Effect of 1,25-$(OH)_2D_3$ on pathophysiology of heart, aorta and lungs in male albino rats exposed to chromium(VI)

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By

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Dedicated to my parents

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LIST OF ABBREVIATIONS

+	Mild
++	Moderate
+++	Severe
^{o}C	Centigrade
%	Per cent
g/cm^3	gram per cubic centimeter
g/dl	grams per deciliter
g/mL	grams per milliliter
m^2	meter square
m^3	meter cube
mg	milligram
mg/dl	milligram per deciliter
mg/kg	milligram per kilogram
mg/L	milligram/Liter
mg/m^3	milligrams per cubic meter
mIU/L	milli International Units per Litre
mm Hg	millimeter of mercury
mm3	cubic millimeter
µg	microgram
$µg/m^3$	microgram per cubic meter
µl	micro litre
µM/L	micro Moles per Litre
µm	micro meter
7-DHC	7-dehydrocholesterol
DHCR7	7-dehydrocholesterol reductase
8OHdG	8-hydroxyguanosine
AGEs	Advanced glycation end-products
ALA-D	δ-aminolevulinic acid dehydratase
ALP	Alkalline. Phosphatase
AMPK	Adenosine monophosphate activated protein kinase
ANOVA	One-way analysis of variance
AP-1	Activating protein-1
ATPase	ATP Synthase)
ATSDR	Agency for Toxic Substances and Disease Registry
bcl-2	B-cell CLL/lymphoma 2
Ca	Calcium
CAT	Catalase
CCSEA	Committee for Control & Supervision of Experiments on Animals
CD4+T	Helper cells
CHER	Cholesterol esterase
CHOD	Cholesterol oxidase
CHOP	Cholesterol peroxidase
COPD	Chronic obstructive pulmonary disease
CYP7A1	Cholesterol 7α-hydroxylase
CYP24A1	25OHD-24 hydroxylase
CYP2R1	25-hydroxylase
CYP27B	25OHD-1α hydroxylase
Cr	Chromium
Cr(III)	Chromium(III)
Cr(IV)	Chromium(IV)
Cr(V)	Chromium(V)
Cr(VI)	Chromium(VI)
CSM	Corrected sample measurement
Cu	Copper
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DBP	Vitamin D binding protein
DF	Degree of freedom
DNA	Deoxyribonucleic acid
ECG	Electrocardiography
ECP	Extracellular matrix
EDTA	Ethylene diamine tetracetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial Nitric oxide synthase
Epo	Erythropoietin
ERKs	Extracellular signal regulated kinases
FAS	Fatty acid synthase
FBG	Fasting blood glucose
FBS	Fasting blood sugar
FEV1	Forced expiratory volume
Fe^{2+}	Ferrous ion
FGF-2	Fibroblast growth factor -2
FGF23	Fibroblast growth factor
FFA	Free fatty acids
GLUT2	Glucose transporter-2
GLUT-4	Glucose Transporter-4
GOD	Glucose oxidase
GPO-PAP	Glycerol-3-phosphate oxidase
G6PD	Glucose-6-phosphate dehydrogenase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH-R	Glutathione reductase
GSH-T	Glutathione S-transferases
GSH	Glutathione
GRO-α	Growth regulated protein alpha
GTF	Glucose Tolerance Factor
H_2O_2	Hydrogen peroxide
H2SO4	Sulphuric acid
Hb	Hemoglobin
HBEC	Human bronchial epithelial cells
HCl	Hydrochloric acid
Hct	Hematocrit
HDL-C	High density lipoprotein cholesterol
HF	High frequency
HIFs	Hypoxia-Inducible Factors
HIF-1	Hypoxia-inducible factor 1
HMG-CoAR	HMG-CoA reductase
HPE	Histopathological examination
HMGR	HMG-CoA reductase
HR	Heart Rate
HRE	Hypoxia-responsive elements
HRP	Horseradish peroxidase
HRV	Heart rate variability
IAEC	Institutional Animal Ethics Committee
ICMR	Indian Council of Medical Research
IF-kβ	Inhibitory factor-kappa beta
IL	Interleukin
IP	Intraperitoneal
iNOS	inducible Nitric oxide synthase

K^+	Potassium channels
$K_2Cr_2O_7$	Potassium dichromate
Kg	Kilogram
Km	Correction factor
LA	Lumen area
LDH	Lactate dehydrogenase
LDL-C	Low density lipoproteins cholesterol
LPO	Lipid peroxide
LPS	Lipopolysaccharide
LF	Low frequency
LF/HF	LF / HF ratio
LFT	Liver function tests
LXRα	Liver X receptor-α
MDA	Malondialdehyde
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MMP	Matrix metalloproteinase
Mn	Manganese
$Na_2Cr_2O_7$	Sodium dichromate
Na+	Sodium
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NE	Norepinephrine
NIBP	Non-invasive blood pressure
nm	nanometers
Nrf2	Nuclear factor erythroid 2 related factor 2
NO	Nitric oxide
NOS	Nitrogen oxygen species
NOX	Nitrogen oxides
nu	normalized units
NWI	Normalized wall index
O_2	Oxygen
OD	Optical density
·OH	Hydroxide ion
·O2–	Superoxide ion
OPN	Osteopontin
OS	Oxidative stress
PARP-1	Poly(ADP-ribose) polymerase 1
PDH	Pyruvate dehydrogenase
PI3K	Phosphoinositide 3-kinase
Akt	Protein kinase B
POD	Peroxidase
$ONOO^-$	Peroxynitrites
PTH	Parathyroid hormone
PDGF	Platelet derived growth factor
PEGME	Polyethylene- glycol- methyl ether
PG's	Prostaglandins
pg/ml	picogram per millimeter
PHDs	Prolyl hydroxylases
PI3-K	Phosphoinositide-3 kinase
PLGF	Placental growth factor
PO2	Partial pressure of oxygen
PPAR-γ	Peroxisome proliferator activated receptor-gamma
PSI	Pulmonosomatic Index
PUFAs	Polyunsaturated fatty acids
PUFA	Polyunsaturated fatty acid
OGTT	Oral glucose tolerance test
PCV	Packed cell volume
PEGME	Polyethylene glycol methyl ether
Plt	Platelets
PVS	Polyvinyl sulfonic acid
RAAS	Renin Angiotensin Aldosterone System
RANKL	Receptor activator of nuclear factor kappa beta
RBC	Red Blood Cells
RNA	Ribonucleic Acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
$RS^•$	Thiyl radical
rpm	Revolutions per minute
Sam	Sample
sec	seconds
SBP	Systolic blood pressures
SD	Standard deviation
SGOT	Serum
SGPT	Serum
SOD	Superoxidase dismutase
SIRT1	Sirtuin 1
SNS	Sympathetic nervous system
SPSS	Statistical Package for the Social Sciences
SREBPs	Sterol regulatory element-binding proteins
Std	Standard
SVCTs	Sodium-dependent vitamin C transporters
TC	Total cholesterol
TGL	Triglycerides
Th17	T Helper cell
TLC	Total leucocyte count
TLR4	Toll-like receptor
TMB	3,3', 5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor-alpha
TVA	Total vessel area
TRPV	Transient receptor potential vanilloid
TVA	Total vessel area
UV	Ultraviolet
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptors
VLDL	Very low- density lipoprotein
VSMCs	Vascular smooth muscle cells
WA	Wall area
Wnt/ß	Wingless-related integration site
WHO	World Health Organization
Zn	Zinc
ZnSO4	Zinc Sulphate

ABSTRACT

Introduction: Exposure to heavy metal Cr(VI) causes generation of free radicals, induction of oxidative and nitrosative stress with depletion of antioxidants leading to sequels of cell injury. Fat-soluble $1,25(OH)_2D_3$ is an anti-oxidant capable of reducing noxious effects of oxidative and nitrosative stress caused by Cr-induced generation of reactive oxygen and nitrogen free radicles.

