.[17] These observations are further supported by neurological deficit scores and serum MDA and NO levels while comparing in both Group 2 and Group 3 rats. Results also showed that subchronic hypoxia preconditioning (Group 3) increases both serum MDA and NO levels at pre-LCCA occlusions as compared to respective normoxic Group 2 experimental animals [Figures 2 and 3]. Interestingly, both serum MDA and NO levels of Group 3 did not further change after occlusion from preocclusion values at the end of 12 h reperfusion (post) period. Nonsignificant changes of both serum MDA and NO between pre- and post-LCCA occlusion in Group 3 (subchronic hypoxia pretreated) reflects lesser LCCA occlusion induced oxidative and nitrosative stresses in Group 3 (subchronic hypoxia pretreated) as compared to Group 2 (normoxia). Some studies on mice showed acute hypoxia preconditioning protect against transient focal cerebral ischemia, but our study demonstrated for the first time that subchronic preconditioning with hypoxia protects the brain injury against transient focal ischemia induced by unilateral carotid artery occlusion.[18]

This hypoxic model could serve as a helpful plan to remodel ischemic brain injury from cerebral ischemia including stroke. The exact mechanism underlying this neuroprotective effect of preconditioned/pretreated subchronic hypoxia remains obscure. Although further studies are needed to establish true cerebrovascular pathophysiology on unilateral common carotid artery occlusion with reference to subchronic hypoxia pretreatment, it may be considered as a possible protective strategy to ameliorate ischemic brain injury from cerebral focal ischemia or stroke. Further, it has been reported from a clinical study that hypoxia preconditioning provides more protective cardiometabolic profile.[19] Hypoxia preconditioning was also found to be beneficial to promote mesenchymal stem cells proliferation which facilitates intrastriatal transplantation and support therapeutically to fight against Parkinson's disease.[20]

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Conflicts of interest

There are no conflicts of interest.

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Dietary Interventions in Liver Disease

Foods, Nutrients, and Dietary Supplements

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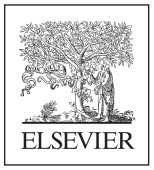

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Chapter 26

Heavy Metals and Low-Oxygen Microenvironment—Its Impact on Liver Metabolism and Dietary Supplementation

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1. INTRODUCTION

The **term heavy** metal refers to any metallic chemical element that has a relatively high density and is toxic or poisonous at low concentrations. The heavy metals constitute major fraction of the periodic table and are generally interpreted to include those metals from periodic table groups IIA through VIA. Examples of heavy metals are nickel, lead, mercury, cadmium, chromium, etc. Heavy metals cannot be degraded or destroyed. It enters in our bodies via food, drinking water, and air and primarily targets liver and other metabolically active tissues. As trace elements, some heavy metals (e.g., copper, selenium, zinc) are essential to maintain the metabolism of the human body. However, at higher concentrations they can lead to poisoning. Heavy metal poisoning may occur through various ways such as drinking water through lead pipe or occupational exposure (lead–cadmium or nickel–cadmium batteries) or stainless steel industries (nickel-chromium), refineries or petrochemicals (nickel, lead, cadmium), jewelry, etc.[1] Hypoxia belongs to the most serious factors that can directly impair the function of metabolic pathways in the animal cell. The exposure of experimental animals to hypoxia has been widely used in many morphological and physiological studies. Physiological hypoxia induces cell signaling process for the formation of new blood vessels (angiogenesis) to regulate vascular tone during developmental stage.[2] Physiological oxygen levels (PO_2) in healthy body varies from −100 Torr in the alveoli to <10 Torr in medulla of kidney and retina.[3] Tissue exposure to low-oxygen tension is observed in several physiological and pathological conditions such as ischemia for shorter duration or in case of the high-altitude inhabitants or any other chronic diseases for longer duration of hypoxic exposure. In both cases, hypoxic cells are programmed to rapid adjustment to maintain O_2 supply to most vital organs such as heart and brain. It is understood that atherosclerosis, stroke, or vascular occlusion leads to tissue ischemia followed by hypoxia. Tissue hypoxia also develops through immune cell infiltration in vascular dysfunction during chronic inflammation process.[3,4] It has been observed that hypoxia absurdly stimulates free radicals release from the mitochondria that control the transcriptional and posttranslational response to low-oxygen conditions.[5] Hypoxia-induced generation of reactive oxygen species (ROS) has been a subject of theoretical and practical dispute as experimental designs able to quantitatively evaluate ROS formation. Under normoxic conditions, ROS (constantly generated in erythrocytes) are mostly counteracted by their endogenous (superoxide dismutase, glutathione peroxidase, catalase or reduced glutathione) or exogenous (vitamin C, vitamin E, etc.) antioxidant defense systems. Studies also show that wild-type human hepatoma cells (Hep3B) increase ROS generation of metal-activated cell signaling pathways during hypoxia.[5,6] Valko M et al.[7] stated that "hypoxia-activated gene transcription via a mitochondria-dependent signaling process induces increased ROS." The mechanisms by which mammalian cells adapt to acute and chronic alterations of oxygen tension are extremely important to understand the exact homeostasis regulation to counteract hypoxia-induced cell damage as a therapeutic strategy. Heavy metals are capable to induce expression of HIF-1 transcriptional factor and vascular endothelial growth factor (VEGF) genes through the

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phosphatidylinositol 3-kinase or Akt pathway or ROS.[8] Heavy metals–induced alteration of the hypoxia signaling system influenced by metal-induced oxidative stresses are responsible for progression of metastasis.[9] This chapter gives a brief understanding of current state of knowledge of chronic hypoxia and its influence on generation of ROS by inducing oxidative stress in the physiological system. The review will also provide recent update of heavy metal nickel toxicities on oxidant and antioxidant balance and molecular interaction of chronic hypoxia and heavy metal nickel (Ni) in the physiological system in vivo. Cellular hypoxia causes an initiation of hypoxia-response genes responsible for angiogenesis, oxygen transport, and metabolism.[10] Chronic hypoxia stimulates NF-κB gene expressions and it reduces KLF4, which further leads to an enhanced NOS2 expression (Fig. 26.1). **Both hypoxia and heavy metal exposure induce generation of ROS and increase expression of p53, NF-kβ, AP-1, MAPK, and HIF-1α. The increase expression of all these transcription factors leads to either cellular adaptation or cell death**.[11] **It is also to be mentioned that** hypoxic injury due to metal assault or hypoxia exposure causes "cell death" by cells swelling, plasma and nuclear membrane disruption, cellular lysis in association with acute inflammation that may exacerbate the initial hypoxic injury response. However, the alternative mode of cell death, apoptosis, is also possible (Fig. 26.1). During apoptosis, the cells use their molecular machinery to shrink or expand into membrane-bound apoptotic bodies, with or without nuclear fragments that are easily phagocytosed by adjacent tissue cells or macrophages and minimize any acute inflammatory response.

Liver is an important metabolically active organ. It stores additional nutrients in the form of glycogen and lipids. During the need of the hour these nutrients yield energy and keep all the vital functions intact. Hepatocytes also synthesize plenty of proteins including albumin and clotting factors. Furthermore, it synthesizes cholesterol and triglycerides. Another important function of liver is to produce bile salts which are essential for digestion and absorption of lipids. The hepatocytes also play an important role as the center of detoxification in the body, influencing drug metabolism and breakdown of hormones. This organ is an important source of storage of vitamins such as B12, A, D, K and folic acid, besides being an important source of iron. To make the liver a well-functioned organ, a considerable amount of oxygen is needed. Altered metabolic functions due to toxic insults or metabolic stress due to hypoxia or heavy metal toxicities disturb oxygen homeostasis in liver and lead to serious liver diseases. Most of the cases, malfunction of liver leads to fatty liver symptoms and the cell signaling pathways greatly affected is oxygen dependent, hence hypoxia may be considered as an important cause of liver malfunction.[12] Interestingly, hypoxia and divalent heavy metals such as nickel (Ni) and lead (Pb) generate ROS and disturbed oxidant/antioxidant balance which is linked to the transcriptional factor HIF-1α. The results from the author's laboratory showed both divalent cationic heavy metal (Ni and Pb) and chronic sustained hypoxia stimulate the production of HIF-1α transcription factor and VEGF gene expression in metabolically active tissues in similar molecular mechanism. Heavy metals cause oxidative stress by inducing the generation of ROS; reducing the antioxidant defense system of cells via depleting glutathione; interfering with some essential metal; inhibiting sulfhydryl (SH), dependent enzyme, or antioxidant enzymes activities; and/or increasing susceptibility of cells to oxidative attack by altering membrane integrity and fatty acid composition.[13,14]

Nutrients such as vitamin C or E are found to be the most effective circulatory antioxidant in human system.[15] Ascorbic acid or vitamin C prevents lipid peroxidation, oxidation of low-density lipoproteins, and advanced oxidation protein products.[16] Vitamin C may comprise the first line of defense system in RTLF against external pro-oxidative assaults.[17] It has been reported that intracellular depletion of ascorbic acid aggravated some heavy metal (nickel, cobalt, etc.)-induced carcinogenicity and acute toxicity.[18] The effect of simultaneously supplemented vitamin C on experimental nickel treatment