Objective: The present study was aimed to evaluate potential role of $1,25(OH)_2D_3$ as an antioxidant against Cr(VI)-induced toxicities in the heart, aorta and lung of experimental animals.

Methods: The experimental animals consisted of twenty-four laboratory bred adult male Wistar strain of albino rats, weighing between 180 to 220 grams, which were randomly sorted into four equal groups; each with six rats. Group-1 served as a control with no interventions. Group-2 was exposed to $K_2Cr_2O_7$ intraperitoneally in a dose of 0.5 mg/kg body weight on alternative days. Group-3 was supplemented with $1,25(OH)_2D_3$ orally in a dose of 12.5 µg/kg daily. Group-4 was given combined $K_2Cr_2O_7$ and $1,25(OH)_2D_3$ simultaneously with respective doses and routes. The intervention was carried for 21 days. Gravimetry (body weight) and cardiac electrophysiologic analysis (ECG, MAP and HRV) was done pre (Day-1) and post (Day-21) -interventions. During the course of intervention blood sugar was estimated on day-1, 7, 14 & 21. After 21-days of interventions, rats were fasted for 10 hours during the night. Collection of blood samples followed by sacrifice of the rats and collections of tissue specimens of heart, aorta, lungs and liver was carried out on day-22.

Results: After 21 days of individual treatments, statistically significant impairments were observed. The Group-2 ($K_2Cr_2O_7$-exposed) had a significant impairment in; gravimetry (loss of body weight and increased organosomatic index), cardiac autonomic functions (increased sympathetic and decreased parasympathetic causing

vasovagal imbalance), hemodynamic (increased HR, MAP, altered HRV) hematologic (reduced Hb, red cell indices, Plt, and increased TLC), glucose homeostasis (increased FBG, OGTT, decreased plasma insulin, increased insulinogenic index and decreased liver glycogen), lipid profile (decreased TC, TGL, LDL and decreased HDL), LFT (increased serum- bilirubin, SGOT, SGPT, ALP, decreased protein and albumin), serum oxidative stress (increased serum MDA), tissue oxidative stress (increased tissue LPO in heart, aorta and lungs), nitrosative stress (increased serum NO), antioxidative markers (deceased SOD, vitamin- C, D & E), molecular marker (increased serum VEGF), chromium (increased serum chromium), histopathogic (morphologic tissue remodeling of heart, aorta and lung tissues) and NWI (increased in coronary arteries and aorta). Supplementation of $1,25(OH)_2D_3$ ameliorated the respective parameters.

Conclusion: The results clearly substantiate that chromium-toxicity enhanced the generation of ROS and NOS, causing oxidative and nitrosative stress and affecting pathophysiologic changes in cardiovascular and pulmonary system in male albino rats. It was manifested with cardiac sympathetic over activity and parasympathetic under-drive, vasovagal imbalance, hypertension, impaired glucose homeostasis, metabolic, oxidative and nitrosative stress synchronously deprivation of antioxidants, up-regulation of vascular functions and tissue remodeling. Supplementation of $1,25(OH)_2D_3$ brought ameliorating reduction in sympathetic over-activity; MAP; metabolic, oxidative and nitrosative stress, cardiovascular and pulmonary remodeling. These effects of $1,25(OH)_2D_3$ could be attributed to its antioxidant property which can possibly be explored as an adjuvant in therapy against Cr(VI) induced toxicity in humans.

Keywords: Chromium(VI) toxicity, cardiorespiratory remodeling, oxidative stress, Vitamin D supplementation.

Chapter-1

Introduction

1. Introduction

Heavy metals are natural elements that accumulate in the environment and cause toxic effects on the health and welfare of living-beings including humans, when exposed to them.

1.1 ***Chromium*** is one of the heavy metals and its compounds have been in ever increasing widespread commercial use for more than a century in various metallurgical production and applications. Environmental contamination of Cr(VI) has become a global concern. According to ATSDR's substance priority list, Cr(VI) is the 17^{th} most common environmental pollutant. The European Chemicals Agency (ECHA) labels Cr(VI) as a "substance of very high concern". An upsurge in concentration of Cr(VI) in groundwater system have also been reported in several parts of India.[1]

Millions of workers are at risk of occupational exposure to chromium compounds; particularly those who are working in the areas of chromium- mining, pigmenting, plating, welding, spray painting, production of stainless steel and processing of chromium alloys where the exposure levels of Cr(VI) can exceed more than 1 mg/m^3.[2]

Elemental chromium is present in ores, primarily as chromite ($FeOCr_2O_3$) with several oxidative states. Cr(III) is the stable form that occurs naturally, formed by denudation of minerals in the earth's crust. Cr(VI) is formed by excessive anthropogenic industrial and urbanization activities, leading to its contamination into the environment through poor unregulated disposal of waste. It is highly unstable and more toxic.[3] Therefore its levels in the drinking water may surpass the acceptable range of 0.05 mg/L set by WHO.[4,5]

Cr(VI) enters the body most commonly through inhalation (~70%), ingestion (~30%) and to a lesser extent, through the skin contact (<5%) and gets distributed to all vital organs of the body.[6] Cr(VI) gets reduced to Cr(III) with intermediary formation of reactive- oxygen and nitrogen species, which target intracellular complexes.[7] Studies have shown that Cr(VI) can enhance lipid peroxidation, alteration of calcium and sulfhydryl homeostasis and DNA damage resulting in oxidative stress with subsequent oxidative deterioration of biological macromolecules. Concomitantly it also inhibits antioxidant enzymes like glutathione causing its depletion in the cells which results in various toxic manifestations in metabolically active tissues. [8]

Extensive review ascertains that Cr(VI) is classified as cardiotoxic,[9] pulmonotoxic,[10] vasculotoxic,[11] mutagenic,[12,13] genotoxic,[14,15] hepatotoxic [16] and class-I carcinogenic.[17] Incidence of occupational exposure to chromium toxicity remains high worldwide.[18] It is a matter of public health concern that nonoccupational exposure can happen through the consumption of food, water or inhalation of air polluted with chromium.[19,20]

Numerous data in humans and laboratory animals have elucidated that Cr(VI) generally exerts greater toxicity on the respiratory and cardiovascular systems. The exact mechanisms by which chromium contributes to cardiovascular diseases are not fully understood, but popular theories link an imbalance between formation of reactive oxygen species formed by oxidative stress and body's inability to eliminate them.

Endogenous enzymes like glutathione peroxidase & reductase, superoxide dismutase and catalase can prevent cellular toxicity caused by different chromium compounds. Analogously, exogenous non-enzymatic antioxidants such as vitamin C, E, A etc. have the radical scavenging ability to stop the harmful effects of Cr.[19-22]

Nevertheless, the protective role of $1,25OH_2D_3$ as an antioxidant has not received much attention in the past.

1.2 ***$1,25(OH)_2D_3$*** is a secosteroid [23] represents the biologically active form of vitamin D[24] and plays defensive role as an antioxidant against oxidative stress of metabolic diseases including cardiovascular and respiratory ailments in the body.[25] $1,25(OH)_2D_3$ though primarily involved with calcium homeostasis[26] but with the virtue of regulating more than 200 genes, it can participate in extra-calcimic activities including production of renin in the kidney,[27] insulin in the pancreas[28] and parathyroid hormone in parathyroid gland etc.[29] Studies have shown that it is a potent immune modulator,[29,30] anti-inflammatory,[31] anti-proliferative,[32] pro-differentiative,[32] cardio-protective,[33] pulmono-protective,[34] vaso-protective[35] and neuro-protective vitamin.[33]

Studies on vitamin- C and E in relation to heavy metals exist, but the influence of vitamin D against chromium(VI)-induced toxicity remains unclear. The aim of this study is to analyze the antioxidative potentiality of $1,25(OH)_2D_3$ supplementation on Cr(VI)-induced cardiovascular and respiratory pathophysiology.

Chapter-2

Review of Literature

2.0 REVIEW OF LITERATURE

Heavy metals are natural heterogenous group of elements which have specific weight of >5 g/cm^3 and 5 times higher density than water. They are either nonessential such as arsenic, lead and mercury or essential such as trace elements- chromium, cobalt, copper, iron, manganese, nickel, selenium and zinc for cellular functions. [36] But when exposed in excessive amounts these elements can cause toxic effects on the health and well-being of living beings including humans.[37] Studies reveal a link between heavy metal exposure and chronic diseases including respiratory, cardiovascular, renal diseases, metabolic disorders and cancers.[38]

2.1 CHROMIUM

Chromium (Cr) is a heavy colored elemental metal and derives its name from the Greek term *chroma*, which means *color*. Cr has been used vastly worldwide, [38] since its discovery in 1798 by the French chemist Nicolas-Louis Vanuquelin. [39]

2.2 Chemistry of chromium

Chromium has an atomic number *24*, weight *51.9961*, specific gravity of *7.19* g/mL, melting point of *1857.0* °C,[40] belongs to group *6* of the periodic table and is insoluble in water.[36,41]

2.3 Physical occurrence of chromium

Chromium with its various oxidative forms ranks as the 21st most abundant element in the earth's lithosphere and exists in several oxidative states ranging from $^{-}$2 to $^{+}$6 valence forms.[42] Chromium occurs in the environment primarily in three most common valence states namely chromium (0/elemental metal), trivalent ($^{+}$3 / Cr(III) and hexavalent ($^{+}$6 / Cr(VI). Cr(0) is not naturally occurring and only Cr(III) and Cr(VI) compounds are detected in the environment in significant quantities.[43]

The most stable Cr(III) is found naturally in chromite ore as ferrochromite ($Fe_0Cr_2O_4$) which is a combined metal oxide containing iron, magnesium and aluminium.[44,45] The Cr(VI) is the second-most stable form that seldom occurs in nature, but it is released through man-made activities.[1]

2.4 Compounds of chromium

2.4.1 *Chromite ore ($Fe_0Cr_2O_4$)* is the only source of chromium that occurs naturally as ferrochromite and is used commercially.[46]

2.4.2 *Ferrochromiums (FeCr)* are intermediate alloy products of chromium combined with iron, nickel and cobalt and are mostly used for polishing the stainless steels. [47]

2.4.3 *Trivalent chromium ((Cr(III)) compounds* include chromic- oxide, acetate, nitrate, chloride, phosphate and sulfate.[48]

2.4.4 *Hexavalent chromium ((Cr(VI)) compounds* include chromates of- ammonium, calcium, potassium, sodium, barium, zinc, lead etc.[8,48]

2.5 Chromium as an essential trace element

The use of chromium compounds is determined by their valence. Cr(III) is a trace element concerned with regulation of insulin and glucose metabolism and used as an essential micronutrient in dietary supplementations.[49]

2.6 Commercial uses of chromium compounds

On the other hand, Cr(VI) compounds are used commercially in multitudinous products as; a hardener, corrosion inhibitors in steel and alloys;[50] a pigmenting agent in glass industry, a mordant in textile industry, a hardener in emulsions;[51] an oxidizing catalyst for quantitative analysis in chemical industries;[45] a tanning agent in leather materials;[41] a rust inhibitor in paints;[52] a catalyst in chromium plating, waterproofing, synthetic perfumes and pyrotechnics;[53] a depolarizer in high-temperature batteries;

a preservative in wood, wool and inks,[54] a stabilizer in cement;[55] jet engines, nuclear plants and acid-resistant equipments.[44]

2.7 Occupational exposures of chromium

Occupational exposure to Cr(VI) compounds can occur during its mining, production and its use in wide range of applications [56,57] through inhalation, ingestion or skin contact.[44] Many epidemiological studies suggest that approximately two million occupational workers get exposed to Cr through handling of various substances such as anti-corrosion agents, welding rods, leather, dyes, textiles, cement, paints, printing inks, cutting oils, photographic materials, etc.[58]

0.1-10% of Cr can be exposed in various occupational workers[46] which can be found in high concentrations in various organs,[57,59] mainly in lungs[60] and body fluids like urine.[61] Leather tanning contains 1-6% of Cr(III) splashes[46] which gets absorbed from the oral ingestion.[62] Elevated levels of Cr(VI) can range from 13-2900 μg/m^3 in air samples of occupational areas involved in chromium plating, welding,[59] spray painting,[63] and cement factories.[64]

Coal contains Cr up to 54 mg/kg[65] and coal power plants contain 2.3-31 mg/kg of Cr [66] and the fly ash contains 1.4-6.1 mg/kg of Cr(VI) in the air,[46] contributing an additional source of exposure for occupational as well as non-occupational individuals staying in the potential exposure zones.

2.8 Environmental contamination and non-occupational exposures of chromium

Cr(III) can be present up to 250 mg/kg in the soil. Negligible quantities of chromium 20-50 μg/kg may be found in most of the vegetables, fruits, grains and cereals.[67] International standard for Cr(VI) in drinking-water is 0.05 mg/l.[68] Chromium contamination of soil, surface, ground and seawaters can occur through

wastewaters and fly ash from chromium industries and improper disposal of municipal incineration wastes.[69]

2.9 Toxicokinetic of chromium

Cr-induced toxicity is influenced by factors like its valence, dose and duration of exposure.

2.9.1 Absorption of chromium

Cr(VI) is more rapidly absorbed from the lungs than Cr(III) through inhalation in exposed individuals[70] and animals.[71] The rate of absorption depends upon their valence and capacities to penetrate into the cells.[1,72] About 85% of Cr(VI) and 5-30% of Cr(III) is shifted into the bloodstream[73] and the remaining unabsorbed Cr enters the gastrointestinal tract through mucociliary transport. Cr(VI) is less water-soluble, has longer retention time and highest absorption fraction in the lungs than the Cr(III).[74,75]

When orally ingested, Cr(VI) is reduced to Cr(III)[76] by ascorbic acid [74] in gastric juice [77] of the stomach, out of which <10% of the ingested dose is absorbed in upper small intestine mainly in jejunum,[75] reaching the peak plasma concentrations in 2 hours.[78]

Cr(III) as an essential element in humans, only 0.5–2.0% is absorbed out of 20-45 μg of daily intake.[79] Cr compounds can be absorbed from the skin[80] and produce systemic toxic effects in the organs.[81] Dermal absorption of Cr(VI) is slightly faster than Cr(III)[82] and extends beyond the dermis reaching lymphatic and blood vessels by 5 hours.[83]

2.9.2 Distribution of chromium

Cr(VI) gets transported into the bloodstream in 3 times greater and much rapidly than Cr(III) due to its particle size.[84] Chromium concentration in plasma and erythrocyte is gradient-dependent and has a plasma elimination half-life of 36 hours.[85]

Cr gets distributed in all parts of the body with highest concentrations in lungs, kidneys, liver, spleen, heart, aorta and hilar lymphnodes[70] including in infants via breast milk.[86,87] Cr(VI) traverses cell membranes substantially[1] and binds to intracellular proteins of various tissues resulting in greater degree of toxicity.[88]

2.9.3 Metabolic stress of chromium

As a trace element Cr(III) is essentially required for metabolism of carbohydrate, proteins, fat and intracellular reduction reactions.[89] *Chromodulin*, also known as Glucose Tolerance Factor (GTF), is an oligopeptide complex of four chromium ions with receptors for insulin within the cells. Erythrocyte membrane is non permeable to Cr(III) and permeable to Cr(VI). The unstable Cr(VI) reduction to Cr(V), Cr(IV) and finally to a stable Cr(III),[90] takes place with the combined actions of ascorbate and glutathione. During this course, chromium-hemoglobin complexes are formed within the cell which cause change in concentration equilibrium, resulting in more membrane diffusion of Cr(VI) into the erythrocytes with effective greater concentration within the cells for the life-span.[91] The rate of intraerythrocytic reduction of Cr(III) determines the rate of diffusion into the eyrthrocytes.[92] The Cr held inside the cell is drawn-out steadily,[71] eluting 1% daily from the cell.[93]