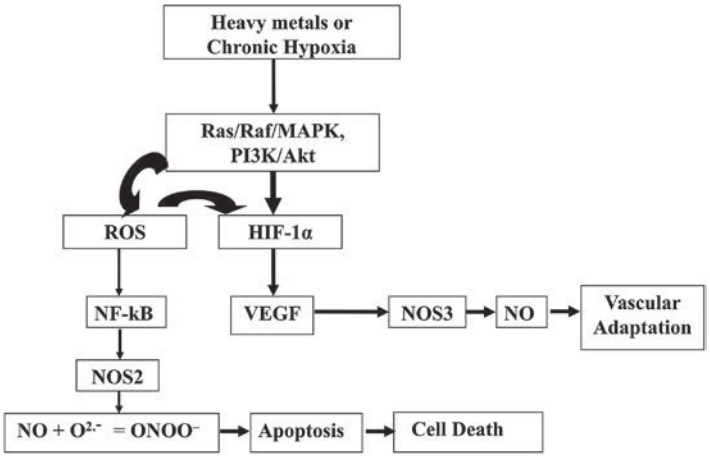

FIGURE 26.1 Graphic representation showing heavy metals or hypoxia-induced common cellular abnormalities. *NF- kB*, nuclear factor-kappa B; *NO*, nitric oxide; *NOS*, nitric oxide synthase; *ONOO⁻*, peroxynitrate; *HIF-1α*, hypoxia inducible factor 1α; *ROS*, reactive oxygen species; *VEGF*, vascular endothelial growth factor.

shows ascorbic acid is capable to reduce intestinal absorption of nickel. The mechanism involves that vitamin C is capable to reduce ferric iron to ferrous iron in the duodenum, thus availability of divalent ferrous ion increases which competes with nickel or lead also as divalent cation for intestinal absorption.[19] Recent reports indicate the capability of ascorbic acid as a regulatory factor may influence gene expression, apoptosis, and other cellular functions of living system exposed to heavy metals.[20] This chapter elaborately explains the role of dietary supplementation of nutrients such as vitamins and other nutrients in heavy metals such as nickel and lead, which induces altered hepatic functions in low-oxygen microenvironments.

2. HEAVY METALS AND ITS INTERACTIONS

Heavy metals interact with the biological system in a complex manner. Even elemental speciation of the metals also matters in its interactions with systems. The **term heavy** metal refers to any metallic chemical element that has a relatively high density and is toxic or poisonous at low concentrations. The heavy metals constitute major fraction of the periodic table and are generally interpreted to include those metals from periodic table groups IIA through VIA. Examples of heavy metals are nickel, lead, mercury, cadmium, chromium, etc.[1]

2.1 Heavy Metal Toxicities: Nickel and Lead

There are five priority substances which are selected by WHO for the nickel risk assessment. They are nickel powder, nickel sulfate, nickel chloride, nickel carbonate, and nickel nitrate. Nickel powder (T; R48-23) has been classified in chronic toxicity classification as per environmental risk assessment report on nickel. $NiSO_4$, $NiCl_2$, $NiCO_3$, and $NiNO_3$ are classified as carcinogen class I (by inhalation), reproductive toxicants class II (may cause harm to unborn children), and chronic toxicants (T; R48-23). If particle size of nickel powder found to be less than 0.1 mm, it is classified as T; R52-53 (harmful to the aquatic environment).[21] Acute toxicity in humans, which results from absorption through the gastrointestinal tract or by inhalation through lungs, was first reported by Sunderman in 1954.[22] Further studies showed that a single dose oral LD50 in rats for the less-soluble nickel oxide and subsulfide was >3600 mg Ni/kg bwt, whereas the oral LD50 for the more soluble nickel sulfate and nickel acetate ranged from 39 to 141 mg Ni kg^{-1} bwt in rats and mice.[23] The metal is not only an allergen but also a potential immunomodulatory and immunotoxic agent in humans.[19] Weischer et al.[24] reported that oral administration of nickel as NiCl2 in male rats over a period of 28 days at concentration of 2.5, 5.0, and 10.0 µg/mL in drinking water (0.38, 0.75, or 1.5 mg/kg day) resulted in significant dose-dependent hyperglycemia, decrease in serum urea, and significant increase in urine urea. At 0.75 mg/kg doses, increased leukocyte count was also observed. It was noticed that exposure of dietary nickel sulfate hexahydrate (100, 1000, or 2500 ppm) to dogs for 2 years failed to produce significant signs of compound-related toxicity.[25] The toxicity of the different nickel compounds is related to its solubility, with soluble nickel sulfate being the most toxic and insoluble nickel oxide being the least toxic. The difference in the toxicity across compounds is probably due to the ability of water-soluble nickel compounds to cross the cell membrane and interact with cytoplasmic proteins.[21]

Lead poisoning can affect almost all parts of the body, but its effects are most pronounced on the central nervous system and kidneys. Lead can impair cognitive development, which can lead to learning disabilities and behavioral problems. Acute lead exposure can cause encephalopathy, severe abdominal pain, vomiting, diarrhea, coma, seizures, and, in some cases, death. Chronic exposure can cause weakness, prolonged abdominal pain, anemia, nausea, weight loss, fatigue, headache, and loss of cognitive function. Chronic, low-level lead exposure can be asymptomatic until kidney function starts to deteriorate.[11] Lead has no known physiologically relevant role in the body, and its harmful effects are myriad. Lead and other heavy metals create reactive radicals, which damage cell structures including DNA and cell membranes. Lead also interferes with DNA transcription enzymes that help in the synthesis of vitamin D, and enzymes that maintain the integrity of the cell membrane. Anemia may result when the cell membranes of red blood cells become more fragile as the result of damage to their membranes. Lead interferes with metabolism of bones and teeth and alters the permeability of blood vessels and collagen synthesis. Lead may also be harmful to the developing immune system, causing production of excessive inflammatory proteins; this mechanism may mean that lead exposure is a risk factor for asthma in children. Lead exposure has also been associated with a decrease in activity of immune cells such as polymorphonuclear leukocytes. Lead also interferes with the normal metabolism of calcium in cells and causes it to build up within them. It is metabolized by CYP450 to trimethyl lead (TML). Mechanisms of its toxicity include damage to membranes, disturbances in energy metabolism, and direct interference with neurotransmitter synthesis. Symptoms of its toxicity include nausea, vomiting, diarrhea associated with nervous system problems such as irritability, headache, and restlessness. Chronic heavy sniffing of leaded gasoline results in signs of dementia and encephalopathy, with cerebellar and corticospinal symptoms. Lead primarily acts by competing with endogenous cations on protein-binding sites. In particular, lead can substitute both calcium and zinc in

numerous proteins. Among stress-response genes that were upregulated by lead treatment, GFAP, microsomal glutathione S-transferase, mitochondrial 10 KDa heat shock protein, and HSP70 are all involved in general cellular responses to stress. Daphnia hemoglobin gene was greatly expressed following lead exposure.[26]

3. HYPOXIA PATHOPHYSIOLOGY

Hypoxia is a pathological condition in which the body as a whole (generalized hypoxia) or a region of the body (tissue hypoxia) is deprived of adequate oxygen supply. Variations in arterial oxygen concentrations can be part of the normal physiology, for example, during strenuous physical exercise. In healthy humans, there is a range of physiological oxygen levels within the tissues of the body, ranging from PO2 values of −100 Torr in the alveoli of the lungs to less than 10 Torr in tissues such as the medulla of the kidney and the retina.[27]

3.1 Hypoxia Microenvironment

Physiological hypoxia is an important microenvironmental signal in a range of processes including new blood vessel formation (angiogenesis) during development and wound healing, the regulation of vascular tone, and the response to exercise. However, tissue hypoxia is also associated with a diverse and wide range of pathophysiological processes including (but not limited to) vascular disease, chronic inflammation, and cancer.[2] In vascular diseases such as atherosclerosis and stroke, vascular occlusion leads to acute or chronic tissue ischemia with resultant hypoxia. In chronic inflammatory diseases, the greatly increased metabolism of inflamed tissue due to immune cell infiltration matched with vascular dysfunction leads to tissue hypoxia.[27] Hypoxia results from conditions such as ischemia, hemorrhage, stroke, premature birth, and other cardiovascular difficulties. Among which hemorrhagic shock is the leading cause of death and complications in combat casualties and civilian settings. It has been shown to cause systemic inflammation response syndrome, multiple organ dysfunctions, and multiple organ failure.[28] Hypoxia has been shown to lead to increases in intracellular free calcium concentration (Ca^{2+}), 5-lipoxygenase, lipid peroxidation, cycloxygenase (COX), constitutive nitric oxide synthase (cNOS), leukotriene B4 (LTB4), prostaglandin E2 (PGE2), interlukins, tumor necrosis factor-α (TNF-α), caspases, complement activation, kruppel-like factor 6 (KLF6), inducible nitric oxide synthase (iNOS), heat shock protein 70 kDa (HSP-70), and hypoxia-inducible factor-1α (HIF-1α). The sequence of their occurrence provides the useful information for studying the mechanisms underlying the hypoxia-induced injury as well as therapeutic targets to prevent or ameliorate the injury.[11] Hypoxia, or inadequate oxygenation, causes various responses within the body. Its effects are usually mediated via the activation of HIF-1. HIF-1 activation can lead to upregulation of various genes such as erythropoietin and growth factors that help tissues adjust to the decreasing oxygen availability. Semenza and Wang defined a binding site critical for the hypoxia-inducible function, which involves a transcription factor induced by hypoxia. Subsequently, they purified a DNA-binding complex bound to the HRE by affinity purification using oligonucleotide with the HRE sequence and thus identified the encoding cDNAs.[29]