Moreover, ascorbate-aided Cr(VI) to Cr(III) reduction[94] occurs in the epithelial lining fluid, alveolar macrophages, peripheral lung cells and liver cells.[95] When ascorbate is depleted, the reduction process is carried over by glutathione which is slower.[84] During this process, there is formation of chromium-DNA; protein covalent, giving rise to generation of reactive intermediates and secondary free radicals.[96]

In gastrointestinal tract, Cr(VI) is reduced to Cr(V) and finally Cr(III)[97] in the gastric juice[77] with action of ascorbate[98] after oral ingestion and dermal exposure.[99]

In addition, various hepatic microsomal enzymes and flavoprotiens are also associated in reducing Cr(VI) to Cr(III).[81,100,101]

2.9.4 Detoxification and elimination of chromium

Majority of the chromium is expelled through the urinary and fecal matter after oral exposure.[102] Normally humans may excrete 0.0247-0.037 mg/L of Cr, but in cases of exposure it can raise up to 0.22-1.8 g/L. [79,103] When compared to Cr(III), which has a lower capacity to enter the cells, Cr(VI) has slower rate of excretion and higher concentrations in urine.[72]

The reduced Cr(III) gets conjugated with DNA, proteins and glutathione in the liver.[104] The so formed stable Cr(III)-glutathione complexes transit through the cell membrane, get discharged into the biliary system and out flown in the feces.[105] Majority of the orally ingested and unabsorbed Cr contributes the large percentage in fecal excretion.

Chromium in the respiratory tract is wiped out by phagocytosis by alveolar macrophages, absorption into lymph / blood circulation and mucociliary transport to the digestive tract. The remaining precipitates may persist in the lung for decades.[70]

Minor routes of excretion include transplacental, lactational,[106] through hair, nails[107] and to lesser extent wearing of joint arthroplasts made up of cobalt-chromium alloys.[35]

2.10 Pathophysiology of chromium

Cr(VI) is potentially toxic than Cr(III) because of its higher oxidizing or reducing redox potential[108] and its greater ability of penetration, retention and slower excretion.[109]

2.10.1 Oxidative and nitrosative stress induced by chromium toxicity

Due to its tetrahedral anionic property, Cr(VI) resembles natural anions (such as carbonates, sulfates & phosphates)[110] and utilizes their nonselective carrier channels to pass through the cell membranes,[100] which is responsible for its higher uptake into the cell. In contrast Cr(III) forms octahedral complexes which are impermeable to these channels resulting in lower uptake and lesser toxicity of Cr(III).[111]

Unstable Cr(VI) compounds are reduced to the stable Cr(III) by certain flavoenzymes such as glutathione reductase in the cell. During this cycle, there is generation of Cr(V) and Cr(IV) intermediates[112] with reduction of molecular oxygen to O_2 and then to H_2O_2 via dismutation.[113] The resultant Cr(V) and Cr(IV) reacts with H_2O_2 to generate reactive oxygen radicals such as dihydrogen dioxide (H_2O_2), superoxide dioxide (·O2–) and hydroxide radical (·OH) ions.[114] The intermediates like superoxide ions further react with nitric oxide and generate reactive nitrogen species (RNS) such as peroxynitrites. These reactions take place by means of Fenton-like oxidative cycling reactions, producing wide range of ROS/RNS.[115]

During one-electron reduction of Cr(VI),[116] a whole spectrum of generated ROS and RNS forms chromium- DNA, protein covalent and generates secondary free radicals.[117] These complexes induce damage to macromolecules such as DNA, lipids, and proteins and activate the nuclear transcription factor NF-kB.[118-120]

Microsomal reduction of Cr(VI) can also lead to formation of Cr(V)[121] with intermediate glutathione thiyl radicals and hydroxyl radicals producing 8-hydroxyguanosine (8-OHdG) DNA adduct.[122]

Furthermore, one of the main generators of cellular ROS is NADPH oxidase (NOX), catalyzes the oxidation of NADPH and reduces molecular oxygen to produce superoxide radicals, which are then dismutated to produce H_2O_2.[123] Additionally,

Cr(VI) phosphorylates NOX, which prevents the key antioxidant enzymes including glutathione peroxidase (GPx) and superoxidase dismutase (SOD).[124] Overall, there is net induction of various cellular signalling processes including apoptosis through bcl-2 genes.[125]

Because of the ensuing oxidative reactions, damage to various intracellular molecules, including proteins, lipids, and DNA, is assumed to be a key factor in the mutagenic, genotoxic, and carcinogenic effects of Cr(VI).[101,126]

2.10.2 Chromium induced mutagenesis and genotoxicity

The free radicals cause oxidative stress that underlies harmful effects including structural damages in the DNA such as; breaks in the strands,[127] and crosslinks with proteins,[126] causing intersections,[128] resulting in chromosomal adducts and aberrations[129] and functional polymerase arrest of DNA & RNA.[105,130,131]

These structural and functional changes cause alteration of gene expression, disruption of cellular and cytoskeletal communication and signaling pathways[132] resulting in lipid peroxidation.[133] Activation of main transcription factors[1] includes NF-κB, AP-1, p53 and HIF-1[1] which can cause overexpression of c-myc resulting in p53-dependent apoptosis,[116,118,134] cell-cycle and growth arrest,[128] interference in DNA replication and transcription[128] and thereby mutagenesis.[135]

Recent studies suggest that Cr(VI) can induce centromere mediated chromosomal instability and cause lung cancers.[1,131,136]

2.10.3 Chromium induced carcinogenicity

Cr-induced carcinogenicity is primarily attributed intracellular generation of Cr(III) from Cr(IV) and intermediary species forming deleterious complexes with target macromolecules.[137]

Extensive studies have reported a correlation between long term exposure to Cr and carcinogenicity. Squamous-cell carcinomas and adenocarcinomas of the lung were reported in intrabronchial administration of 3-5 mg calcium chromate[138] and bronchial squamous metaplasia in a dose of 2 mg of chromium.[139] Adenocarcinoma of the lung was reported in intrapleural administration of 2 mg sodium dichromate and 12.5 mg developed malignant tumours at the site of implantation,[140] a spindle-cell sarcoma was reported in subcutaneous administration of 10 mg chromium[141] and sarcoma in a dose of 30 mg of lead chromate.[142]

2.11 Effects of Cr-induced toxicity in humans and experimental animals [143-147]

Cr(VI) causes anemia due reduction in Hb and RBC counts,[143] cardiovascular changes due to heterostasis of glucose and lipid metabolism[144-146] and vital organ cellular injuries due to enhanced activities of SGOT/AST.[8,147]

Multiple studies in Cr-exposed experimental animals have demonstrated higher levels of MDA, LPO and lower levels of SOD, GSH and CAT in erythrocytes and liver tissue homogenates indicating an imbalanced oxidant and antioxidant equilibrium. [8,146,148-151]

2.12 Effects of Cr-induced pulmono-toxicity in humans and experimental animals

Studies in occupational workers humans as well as in animal attest that acute Cr(VI)-exposure in high doses can cause respiratory sensitization.[1] Acute respiratory manifestations include allergic and asthmatic attacks, dyspnoea and rhinorrhea, and a reduced forced expiratory volume (FEV1)[143,152] whereas septal perforation, epistaxis, bronchitis, pneumonia and other cardiorespiratory effects like cor-pulmonale can occur from that of chronic exposure.[152,153] A retrospective mortality study in chrome plating

workers revealed a greater risk of death from respiratory diseases like asthma and respiratory distress are more prevalent than cancer.[154]

A large Cohort health review of chromate workers in seven US chromate production plants, found that the lungs were enlarged, with no significant changes in hematological parameters.[1] Studies of workers in the ferrochromium pigment and plating, have shown a statistical association between workers with Cr(VI) exposure and lung cancer.[155,156] In a similar review of stainless steel workers, no deaths due to cardiovascular diseases were reported.[157,158]

Exposure of male Wistar rats to a $Na_2Cr_2O_7$ aerosol in different doses for different durations was evaluated and it was found that there was a significant increase in weight of lungs and leucocytic counts of the blood.[159,160]

An animal study in which inhalation to 11-23 mg/m^3 Cr(VI) for few days found bronchitis, pneumonia with fibrosis.[12] Another study reported morphological changes in alveolar structures and recruitment of macrophages in the lavage fluid of Cr(VI) inhaled animals than in those inhaled Cr(III).[100] Human studies have clearly shown that Cr(VI) is a class-I human carcinogen and increases the incidence of lung cancers.[1] Animal studies have shown that exposure to chromium(VI) by inhalation can cause lung tumors.[30]

2.13 Effects of Cr-induced cardiovascular toxicity in humans and experimental animals

A study reported that ingestion of Cr(VI) compounds leads to hypotension, ventricular arrhythmias, severe respiratory distress, metabolic acidosis, cardiopulmonary arrest and death, which were confirmed by autopsy. In another study, of 230 middle-aged workers involved in chromate production, found chromium

poisoning manifestations along with changes in electrocardiography and myocardial markers.[1,161,162]

The exact mechanisms by which chromium contributes to cardiovascular disorders are not fully understood, but popular theories link an imbalance between formation of reactive oxygen species formed by oxidative stress and body's inability to eliminate them. Radical scavengers can prevent cellular damage caused by different chromium compounds [17,18] which can be endogenous antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) or exogenous antioxidants such as vitamin C, E, A etc.[163]

Numerous investigations have examined the impact of exogenous antioxidant vitamins such as vitamin C and E on heavy metals (nickel, lead and cadmium). Uncertainty exists regarding the antioxidant role of $1,25(OH)_2D_3$ against Cr(VI)-induced cardiovascular and respiratory toxicities.

2.14 ROLE OF $1,25(OH)_2D_3$ IN CHROMIUM TOXICITY

$1,25(OH)_2D_3$, an active form of vitamin D, is mainly engaged in calcium homeostasis and bone formation, but it also plays a secondary role in a number of nonskeletal biological processes[164] through the vitamin D receptor (VDR), which is a part of the nuclear hormone receptor superfamily.[165]

2.15 Brief history

Vitamin D was discovered by McCollum and Davis in 1913, the structure of vitamin D_2 was deduced in 1931 by Askew[166] and the structure of vitamin D_3 synthetically in 1968 by Windaus.[167] $1,25(OH)_2D_3$ was discovered as an active form of vitamin D in 1968,[168] and its biologic activity[169] was confirmed in 1971[169] by multiple studies, which demonstrated that vitamin D functions as a prohormone rather than a vitamin.[170,171]

2.16 Sources

Vegetables, fruits or grains are poor sources of vitamin D. Animal food sources, except fish liver oils contain low quantities of vitamin D.[30]

2.17 Metabolism of Vitamin D

Vitamin D exists in two different forms; D_2 which is obtained through the UV irradiation of ergosterol, in plankton and D_3 synthesized in skin.[30]

2.17.1 Cutaneous phase of Vitamin D_3

Precursor of vitamin D, 7-Dehydrocholesterol (7-DHC) is converted into cholesterol by 7-dehydrocholesterol reductase.[172] A photolytic process (wavelength of 290-310) irradiates 7-dehydrocholesterol (7-DHC) to create provitamin D, which is then converted to vitamin D_3 over a time.[173]

2.17.2 Hepatic phase of 25OHD

Vitamin D is converted to 25OHD by 25-hydroxylase (CYP2R1). 25OHD serves as an indicator of vitamin D levels.[174]

2.17.3 Renal phase of $1,25(OH)_2D$

25OHD-1α hydroxylase (CYP27B) converts 25OHD to $1,25(OH)_2D$ which is responsible for carrying out hormone-like actions of vitamin D.[176] The renal synthesis of 1,25-dihydroxycholecalciferol is regulated by activation of parathyroid hormone (PTH) and suppression by calcium, phosphate and FGF23.[175,100] Subsequently, the kidneys also convert second metabolite of 25OHD, namely $24,25(OH)_2D$ by 25OHD-24 hydroxylase (CYP24A1) to $1,24(OH)_2D$.[176]

2.18 Circulatory transportation

Most of the vitamin D and its metabolites are transported in the blood stream by coupling maximally with vitamin D-binding protein (DBP) and minimally by albumin, both of which are processed by the liver. [176-178]

2.19 Mechanism of action

$1,25(OH)_2D_3$ forms a ligand complex with its transcription factor vitamin D receptor (VDR) present in most of the cells.[177] Subsequently, this complex binds to DNA sequences called vitamin D response elements (VDRE), amplifying wide spread actions of $1,25(OH)_2D_3$ in physiologic as well as pathologic processes.[176-178]

2.20 Calcium homeostasis

$1,25(OH)_2D_3$ regulates absorption of calcium in duodenal and caecal part of the intestine,[178, 179] by stimulating calcium entry at calcium specific channel called TRPV6 and TRPV5 located at the brush border of intestinal cell membrane.[179] Calcium binding proteins *Calbindins* regulate calcium transport through the cellular vesicles. Ca-ATPase pump mediates the calcium removal at the basolateral membrane of the cell. [179,180]

Similarly, $1,25(OH)_2D_3$ regulates calcium reabsorption in the renal tubule of the kidney through similar mechanisms observed in the intestine. About 98% of calcium is reabsorbed in the proximal tubule by a paracellular, sodium-dependent process (without the regulation of $1,25(OH)_2D_3$ and PTH) in; ascending loop of Henle, distal tubule and collecting duct in a range of 20, 10-15 and 5% respectively.[180,181] Calcium moves against an electrochemical gradient in a sodium independent fashion in the distal tubule transcellularly under the regulation by $1,25(OH)_2D_3$ by inducing the VDR molecule *Calbindine*.[182] In contrast, 80% of phosphate is reabsorbed in the proximal tubule under the regulation of PTH.[176,183]

$1,25(OH)_2D_3$ induces RANKL (Receptor activator of nuclear factor kappa beta), a membrane bound protein in osteoblasts to stimulate the formation of osteoclasts and promotes the production of collagen, alkaline phosphatase, osteocalcin and Fibroblast Growth Factor (FGF23), thus regulates both bone formation as well as bone

resorption.[176,184] In addition $1,25(OH)_2D_3$ stimulates calcium transport across the plasma membrane, primarily involving the bone, gut and kidney.