3.2 Hypoxia and Heavy Metals (Nickel and Lead)

Over the recent years, induction of signaling pathways that regulate key cellular responses related to cancer growth and progression by metals has been the focus of many studies. The unraveling of these pathways and the deciphering of their interplay with metals should allow a better understanding of metal toxicity and hopefully will enable development of prophylactic strategies and therapeutic approaches. Authors' laboratory and works of Leonard (2004) have shown the mechanisms of toxicities caused by heavy metals such as nickel and lead, emphasizing on the involvement of the hypoxia signaling pathway by metal-induced generation of ROS and oxidative stress generation.[20,30] Hypoxia-induced factor HIF-1 controls precise oxygen homeostasis by modulating expression of several cancer-related genes, including heme oxygenase 1 and vascular endothelial growth factor. The carcinogenic metals such as nickel, lead (Pb), or chromium have been known to activate HIF-1.[8,31] It has been observed that heavy metal–induced ROS generation during the exposure of cells to metals mimic hypoxia-like symptoms.[32] The mechanisms of carcinogenesis caused by heavy metals such as nickel emphasizes on the involvement of the hypoxia signaling pathway by metal-induced generation of ROS and oxidative stress generation in cancer progression.[9] One of the pathways by which heavy metals such as nickel and lead induce intracellular hypoxia is by reducing heme biosynthesis. Low level of heme reduces intracellular oxygen tension and simply intracellular low Fe^{2+} and low oxygen tension inhibit PHD_2 (prolyl hydroxylases). Under normoxic conditions, HIF-1-prolyl hydroxylases (PHD) hydroxylate the prolyl residues at amino acids 402 and 564. These enzymes require dioxygen, Fe^{2+}, ascorbate, and two oxoglutarates for activity. The hydroxylated peptides interact with an E3 ubiquitin-protein ligase complex composed of

pVHL (von Hippel–Lindau tumor suppressor protein), elongin B and C, and Cullin 2 (CUL2), and then poly-ubiquitinized, resulting in HIF-1α degradation by the 26S proteasome. Under hypoxic conditions, HIF-1α is not hydroxylated because the major substrate, dioxygen, is not available. The unmodified protein escapes the VHL-binding, ubiquitination, and degradation, and then dimerizes HIF-1α and stimulates the transcription of its target genes.[33]

4. HEAVY METALS IN LIVER DISEASES

Heavy metals related to cardiovascular and pulmonary disorders are quite common and reported elsewhere, but currently, heavy metals and its impact on liver disease are considered as serious as before.[34]

4.1 Heavy Metals and Liver Pathophysiology (Nickel and Lead)

Fatty liver disease is considered as one of the important causes of chronic liver disease, and it is manifested by a complicated etiology. Heavy metal–induced changes in liver pathophysiology including fatty liver changes are under non–alcoholic fatty liver disease (NAFLD) category. Fatty liver induces a prolonged inflammatory response which leads to fat accumulation in the liver due to hepatocellular damages. One study showed that heavy metals caused NAFLD in men under 24 BMI. In case of overweight and obese, it becomes more serious. It was also observed that lead (Pb) causes more liver damage than nickel (Ni).[35] Heavy metals such as nickel and lead cause hepatocellular hyperplasia, which may lead to even carcinoma of liver. Studies on nickel clearly showed elevation of liver aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyltranspeptidase.[6,36] Furthermore, it has been found that at advanced stages of hepatic cirrhosis, there was a significant increase of hepatic levels of nickel.[37] Another study also showed lower serum nickel concentration in liver cirrhosis patients which attributes a possible reduction of hepatic synthesis of nickel transport protein, i.e., nickeloplasmin and albumin.[38] Similarly, study also showed that lead and mercury are linked with NAFLD.[39] Furthermore, it was observed that lead (Pb) become conjugated in liver and stored there in highest concentration. Lead exposure on experimental animal showed an elevation of AST, ALT, and alkaline phsophatase, which clearly indicate a possible liver failure.[40] The most common pathways for metal-induced hepatotoxicity is through free radicals due to oxidative stress. The free radicals which are generated due to heavy metal exposure damage cell membrane lipid bilayers, nucleic acids, and enzymes. These in turn causes functional impairment of cell integrity and disturbs cytoprotective systems. Furthermore, it imparts oxidant and antioxidant imbalances and leads to cellular injuries. The mechanisms of hepatotoxicities are through affecting hepatic mitochondrial respiratory systems by reducing cytochrome *c* oxidase activity. Excessive accumulation of heavy metals also disturb hepatic calcium regulatory system by damaging microsomal calcium sequestration and damaging hepatocellular DNA, which further leads to carcinoma of liver.[41]

4.1.1 Nickel and Hepatotoxicities

A transient increase in serum bilirubin was observed in 3 out of 10 workers who were hospitalized after drinking water from a water fountain, contaminated with nickel sulfate.[41] In rats, decreased liver weight was observed following exposure for 28 days to 2 year to 0.97–75 mg/kg day of nickel chloride or nickel sulfate.[42] Recent studies on rats by Das et al.[43] revealed a nickel sulfate–induced degenerative effect on hepatic tissue They have observed that after the intraperitoneal injection of nickel sulfate, normal hepatic architecture was greatly altered, along with appearance of vacuolated cytoplasm (fatty liver), eccentric nuclei, and Kupffer cell hypertrophy. One report described decreased hepatic and renal transaminase activities after nickel treatment in rats, which was found more deleterious in a protein-restricted dietary regimen.[44] Nickel sulfate also decreases the liver ascorbic acid and cholesterol levels in rats.[6] Misra and coworkers showed that a single intraperitoneal injection of nickel (II) acetate increased lipid peroxidation and glutathione-S-transferase activity in rat liver and kidney while concomitantly decreasing the glutathione concentration and glutathione reductase activity.[45] The same group found that the nickel-induced hepatic lipid peroxidation in different strains of mice was concurrent with nickel's effect on antioxidant defense systems in liver and kidney.[46] The magnitude of nickel-induced lipid peroxidation showed a reverse correlation with the extent and direction of its effect on glutathione and glutathione peroxidase glutathione reductase but not on CAT, SOD, or glutathione-S-transferase.[46] Das et al.[47] showed, after the nickel treatment of rats, a significant rise in hepatic lipid peroxides and a decrease in antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activities and in the hepatic glutathione concentration. The alteration of oxidant and antioxidant balance due to hepatic lipid peroxidation indicates an elevation of enzyme phospholipase activities while peroxidic disintegration of various subcellular organelles and membrane lipid layers with nickel exposure. Furthermore, it may be postulated that nickel causes Kupffer cell hyperactivity through inflammatory cytotoxic mediators along with fatty liver changes and

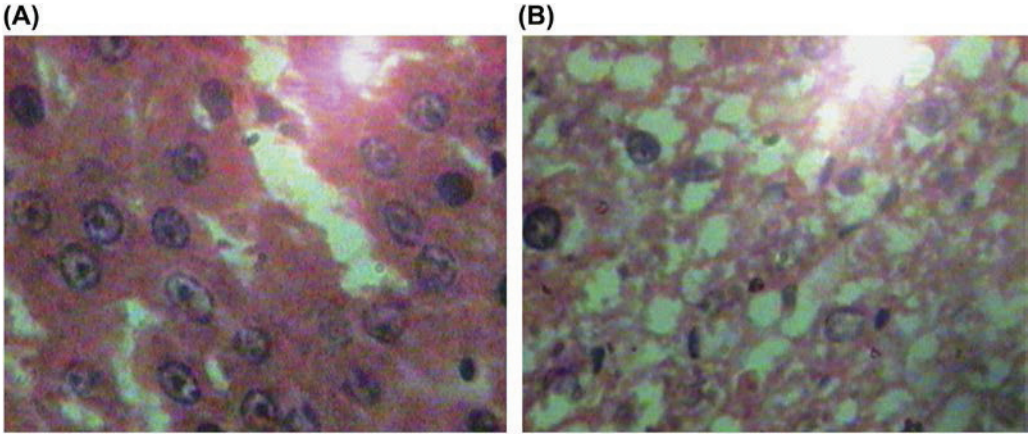

FIGURE 26.2 Normal (A) and nickel sulfate (B)–treated rat liver histopathology (45×).