2.21 Antioxidant role of vitamin D in Cr-induced toxicity

The generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) react with proteins, nucleotides and polyunsaturated fatty acids that lead to oxidative stress.[185] This reaction causes lipid peroxidation, inactivation of proteins, DNA and RNA damage causing change in signaling pathways that results in cell injury in the affected organs.[186,187] The condition may be harmful to cellular processes if enzymatic and non-enzymatic antioxidants are unable to provide an adequate defense against ROS and RNS.[187] Studies on experimental animals suggested that due to structural similarities with cholesterol, vitamin D could be considered an antioxidant[188] and its effects are higher than that of vitamin E.[189] The studies have shown that Vitamin D can suppress proinflammatory cytokine in the inflammatory cells and reduce the effects of reactive oxygen species (ROS), which would otherwise aggravate the oxidative stress.[100,190]

2.22 Vitamin D in Cr-induced respiratory oxidative stress

Vitamin D can decrease oxidative stress primarily in human bronchial epithelial cells (HBEC)[100] which is a barrier and regulator of inflammation,[191] resulting in reduction of pro-inflammatory cytokines and matrix metalloproteinase (MMP) in the airway smooth muscle cells, which would otherwise contribute to bronchial inflammation and pulmonary remodelling.[192,193] Vitamin D increases interleukin (IL)-10 levels [194] in allergic lung diseases and inhibits phospholipase A2 production which is involved in the degradation of surfactants and inhibits B cell proliferation and differentiation to plasma cells.[195]

In an experimental study, bronchial lung epithelial cells treated with vitamin D produced more IL-8 and reduced phagocytosis in macrophages after LPS stimulation compared to cells not treated with vitamin D. [100,196] In another human study, high doses of vitamin D in patients reduced pulmonary exacerbations which may be attributed to VDR present in the HBEC that could activate $1,25(OH)_2D_3$.[100,197]

$1,25(OH)_2D_3$ blocks the translocation of transcription factor p65, involved in transformation of CD4+T helper cells into the Th17 lineage.[100,198] Molecular mechanisms have proved, regulation of *p*38 MAPK activity and the nuclear translocation of NF-κB regulation by vitamin D in anti-inflammatory actions.[100,199]

A review of 22 meta-analysis studies found a correlation between vitamin D intake and inhibition of cell growth, improved prognosis, lower metastasis and longer survival in lung cancer patients.[200]

2.23 Vitamin D in Cr-induced cardiovascular oxidative stress

Research studies indicate a strong connection between vitamin D_3 deficiency and incidence of cardiovascular events.[201,202] Interventional studies suggest $1,25(OH)_2D_3$ deficiency can lead to endothelial dysfunction, [203,204] but this can be reversed with vitamin D supplementation, improving cardiovascular health. [205]

2.24 Vitamin D in Cr-induced vascular oxidative stress

A study proposed that $1,25(OH)_2D_3$ exerts its antioxidant effects in endothelial cells of human coronary artery by inhibiting NADPH enzyme expression.[206] Another study explained vitamin D's vasoprotective effect for its ability to inhibit advanced glycation end-products (AGEs) buildup in the aortic tissue.[207]

After oxidative stress, the Ras-MAPK signaling pathway regulates cell apoptosis.[208] The pathway also controls transcription factors[209] like SIRT1,[210] crucial for vascular endothelial homeostasis[211] and in preventing endothelial cell

death.[212] A study found that $1,25(OH)_2D_3$ inactivates the caspase cascade by turning on the MEKs/ERKs/SIRT-1 axis in endothelial cells under oxidative stress, thereby reducing superoxide production and apoptosis.[213] Pretreatment with $1,25(OH)_2D_3$ also prevented superoxide production, regulated the autophagy-apoptosis interaction, preserved the mitochondrial function and enhanced cell survival.[214] Vitamin D may impact endothelial cell function by improving oxidative stress response. Deficiency has been linked to chronic conditions like cardiovascular disease. [201]

Oxidative stress is linked to chronic diseases including cardiovascular and respiratory diseases.[186] Studies suggest that vitamin D may have positive effects on various conditions in animals, but evidence in humans is lacking.

Chapter-3

Aim and Objectives of the Study

3.1 AIM OF THE STUDY

The study was aimed to analyze pathophysiologic changes in the cardiovascular and respiratory system on hexavalent chromium exposure and the antioxidative role of $1,25(OH)_2D_3$ in Cr-induced cardiovascular and respiratory changes.

3.2 OBJECTIVES OF THE STUDY

3.2.1. To evaluate effect of hexavalent chromium on heart, coronary artery, aorta and lungs in Wistar strain of male albino rats by studying various hematological, biochemical, molecular and histopathologic alterations in heart, coronary artery, aorta and lungs.

3.2.2. To evaluate effect of supplementation of $1,25(OH)_2D_3$ on heart, coronary artery, aorta and lungs in Wistar strain of male albino rats exposed with hexavalent chromium by studying hematological, biochemical, molecular and histopathological alterations in heart, coronary artery, aorta and lungs.

3.3 HYPOTHESIS

3.3.1 Null Hypothesis:

There will not be any significant effect of $1,25(OH)_2D_3$ supplementation on cardiovascular and respiratory pathophysiology in male albino rats exposed to Chromium(VI).

3.3.2 Alternate Hypothesis:

$1,25(OH)_2D_3$ supplementation may improve cardiovascular and respiratory pathophysiology in male albino rats exposed to Chromium(VI).

Chapter-4

Materials and Methods

4.0 MATERIALS AND METHODS

4.1 Sources of data collection

4.1.1 Study design : Experimental animal model study.

4.1.2 Animals used : Adult Wistar strains of male albino rats.

4.1.3 Period of study : 3 years (From October 2015 to November 2018).

4.1.4 Treatment period : 21 days.

4.2 Ethics on animal research

The research protocol was followed as per the guidelines of "ICMR" and "Committee for Control and Supervision of Experiments on Animals" [215] (CPCSEA) on animal research, Government of India. Institutional Animal Ethics Committee (IAEC) clearance was approved by vide letter No: O/w No. *AMC/IAEC/ 2014-15/02/* dated *29/06/2015*.

4.3 Sampling design and sample size [216]

The sample size was calculated using the resource equation approach. [217, 218]

$$\text{Sample size}^{[218]} = \frac{\text{Degree of freedom}}{\text{Number of groups}} + 1$$

$$\text{Minimum numbers per group} = \frac{10}{4} + 1 = 3.5$$

$$\text{Maximum numbers per group} = \frac{20}{4} + 1 = 6$$

Total No. of rats = No. of groups (4) X No. of rats in each group (6)

= 24

4.4 Animal selection criteria

Table 4.4 showing selection criteria of experimental animals.

Criteria	Inclusion criteria	Exclusion criteria
Wistar rats	Healthy and male	Unhealthy and female
Age (in weeks)	8-10	<8 / >11
Body weight (in gms)	180-220	<180 / >220

4.5 Research methodology

4.5.1 Experimental animals

Adult laboratory bred Wistar strains of male albino (*Rattus norvegicus*) rats were used for the experiment. The experimental rats were adequately acclimatized for 7 days to the laboratory conditions with natural lighting schedule *i.e.* 12h light and 12h dark (circadian rhythm), at constant room temperature (22±4 ^{o}C) and humidity of ~70%. They were fed with sufficient laboratory stock diet, water *ad libitum*.[219] The rats were then randomly allocated into 4 groups; each with 6 rats. Three rats were kept in each metabolic wire cage measuring 60 X 30 X 20 cm.[220] All the animals were in docile condition and no mortality was observed. [221]