eccentric nuclei in hepatocellular architecture. Nickel-induced changes of hepatic SOD, CAT, and GSH-Px reveal possible interaction of free radicals and hepatic enzymes and damaging SH protective mechanism against lipid peroxidation.[47] Study also revealed that nickel or some other heavy metals cause alteration in hepatic HMG-CoA reductase activities and disturb LDL-receptor gene expression. It ultimately changes the lipid profile of physiological system.[48,49] Another study showed that nickel induced severe liver damage as indicated by rise of SGOT, SGPT, and ascorbate-cholesterol metabolism in experimental rats. The study also showed that nickel sulfate causes decrease in absolute liver weight without altering hepatosomatic index which is indicative of hepatic degenerative changes.[50] Increased activity of SGOT and SGOT after nickel exposure reflects possible leakage of hepatic enzymes from liver cytosol in circulatory system due to nickel-induced cellular damages.[51] Another observation on nickel-induced hyperglycemia in experimental animals indicates a marked reduction in hepatic fructose-2-6-bisphosphate, which is an indicator of gluconeogenic and glycolytic pathways suggestive of increase of liver gluconeogenesis.[52]

In histopathological studies in the author's laboratory, the liver showed congestion of central veins and sinusoids and some hepatocytes suffered from vacuolar degeneration, fatty changes, etc. (Fig. 26.2A and B). Mathur et al. also observed the same in nickel sulfate–treated rats.[53] Results from the author's laboratory are in agreement with those obtained by El-Saeed and Mekawy[54], Ptashynski and Klaverkamp,[55] and Sobecka[56]. Nickel intoxication causes a vacuolization of the cytoplasm, the increase in numbers of pyknotic nuclei, and the decrease in glycogen content in hepatocytes.[57] The hydropic degeneration of hepatocytes may be due to the irritation of toxic metabolites and impairment of potassium sodium pump that disturbs the ion exchange through the cell wall. The increased oxidative stress, the formation of ROS as well as depletion of cellular antioxidant level may be resulted in histopathological changes of liver. Heavy metal–induced interstitial fibrosis, increased numbers of pyknotic nuclei, as well as necrosis in hepatocytes have also been reported earlier.[58]

4.1.2 Lead and Hepatotoxicities

Like nickel, lead too raises serum LDL-cholesterol, VLDL-cholesterol, total cholesterol and triglycerides, and decreases serum HDL-cholesterol and HDL/LDL ratio. It may be due to changes of the gene expression of hepatic enzymes and LDL receptor synthesis. Defects in the LDL-receptor interfere with cholesterol uptake from the bloodstream, which in turn causes excess cholesterol synthesis in the liver and high levels of serum total cholesterol and LDL-cholesterol.[48] The improvement of serum lipid profile also reflects normalization of liver P450 enzyme system function by α-tocopherol.[59] Lead generates long-lived ROS. These might cause oxidative stress that results in oxidative deterioration of biological macromolecules leading to oxidative damage to the hepatic cells.[60] In an experimental study, lead acetate induced increase plasma MDA with decreased hepatic SOD, CAR, and GSH-Px were noticed which are indicative of hepatic oxidative stress.[61] During hepatotoxicity, these enzymes are structurally and functionally impaired by free radicals, resulting in liver damage. Glutathione comprises up to 90% of the nonprotein thiol content of mammalian cells and performs a pivotal role in maintaining their metabolic and transport functions. It acts as a nucleophilitic "scavenger" of many compounds and their metabolites via enzymatic and chemical mechanisms, converting electrophilic centers to ether bonds. Glutathione depletion to about 20%–30% of total glutathione levels can impair cell defenses against toxic actions, which may lead to cell injury

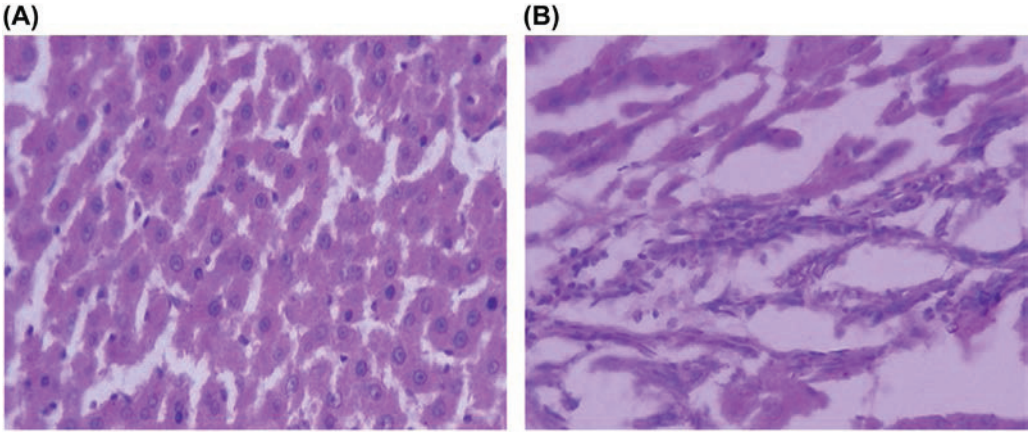

FIGURE 26.3 Normal (A) and lead acetate– (B) treated rat liver histopathology (45×).

and death.[21] Furthermore, glutathione is considered a crucial factor in maintaining the structural integrity of cell membranes, largely through reactions that protect the membrane against free radical formation.[62] Lead interacts with negatively charged phospholipids in membranes and through the induction of changes in membrane physical properties could facilitate the propagation of lipid oxidation in liver. Lead affects membrane-related processes such as the activity of membrane enzymes, endo- and exocytosis, transport of solutes across the bilayer, and signal transduction processes in hepatocytes by causing lateral phase separation.[30,61] Lead-induced oxidative stress in liver caused increase of rate of production of hydroxyl radicals which may lead to lysosomal and mitochondrial damages. Besides these direct hepatocellular damaging by lead-induced ROS and reactive nitrogen species (RNS), it may also interfere cell signal transduction by reversible oxidation and nitrosation of protein SHs in the hepatic sinusoid.[63]

Histopathological studies of lead-treated rat liver from the author's laboratory indicated little swollen hepatocytes with ill-defined cell borders with variation in cellular size and shape. The nuclei are large, more vesicular with variable size and shape, and contain multiple three to four prominent nucleoli. The cytoplasm is vacuolated and microvesicular. There are foci of fatty change and ballooning degeneration and necrosis of hepatocytes in zone 3 (centrilobular) areas.

The portal area appears mildly enlarged with mild proliferation fibrous tissue with infiltration of mixed acute and chronic inflammatory cells. The sinusoidal spaces are variably widened with increase in number of Kupffer cells. Central vein shows features of dilatation and congestion (Fig. 26.3B). Results clearly indicate hepatocellular damage by lead exposure.

4.2 Possible Mechanism of Altered Hepatocellular Architecture by Heavy Metals

It was found that most of the divalent heavy metals such as nickel, lead, and cadmium enter into systemic circulation from intestine through metal transporter proteins (MTP 1). Through circulation these metals enter first to liver via portal circulation where it is absorbed through sinusoidal capillaries. In hepatocytes, these heavy metals are penetrated through specific membrane transporters such as DMT1, ZIP8, and ZIP14t.[64,65]

Heavy metals such as nickel or lead accumulate in liver and resulting hepatocellular damages induce infiltration of polymorphonuclear neutrophils. This in turn causes activation of Kupffer cells followed by necrosis. Usually heavy metal–activated Kupffer cells secretes several inflammatory cytokines and causes secondary liver damage.[66] The exact mechanism of hepatocellular damages by Kupffer cells is yet to be cleared, but a possible role of free radicals, nitric oxide, tumor necrosis factor α (TNF-α) cannot be ruled out[67] (Fig. 26.4). Studies on nickel showed hepatic apoptosis due to overexpressions of caspase-3, caspase-9, and PARP mRNA.[68]

5. HYPOXIA AND LIVER DISEASES

Liver pathophysiology is oxygen dependent. As it is an important organ for metabolism, it is always in demand for oxygen. Hepatic artery, portal veins, and central veins play the pivotal role to maintain liver oxygen homeostasis.[69] It was found that liver always make oxygen microenvironment differentially than other organs. The important change

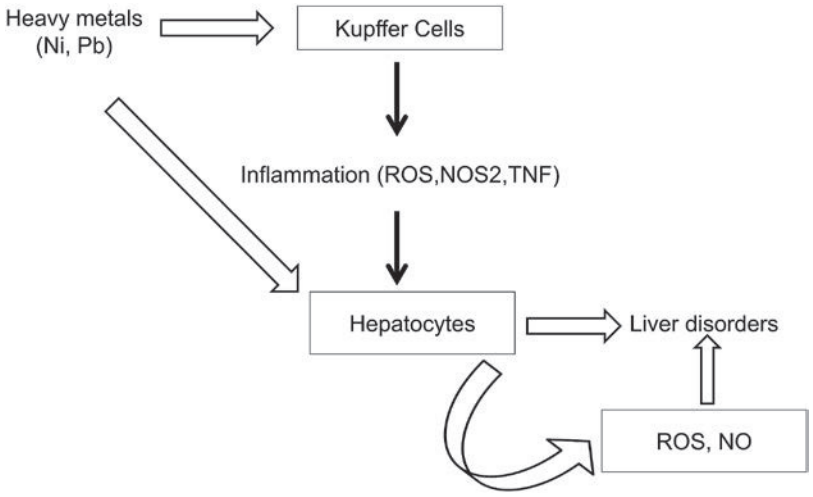

FIGURE 26.4 Heavy metal–induced liver malfunctions.