4.5.2 Experimental animal groups

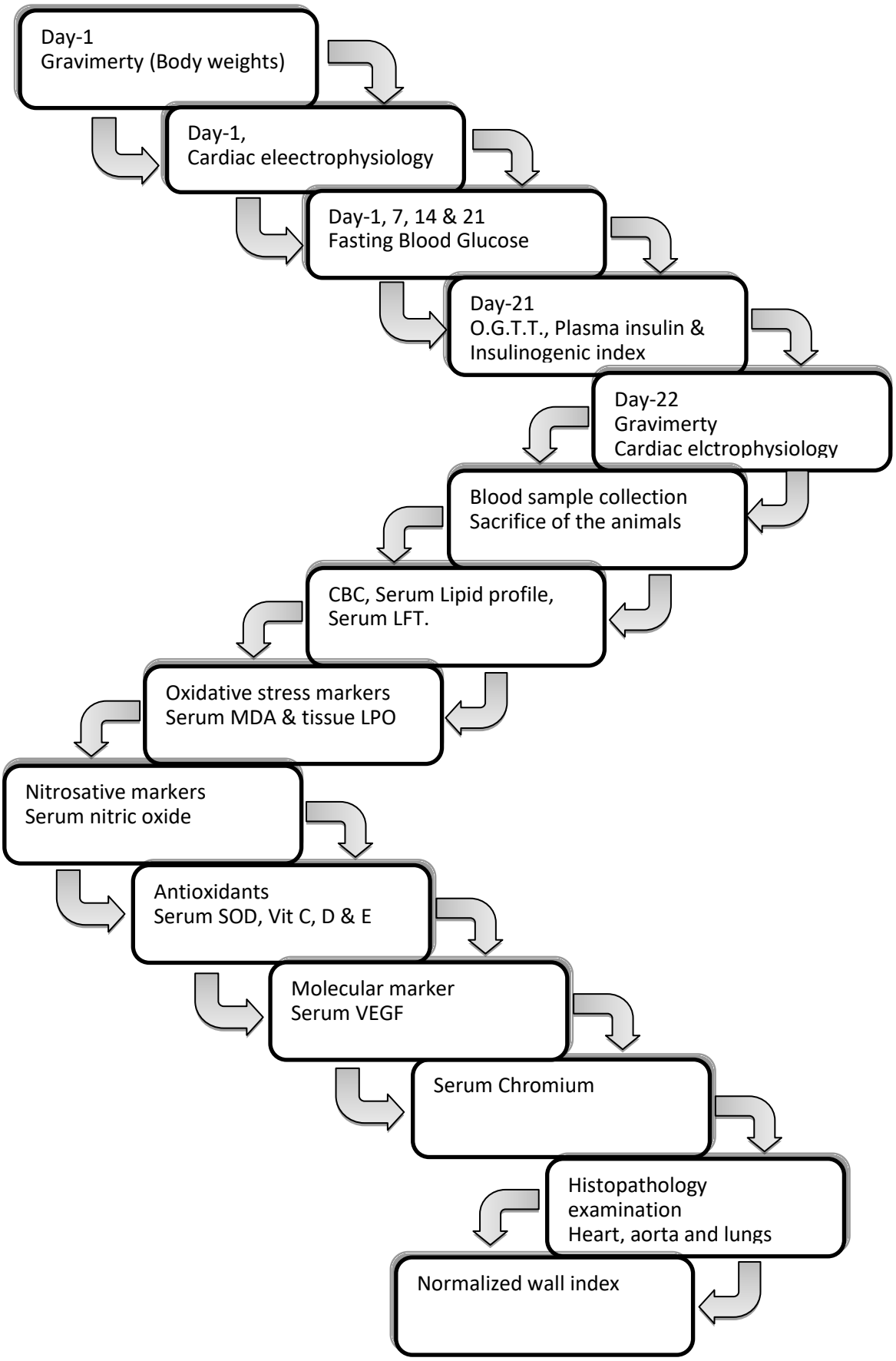
Table 4.5.2 showing experimental animal groups with respective interventions. [222]

Groups	Interventions
Group-1 (n=6)	Untreated normal control
Group-2 (n=6)	Exposure to Potassium dichromate ($K_2Cr_2O_7$) in double distilled water (Qualigens Fine Chemicals, Mumbai, India) in a dose of 0.5 mg/100 gm body weight intraperitoneally on alternate days for a total of ten treatments.[223]
Group-3 (n=6)	Supplementation of $1,25(OH)_2D_3$ (Centaur pharmaceuticals Mumbai, India) in daily dose of 1mcg/gm body weight orally.[224]
Group-4 (n=6)	Exposure to $K_2Cr_2O_7$ (0.5 mg/100 gm b.wt. I.P.) and supplementation of $1,25(OH)_2D_3$ (1 mcg / gm b.wt. orally) simultaneously.

4.5.3 Study Place

The animals were maintained in Central Animal House, Al Ameen Medical College Vijayapura, Karnataka. All the experimental procedures and evaluations were done in Department of Pathology, A.M.C. and "*Laboratory of Vascular Physiology and Medicine*", Department of Physiology, Shri B. M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura.[225] Estimation of serum chromium was done at Shivaji University, Kolhapur, Maharashtra.

4.6 Experimental protocol:



4.7 Sacrifice of animals and collection of tissues:

After the collection of blood samples, the animals were sacrificed by overdose of ketamine (150 mg/kg, ip)[226] in the morning hours to avoid the effects of circadian rhythms on the rats, [227] followed by collection of heart, aorta, lungs and liver for organ somatic index and histopathological evaluation and tissue biochemical analysis.

METHODS OF COLLECTION OF DATA:

Experimental Procedures

4.8 Gravimetry

4.8.1 Change in body weight percentage

The pre-interventional first body weights on day-1 and post-interventional on day-22 of all the rats were measured with a single pan 'Practum 1102-10IN' electronic balance. Percentage body weight change was calculated by using the following formula;[225]

$$\text{Percentage change in body weight} = \frac{\text{(Final body weight) - (Initial body weight)}}{\text{(Initial body weight)}} \text{ X } 100$$

4.8.2 Organ somatic index

After the sacrifice of the animals, lungs and heart were dissected out, trimmed off the fat, wiped clean and were weighed immediately. Organ somatic index was calculated using the formula;

$$\text{Organ somatic index} = \frac{\text{Weight of the organ}}{\text{Weight of the body}} \text{ X } 100$$

4.9 Evaluation of cardiac electrophysiology [228-230]

The following parameters were measured on day-1 before commencement of the intervention and on day-22 after the completion of the intervention.

4.9.1 Measurement of Blood Pressure

Animals were placed on a restrainer, acclimatized for 20 min prior to recording the BP. A non-invasive tail cuff sensor (NIBP / BioPac 200A) was placed which was connected with BioPac MP 100 (a PC windows-based animal electrophysiology system) and analysed by BioPac Student Lab 4.1 software. Systolic (SBP) and diastolic blood pressures (DBP) were measured thrice and a mean was considered for each rat. Mean arterial pressure (MAP) was calculated using the following formula;[225]

$$\text{Mean arterial pressure (MAP)} = \text{DPB} + \frac{1}{3}(\text{SBP - DPB})$$

4.9.2 Recording of ECG (lead II)

After an overnight fasting, the animals were anaesthetized in the morning hours, using Ketamine 60 mg/kg and xylazine 6 mg/kg.[231] ECG was recorded using needle electrodes connected to windows based Biopac MP 45 (a PC windows based animal electrophysiological system and Biopac Student Lab 4.1 software. USA).

4.9.3 Heart Rate

Heart rate was calculated from the recorded ECG.

4.9.4 Analysis of Heart Rate Variability (HRV)

R-R intervals obtained from the recorded ECG (using Biopac Student Lab 4.1 software) 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.[226]

Components of the frequency domain method of HRV analysis [232,233]

Components		**Indicator**
Low frequency component (LF) (nu)	:	Sympathetic activity
High frequency component (HF) (nu)	:	Parasympathetic activity
LF / HF ratio	:	Sympathovagal balance

4.10 Collection of Blood

At the end of 21 days of intervention, the animals were kept on overnight fasting, anesthetized on day-22 and 4 ml of blood from the sublingual vein was collected in commercial EDTA for hematology and plain bulbs with clot activator for biochemical investigations.[233]

Serum preparation: Blood samples were collected in plain test tubes and allowed to stand for two hours at laboratory temperature and then centrifuged for 15 minutes at 1500 rpm. Serum was separated and stored in the labeled eppendorfs tubes -20°C for further analysis.[234]

4.11 Hematological analysis

0.5 ml of blood samples were collected in commercially available potassium EDTA tubes and were analysed by an automated haematology cell counter (Sysmex-KX 21, Transasia Ltd.). The complete blood counts; Red blood cell count (RBC $x10^6/\mu L$), hemoglobin concentration (Hb xg/dL), hematocrit (hct x%), erythrocytic indices (MCV xfl, MCH xpg, MCHC xg/dL), total white blood cell count (WBC x $10^3/\mu L$) and total platelet count (Plt $x10^3/\mu L$) were analysed.[235]