in hypoxia-induced hepatocellular architecture is the formation of plasma membrane protrusion. This formation has numerous cytosol and endoplasmic reticulum. It ultimately causes swelling of mitochondria and near 30%–50% increase of cell volume. These changes can be reversed if reoxygenation to hepatocytes occurs. The hepatocytes may be permanently injured if hypoxia sustains, and it will lead to complete damage of plasma membrane transport system which will cause release of intracellular ingredients of hepatocytes.[70] Oxygen tension in periportal and perivenous part of liver is 60–75 mmHg and 30–35 mmHg, respectively, which clearly indicates a persistent hypoxia in liver due to high metabolic functions.[69] Study reveals that hypoxia is linked to several types of liver diseases. The mechanism by which hypoxia is able to change liver pathophysiology is mainly through HIF-1 and NOS2 expressions. Both these factors are involved in hepatocytes, Kupffer cells, and immune cells. Hypoxia in liver increases the level of TNF-α, IL-1 from hepatocytes which further promote ROS. These ROS in liver are found to have decreased glutathione levels and elevated oxidized glutathione.[71] Although a direct hypoxia response to liver was not found in healthy individuals, in the case of viral hepatitis, metabolic diseases, steatohepatitis, and cancer, an elevation of HIFs is noticed. It has been observed that HIFs induce pathogenesis of hepatocellular carcinoma, and both HIF-1 α and VEGF levels were increased in hepatocellular carcinoma.[72] Many chronic liver diseases due to vital infection, metabolic disorders, or alcoholism are found to be connected with HIFs.[73] Actually HIFs act as protective agents from liver injuries due to hypoxia. HIFs induce generation of VEGF, adenosine, nitric oxide, and Akt signaling pathways to prevent hepatocellular injuries from hypoxia.[74,75] It is observed that Dec1 expression increases in alcoholic liver which indicates HIF-1α regulatory gene involvements to protect liver of alcohol toxicities. Hypoxia region of liver shows alteration of parenchymal vasculature, which leads to fibrosis.[76] HIF-1α expression stimulates hepatic stellate cells (HSCs) and fibroblasts. Another study on NAFLD phenotype showed hypoxia accelerated the NAFLD phenotype with higher level of lipogenesis and inflammation.[77] Another important phenomenon of hypoxia-induced liver injury is through ATP depletion during hepatic ischemia which also leads to necrotic cell death. Hypoxic liver enhances glycolytic metabolism and prevents its best against hypoxia injuries. In case of low glycogen in liver, hypoxia leads to rapid cellular ATP depletion and necrosis.[78] Hypoxia-exposed liver also shows alteration of pH microenvironment. Hypoxia leads to acidosis in liver, which prevents necrotic cell death in liver in spite of low ATP levels.[79]

5.1 Hypoxia—Liver Histopathology

In histopathological studies in the author's laboratory, the subchronic hypoxia–exposed rat liver showed endothelial cells surrounded by a ring of collagen fibers in the central vein. The sinusoids are lined by both endothelial cells and Kupffer cells both of which have inconspicuous flattened nuclei and ill-defined cytoplasmic margins. The hepatocytes are polygonal in shape with well-defined borders and appear to be little swollen with mild narrowing of the sinusoidal spaces. The nucleus is single, is round, and has a fine chromatin pattern with one to two clearly defined amphophilic prominent nucleoli. More or less it reflects normal architecture with insignificant changes in hypoxia-exposed rat liver (Fig. 26.5B).

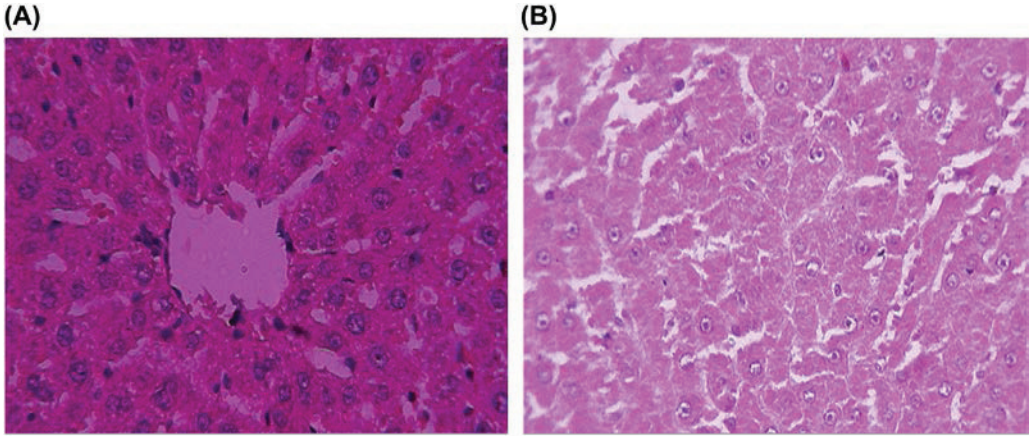

FIGURE 26.5 Normal (A) and chronic hypoxia- (B) exposed rat liver histopathology (45×).

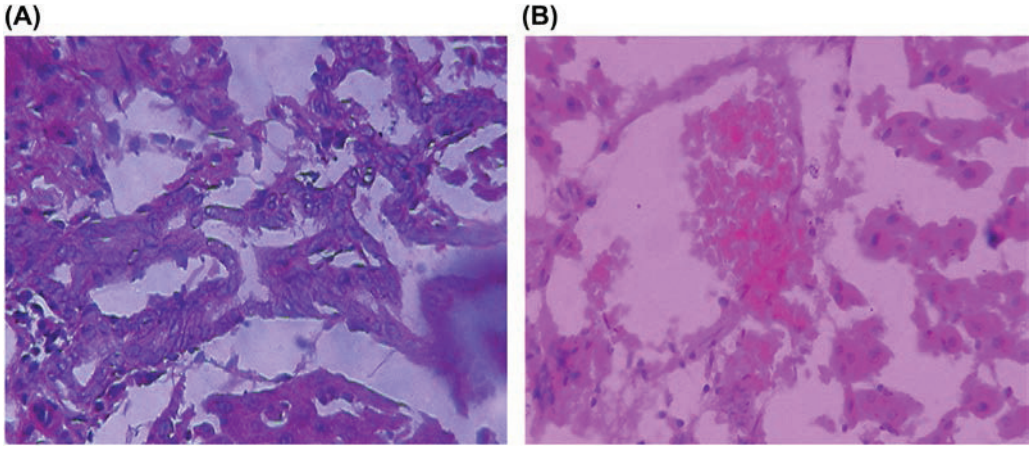

FIGURE 26.6 Hypoxia exposed with nickel sulfate– (A) and lead acetate– (B) treated rat liver histopathology (45×).

5.2 Hypoxia and Heavy Metals (Nickel and Lead)—Liver Histopathology

There is evidence of fatty change and ballooning degeneration and necrosis of hepatocytes. The portal area appears enlarged with severe proliferation fibrous tissue with infiltration of mixed acute and chronic inflammatory cells in nickel sulfate–treated subchronic hypoxia-exposed rats (Fig. 26.6A). In the case of subchronic hypoxia–exposed lead acetate–treated rats, distorted "lobular" architecture of liver parenchyma is noticed. Hepatocytes appear to be little swollen and cytoplasm is vacuolated, microvesicular, and eosinophilic (Fig. 26.6B). It also shows increase in number of mitotic figures along with foci of fatty change and ballooning degeneration and necrosis of hepatocytes in zone 3 (centrilobular) areas. Moderate proliferation of a portal area with fibrous tissue with infiltration of mixed acute and chronic inflammatory cells and variable widening of sinusoidal spaces along with Kupffer cell hyperplasia, dilatation, and congestion of central vein are also observed.

6. HEAVY METALS (NICKEL AND LEAD), HYPOXIA, AND LIVER FUNCTIONS—ROLE OF DIETARY SUPPLEMENTATIONS

Dietary supplementation of protein and other antioxidants including chelators are found to be effective against metal-induced hepatotoxicities. It has been found that metal ions interact with protein in a coordinated manner and chelate. These protein chelator compounds change the toxic characteristics of heavy metals by degrading it. The mode of hepatotoxicities by toxic metals such as nickel and lead are similar with hypoxia exposure. The cell signal pathways of nickel or lead and hypoxia

usually take place through HIF-1α expressions and further manifestation of expression of hepatic VEGF and NOS2. Both nickel or lead and hypoxia exposure induce ROS and inflammatory cytokines and damages hepatocytes, and interestingly, dietary supplementation of antioxidants such as vitamins C or E and high proteins combat the toxicities from these exposures.

6.1 Heavy Metals, Liver Functions, and Dietary Supplementation

The most common therapeutic way to combat heavy metal toxicity is chelation therapy which leads to metal excretion, but chelators themselves have many contraindications. Chelators such as EDTA and meso-2,3-dimercaptosuccinic acid (DMSA) are routinely used against Pb poisoning, but no such chelators are found to detoxify nickel poisoning so far. Hence alternative therapy, especially dietary supplementation, is now gaining momentum against heavy metal poisoning. As per WHO and the US Dietary Supplements Health and Education Act (DSHEA) of 1994, vitamins, minerals, herbs, amino acids, or other food substances additionally supplemented in diets are considered as dietary supplementation.[80] Most of the cases these dietary supplementations are found to be safe for health.[81] Some studies showed that Zn or Se are protective against Pb and Ni toxicity in liver, kidney, and brain. These micronutrients facilitate antioxidant defense mechanisms of metabolically active tissues including liver by acting as cofactor for synthesis of glutathione peroxidase (GSH-Px).[82] One interesting observation is the beneficial role of iron supplementation in metal exposure. In presence of dietary supplemented iron, it competes with other divalent cations derived from metals such as Ni, Cd, or lead at the level of its transporter proteins such as divalent metal transporter-1 (DMT1) and metal transporter protein 1 (MTP1) in the intestine and reduced uptake of these heavy metals.[83] Dietary supplementations of some elements such as calcium or magnesium are also found to be effective against Pb or Ni toxicity. The elements usually decreases heavy metal absorption from intestine or competitively binds with active sites of intracellular metal-binding protein in hepatic tissues and prevent heavy metals such as nickel, cadmium, and lead to exert hepatic tissue damages.[84,85] Some dietary supplementations such as *Allium sativum* Linn (garlic) were found to be hepatoprotective against heavy metals such as nickel and chromium VI.[86] Garlic has been found to be effective against heavy metal toxicities in liver through a number of mechanisms, such as scavenging radicals, increasing glutathione levels, increasing the activities of enzymes such as glutathione S-transferase and catalase, and inhibiting cytochrome p4502E1. Studies of Vimal and Devaki[87] showed that allicin (diallyl thiosulfinate) which is the main biological active compound derived from crushed garlic is highly protective against Cr VI– or lead-induced hepatic lipid peroxidation. Garlic also contains a number of amino acids that are required for the formation of an enzymatic antidote to free radical pathology, which is created by various pollutants including heavy metals. Cysteine, glutamine, isoleucine, and methionine found in garlic help to protect the liver cells from such free radical damage.[88,89] Raw garlic extract can effectively protect the body from metal toxicity. Garlic contains the highest level of the antioxidant selenium, which affords excellent hepatocellular protection.[89] Vitamin supplementations in diet are extremely popular against heavy metal toxicities as low concentration of vitamins C, B1 and B6 are found to have increased sensitivity toward Cd, Ni, and Pb toxicity in hepatic tissues.[90] It is further observed that vitamins C and E are natural exogenous nonenzymatic antioxidants which prevent liver from oxidative stress by preventing hepatic lipid peroxidation.[47] Besides antioxidant actions, vitamin C also acts as a chelating agent like EDTA against Pb toxicities in hepatic tissues.[91] Experimental study in rats has shown a beneficial effect of vitamin E pretreatment against heavy metals induced an alteration in liver antioxidant defense mechanisms.[20,92] Supplementation of vitamins B1 and B6 were found to be effective in decreasing Pb concentration in liver by reversing ALAD activity. Vitamin B1 facilitates Pb excretion and reduces the Pb toxicity.[93] Other good hepatoprotective agents against heavy metal toxicity are black tea or green tea, grapes, and tomatoes. The bioactive constituent of these edibles are mainly catechins, flavonoids, and polyphenols. These compounds are antioxidants by nature and act as chelators against Pb-, Ni-, or Cd-induced hepatotoxicity.[94–96] Some other plants such as liquorice (*Glycyrrhizae radix*) and ginseng (*Panax ginseng* Meyer) are also found to be effective against Cd-, nickel-, and Pb-induced hepatotoxicities. These plants are routinely used in diet by Chinese, Malaysians, and Africans.[97,98] Currently, some probiotics such as *Lactobacillus rhamnosus*, *L. plantarum*, and *Bifidobacterium longum* are found to be effectively neutralizing heavy metals in vitro. Besides this, these probiotics also act as antioxidants. Probiotic such as *L. plantarum* CCFM8610 is capable to reduce intestinal absorption of heavy metals and reduces metals deposition in liver and reversing hepatocellular damages due to heavy metal toxicities.[99,100] Another dietary option against heavy metal–induced hepatotoxicity is use of algae as it contains good amount of vitamin C, vitamin E, carotene, etc. which help to reduce heavy metal–induced oxidative stress.[101] High dietary supplementation of protein was also found to be effective in liver metabolism against nickel-induced toxicities.[6]

6.2 Hypoxia, Liver Function, and Dietary Supplementation

It has been found that supplementation of vitamins C and E on hypoxic rats improves hepatic glutathione level as compared with only hypoxia-exposed rats. It may be due to antioxidant vitamins C and E protect thiol status in the liver from hypoxia

injuries.[20] Antioxidant vitamins such as vitamins C and E also protect hepatocellular reduction of GSH-Px due to hypoxia exposure by decreasing phospholipid hydroperoxides in the cell membrane and prevent further lipid peroxidation.[20] These two antioxidant vitamins usually conjugate with GSH-Px and are able to decrease phospholipid hydroperoxide in liver to inhibit lipid peroxidation.[102] Results show that vitamin C and vitamin E are oxidized by ROS and RNS generated by hypoxia exposure in liver tissues. During combating with these TOS and RNS, tissue produce superoxide, hydroxyl, peroxyl, and nitroxide radicals, as well as nonradical reactive species such as singlet oxygen, peroxynitrite, and hypochlorate. These scavenging actions truly prevent lipid peroxidation, DNA and protein damage in liver. These antioxidants further enter into mitochondria and guard it from oxidative stress–induced damages. It must be noted that mitochondria of living cells including hepatocytes generate lots of intracellular ROS, and vitamins C and E supplementation defend mitochondrial genome.[103] Study shows that hypoxia exposure leads to decrease of hepatic concentration of vitamin C which may be due to overutilization of vitamin C by the liver tissues to counteract altered oxygen microenvironment in liver.[11] It was noticed that supplementation of vitamin E modifies altered oxygen-sensitive VEGF protein expression in hepatic tissues of hypoxic rats which may be through NOS2. In addition to direct cellular oxidant injury by hypoxia exposure, ROS and RNS may constitute signals regulating by either protein function through reversible oxidation and/or nitrosation of protein SHs or gene expression, in the hepatic sinusoid. Normally nitric oxide (NO) is synthesized by NOS2 gene expression and produced RNS in liver due to hypoxia-exposed low-oxygen microenvironment. Such low-oxygen microenvironment in hepatocytes, Kupffer cells, and endothelial cells generate redox-sensitive transcription factor NF-Kβ.[104] Study also revealed that the hepatoprotective effect of vitamins C and E under conditions of hypoxia appears to be due to its influence on the functional activity of adrenal glands. It was reported that these antioxidant vitamins enhanced noradrenaline-mediated activity in hypoxia through an iodoacetic acid–sensitive pathway.[105]

6.3 Heavy Metals, Hypoxia, and Liver Functions—Dietary Supplementation

Heavy metals such as nickel or lead and hypoxia exposure in liver tissues damage its integrity and develop hepatic malfunctions through ROS and RNS regulatory system. Interestingly, the toxic manifestation for both heavy metals and hypoxia in hepatic tissues are common by nature, i.e. increasing production of ROS and RNS subsequently increase expression of HIF-1 α. It was found that HIF-1α which expresses in tissue exposed to hypoxia as adaptive mechanism is important to regulate metabolism in liver and kidney.[106] Furthermore, it is noticed that HIF-1 plays an important role to develop fatty liver and hepatic fibrosis. It is also noticed that hypoxic area in fibrotic liver due to heavy metals or chronic hypoxia exposure localized with VEGF expression in hepatocytes and HSCs.[107]

The possible mechanism by which vitamins C and E counteract HIF-1α transcription factor expression may be through regulating/inhibiting ROS formation and indirectly controlling over production of RNS**. Hypoxia- and heavy metal treatment–induced HIF-1α gene transcription actually facilitate VEGF gene expression in hepatocytes to improve adaptability against chronic sustained hypoxia and metal-induced cellular hypoxia in physiological system.[108] Reports suggested existence of a feedback mechanism between ROS production and HIF-1 α in metabolically active tissues, although this link is a complex phenomenon which involves oxidative phosphorelation in response to hypoxia or heavy metals**.[109] Furthermore, it is noticed that oxidative phsophorylation in metabolic tissues modulate not only ROS but also oxygen redistribution and consumption by interfering HIF-1α degradation pathways and over expression of endogenous antioxidant enzymes.[110]

6.3.1 Heavy Metals, Hypoxia, Vitamin C and E Supplementation—Liver Histopathology

The author's laboratory shows histopathological changes in nickel- and lead-treated rat liver with or without supplementation of vitamin C and E (Fig. 26.7A–G).

Fig. 26.7C and D shows the effect of vitamins C and E supplementation on nickel-treated rat liver histopathology. It shows that the hepatic parenchymal tissue architecture is maintained normal, which is composed of numerous hexagonal to pyramidal "*lobules*." Each lobule consists of a central vein from which the hepatic plates radiate outward toward the portal areas; three to five portal triads are located at the periphery of the lobule containing branches of bile duct, portal vein, and hepatic artery; and occasional mononuclear cells. Cords of hepatocytes and blood-containing sinusoids radiate from central vein to the peripheral portal triads. The hepatocytes are large having well-defined cell borders with mild variation in cellular size and shape. The nuclei are round, regular, and vesicular with one to two prominent nucleoli. The cytoplasm is eosinophilic and hypergranular. The portal area appears mildly enlarged with mild proliferation fibrous tissue. The sinusoidal space appears normal with moderate number of Kupffer cells. Central vein shows features of mild dilatation and congestion. No foci of fatty change/hyaline change/degeneration/necrosis/inflammatory reaction are found in vitamins

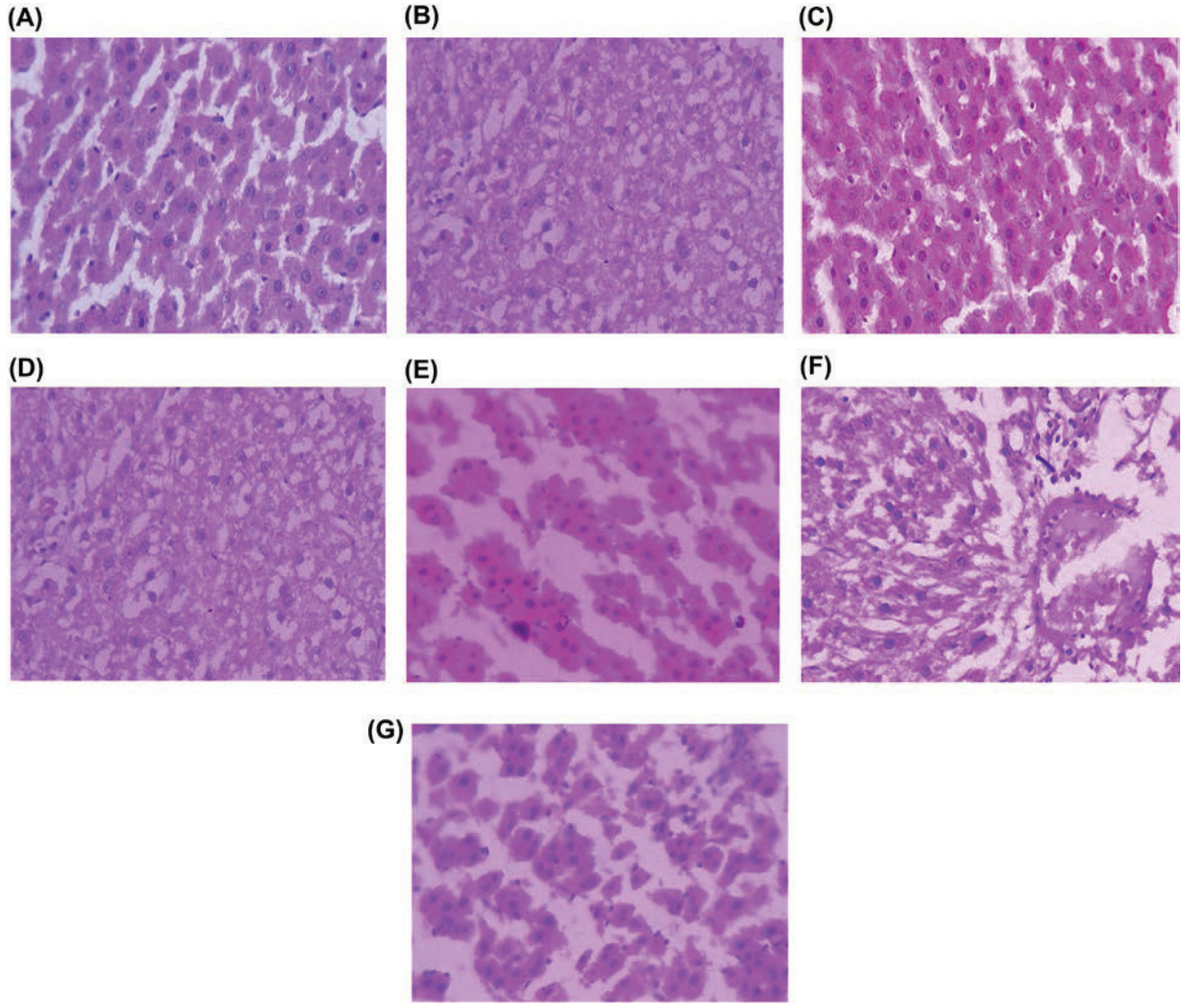

FIGURE 26.7 Normal (A), nickel sulfate– (B), nickel sulfate with vitamin C– (C), nickel sulfate with vitamin E– (D), lead acetate– (E), lead acetate with vitamin C– (F), and lead acetate with vitamin E– (G) treated rat liver histopathology (45×).

C and E supplemented nickel-exposed rats. Results clearly indicate an improvement of hepatic architecture in nickel- or lead-exposed rats supplemented with either vitamin C or vitamin E. Fig. 26.7F shows hepatic parenchymal tissue with mild distortion of "lobular" architecture which is consisting of a central vein, hepatic plates, and portal areas containing branches of bile duct, portal vein, and hepatic artery in vitamin C–supplemented lead treated rat. The hepatocytes are large having ill-defined cell borders with mild variation in cellular size and shape. The nuclei are round, regular, and vesicular with one to two prominent nucleoli. The cytoplasm is vacuolated to clear type with decreasing eosinophilia containing micro/macro vesiculations. There are mild foci of fatty change and ballooning degeneration and necrosis of hepatocytes in zone 3 (centrilobular) areas. The portal area appears mildly enlarged with mild proliferation fibrous tissue with infiltration of mixed acute and chronic inflammatory cells. The sinusoidal spaces are variably widened with pronounced increase in number of Kupffer cells. Central vein shows features of dilatation and congestion. Fig. 26.7G shows near normal architecture of liver parenchyma. Hepatocytes appear normal with sinusoidal spaces that appear normal with moderate number of Kupffer cells. Cytoplasm is eosinophilic and central vein appears normal. Results indicate vitamin E is a better protector than vitamin C against lead-induced hepatotoxicities.

The study on experimental rats in the author's laboratory shows the liver histopathology in hypoxia-exposed and vitamins C and E–supplemented hypoxia-exposed rats (Fig. 26.8A–D).

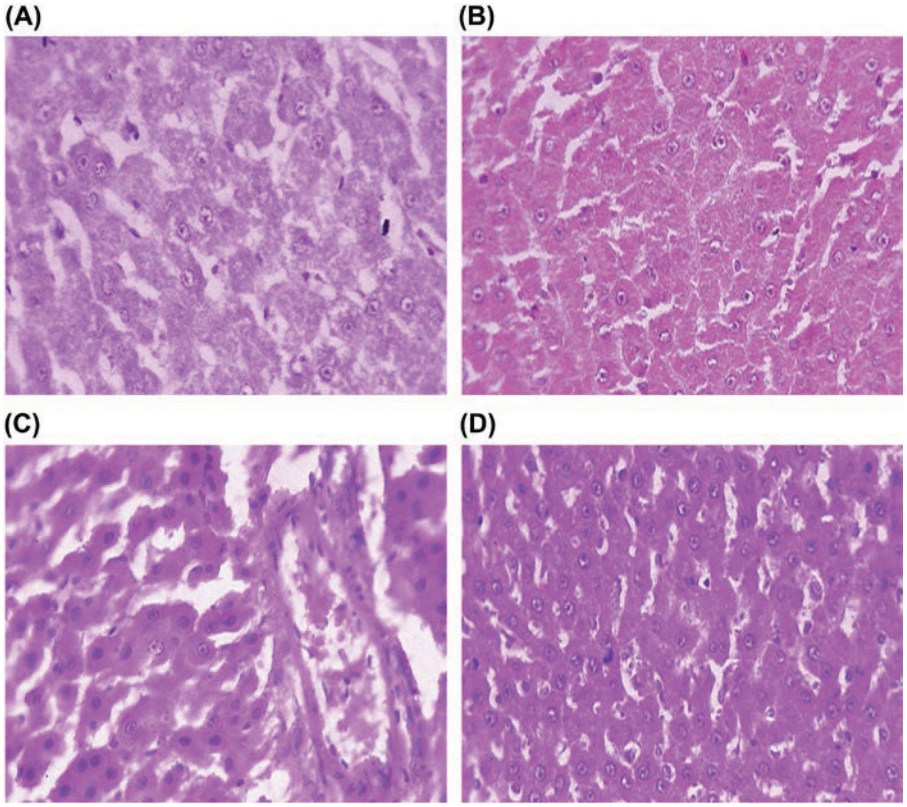

FIGURE 26.8 Normal (A), hypoxia exposed (B), hypoxia exposed with vitamin C– (C), and hypoxia exposed with vitamin E–supplemented rat liver histopathology (45×).

Fig. 26.8B shows hypoxic liver architecture. The central veins are lined by endothelial cells surrounded by a ring of collagen fibers. The sinusoids are lined by both endothelial cells and Kupffer cells both of which have inconspicuous flattened nuclei and ill-defined cytoplasmic margins. The hepatocytes are polygonal in shape with well-defined borders and appear to be little swollen with mild narrowing of the sinusoidal spaces. Microscopic profile shows normal architecture of liver parenchyma maintained with mild narrowing of the sinusoidal spaces. Cytoplasm is more eosinophilic and hypergranular. Fig. 26.8C showed vitamin C shows normal architecture of liver parenchyma is maintained. Mild narrowing of the sinusoidal spaces with portal triad shows mild proliferation with mild thickening of basement membrane of the blood vessels. No obvious significant changes are noticed. In the case of vitamin E–supplemented hypoxic rat liver, normal architecture of liver parenchyma is maintained but hepatocytes appear to be little swollen with mild narrowing of the sinusoidal spaces. The nucleus is single, is round, and has a fine chromatin pattern with zone 1 to 2 clearly defined amphophilic-prominent nucleoli (Fig. 26.8D). Fig. 26.9A shows effect of vitamin C supplementation on hypoxia-exposed nickel (Ni)-treated rat liver. The experimental studies from the author's laboratory on histopathology of liver clearly indicate near normal architecture of liver parenchyma. Hepatocytes appear normal, and sinusoidal spaces appear also normal with moderate number of Kupffer cells. Cytoplasm is appeared to be eosinophilic. Histopathology also indicates normal central vein with normal portal triad. Results show beneficial effect of vitamin C on nickel-treated hypoxic rat liver as compared with rats without vitamin C supplementation (Fig. 26.6A). In case of vitamin E supplementation on nickel-treated hypoxic rats, mild distortion of "lobular" architecture of liver parenchyma and large hepatocytes with mild variation in cellular size and shape are observed (Fig. 26.9B). Furthermore, liver histopathology reveals that cytoplasm is vacuolated to clear type with decreasing eosinophilia containing micro- and macrovesiculations. There are foci of fatty change and ballooning degeneration and necrosis of hepatocytes in zone 3 (centrilobular) areas and mild proliferation of portal area with fibrous tissue with infiltration of mixed acute and chronic inflammatory cells. Variable widening of sinusoidal spaces are also seen (Fig. 26.9B). Although results indicate a relative beneficial effect of vitamin E supplementation on nickel-treated hypoxic rat liver when compared with nickel-exposed hypoxic rats (Fig. 26.6A), it looked relatively less beneficial when compared with vitamin C–supplemented nickel-treated hypoxic rats (Fig. 26.9A).

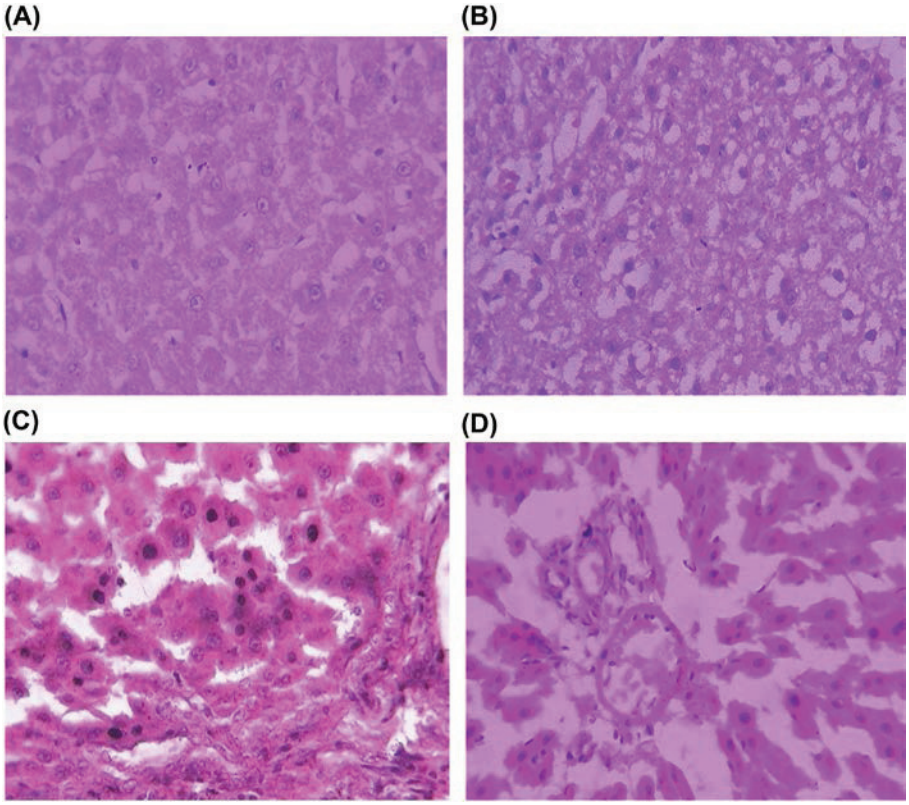

FIGURE 26.9 Nickel and hypoxia exposed vitamin C– (A) and vitamin E– (B) supplemented; lead and hypoxia exposed vitamin C– (C) and vitamin E– (D) supplemented rat liver histopathology (45×).

In authors' laboratory, liver histopathology of lead (Pb)-treated hypoxic rats supplemented with vitamins C and E was also done. Results show a normal architecture of liver parenchyma with mild swollen hepatocytes. Mild narrowing of the sinusoidal spaces was also observed. Portal triad shows mild proliferation with mild thickening of basement membrane of the blood vessels. There were no foci of fatty change or necrosis or inflammatory reaction in histopathology of liver observed (Fig. 26.9C). Fig. 26.9D shows the liver histopathology of Pb-treated hypoxic rats supplemented with vitamin E. Mild distortion of "lobular" architecture of liver parenchyma and vacuolated cytoplasm with decreasing eosinophilia containing micro-and macrovesiculations is noticed. Furthermore, mild proliferation of portal area with fibrous tissue with infiltration of mixed acute and chronic inflammatory cells is also found. Central vein shows features of dilatation and congestion. Results definitely indicate a relative beneficial role of vitamin E supplementation on liver histopathology in lead (Pb)-treated hypoxic rats as compared with lead-treated hypoxic rats without vitamin E supplementation (Fig. 26.6B).

7. CONCLUSION

It may be postulated that heavy metals such as nickel (Ni) or lead (Pb) cause serious cellular damages including hepatocellular damages. Interestingly, chronic sustained hypoxia also induces hepatotoxicities. The molecular mechanisms involved in both the cases are similar by nature.

Heavy metals such as nickel or lead induce hypoxia over expressions of HIF-1α in hepatocellular environment which will be followed by generation of ROS and further expression of VEGF and NOS2 gene in liver. This overexpression of HIF-1α also alters hepatic glycolytic pathways by changing Glut1, LDH, and PFR genes. All these changes lead to hepatocellular damages (Fig. 26.10). Dietary supplements, especially antioxidants such as vitamins C and E are found to be beneficial as they suppress either metal- or hypoxia-induced hypoxia gene expressions in hepatocytes.

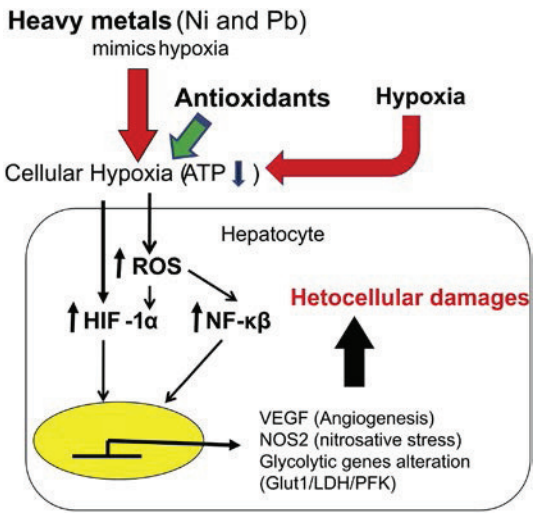

FIGURE 26.10 Heavy metal– and hypoxia-induced molecular mechanisms of hepatotoxicities. *Red arrow* indicates toxicities and *green arrow* indicates protection.

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