4.12 Biochemical analysis of glucose homeostasis

4.12.1 Fasting blood glucose estimation

Blood samples were collected from the caudal tail vein and fasting blood glucose was estimated by Trinder's method performed on day- 1, 7, 14 & 21 after an acute exposure. [236, 237]

Assay principle

The enzyme Glucose oxidase oxidizes glucose to form D-gluconic acid along with dihydrogen dioxide. The latter is made to oxidize a chromogen phenol aminophenazone. Enzyme peroxidase reacts with 4-aminoantipyrine in presence of

phenol producing a colored coordinate compound quinonemine. The intensity of color developed at 505 nm which is proportional to the glucose levels in the sample.

$$[\alpha\text{-D-glucose}] \xrightarrow{\text{Mutarotase}} [\beta\text{-D-glucose}]$$

$$[\alpha\text{-D-glucose}] + [H_2O] + [O_2] \xrightarrow{\text{Glucose Oxidase}} [\beta\text{-D-gluconic acid}] + [H_2O_2]$$

$$[H_2O_2] + [\text{Phenol}] + [\text{4-aminophenazone}] \xrightarrow{\text{Peroxidase}} [\text{Quinonemine (dye)}] + [4\ H_2O]$$

Reagents and preparation

Glucose oxidase-peroxidase reagents were prepared using solution of 25 mg O-dianisidine.

Table 4.12.1 shows assay procedure of fasting blood glucose estimation.

	Reagent blank	Standard (Cal.)	Sample
Reagent (µl)	1000	1000	1000
Sample (µl)	---	---	10
Standard (Cal.) (µl)	---	10	---
Distilled water (µl)	10	---	---
Incubation for 40 min at 35ºC			

Absorbance of sample A_{Sam} and standard A_{Std} against reagent blank at 540 nm was measured by using UV-VIS Spectrophotometer. The following equation was used to express blood glucose level in terms of mg/dL;

$$\text{Blood Glucose (mg/dL)} = \frac{\Delta A\ Sam}{\Delta A\ Std} \times 100\ (\text{Conc. of Std.} / \text{Conc. of Cal.})$$

4.12.2 Oral glucose tolerance test estimation (OGTT)[238]

Blood samples were collected from the lateral caudal tail vein and OGTT was estimated on day-21. A 2 hr Oral Glucose Tolerance Test was performed at 0.0hrs, followed by oral glucose load of 0.35 gm/kg b.wt. and then at 0.5hrs, 1.0hrs, 1.5hrs & 2.0hrs. Blood glucose level was determined by Accu-chek glucometer.

4.12.3 Plasma insulin and insulinogenic index [225,239]

On day-21, lateral caudal tail vein of all the rats was used to collect blood samples in heparin microtubes, wrapped in frozen gel packs and centrifuged at 4000 rpm for 7 minutes. Concentrations of insulin in the plasma were measured on day-21 at 0.0 hr, followed by oral glucose load of 0.35 gm/kg b.wt, and then at 0.5, 1.0, 1.5 & 2.0 hrs, by using ELISA kits (ERINS, Thermo Fischer Scientific, Life technologies).[225]

4.12.4 Plasma insulin estimation

Reagents and preparation

1X Wash Buffer, Diluent B, Diluent C, biotin conjugate, 1X Streptavidin-HRP standard solutions and samples were prepared and diluted as per the manual instructions.

Table 4.12.4 shows assay procedure for plasma insulin estimation.

Total assay time by ELISA : 4 hours and 45 minutes.

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	Std 8
	300 IU/mL	150 IU/mL	75 IU/mL	37.5 IU/mL	18.7 IU/mL	9.38 IU/mL	4.69 IU/mL	0.0 IU/mL
Standards (µL)	100	100	100	100	100	100	100	100
Samples (µL)	100	100	100	100	100	100	100	100
Covering and incubation for 2.5 hrs at room temp.								
4 times washing with 1X Wash Buffer.								
Biotin conjugate (µL)	100	100	100	100	100	100	100	100
Incubation for 1 hour at room temp.								
Washed 4 times with 1X Wash Buffer.								
Streptavidin-HRP (µL)	100	100	100	100	100	100	100	100
Incubation for 45 minutes at room temp.								
4 times washing with 1X Wash Buffer.								
TMB Substrate (µL)	100	100	100	100	100	100	100	100
The substrate began to turn blue.								
Stop solution (µL)	50	50	50	50	50	50	50	50
Change of color of the solution in the wells from blue to yellow.								
Reading the absorbance at 450 nm in ELISA reader within 30 minutes.								
Drawing a standard curve of concentrations of unknown samples and controls								

Rat plasma insulin concentration was expressed as….. µIU/ml and the insulinogenic index were calculated using the following formula;

$$\text{Insulinogenic index} = \frac{(30 \text{ min plasma insulin}) - (\text{fasting plasma insulin})}{(30 \text{ min blood glucose}) - (\text{fasting blood glucose})}$$

4.12.5 Liver glycogen estimation [225, 240,241]

After sacrifice of animals, estimation of liver glycogen was carried by using Abnova ELISA kit. [225, 240, 241]

Assay principle

Enzyme amyloglucosidase catalyzes glycogen to produce β-D-glucose, which subsequently gets transformed to hydrogen peroxide by glucose oxidase. In mediation of horseradish peroxidase, hydrogen peroxide reacts with 10-acetyl-3, 7-dihydroxyphenoxazine (ADHP) forming fluorescent resorufin.

Reagent and preparation

Kit contained ready to use glycogen standards, buffer, enzyme mixture and fluorometric detector. Reagent were prepared and diluted as per the manual instructions.

Tissue homogenate sample preparation

100-150 mg of the frozen tissue was homogenized in 2 mL of protease inhibitor assay buffer. Centrifuged at 800 xg for 10 minutes at 4°C and the supernatant was stored on ice. Tissue samples were diluted at 1:50 using Assay Buffer (1X) and standard curve was prepared.

Table 4.12.5 shows assay procedure for liver glycogen estimation.

6-well solid plate was used for the colorimetric assay.

	Standards (µg/ml)							
	200	100	50	25	12.5	6.25	3.13	0.0
Standards (µL)	10	10	10	10	10	10	10	10
Sample (µL)	10	10	10	10	10	10	10	10
Glycogen Hydrolysis Buffer (µL)	50	50	50	50	50	50	50	50
Hydrolysis Enzyme Solution (µL)	50	50	50	50	50	50	50	50
Incubation at 37°C for 30 minutes under cover.								
Developer	100 µL of DMSO Assay Reagent to Glycogen Fluorometric Detector							
400 µL of diluted Assay Buffer to the fluorometric detector and vortex.								
Developer (µL)	150	150	150	150	150	150	150	150
Incubation at 37°C for minutes under cover.								
Absorbance (colorimetric): at 570 nm.								
Streptavidin-HRP (µL)	100	100	100	100	100	100	100	100
Incubation at room temperature for 45 minutes.								
Washing with 1X Wash Buffer 4 times.								
TMB Substrate (µL)	100	100	100	100	100	100	100	100
Change of color of the substrate to blue.								
Stop solution (µL)	50	50	50	50	50	50	50	50
Change of color of solutions from blue to yellow in the wells.								
Absorbance at 450 nm in ELISA reader within 30 minutes.								
Standard curve drawn from concentrations of unknown samples and controls.								

Calculation of results

Absorbance of each standard and sample was determined. Absorbance value of zero standards from all other standards was subtracted. Values were plotted. Absorbance of the sample minus the absorbance of the sample yielded the corrected sample measurement (CSM). Glycogen concentration of the samples was calculated as follows;

$$\text{Glycogen (µg)} = \text{CSM} - \frac{\text{(y - intercept)}}{\text{Slope}} \text{ X Sample dilution}$$

4.13 Biochemical estimation of serum lipid profile

4.13.1 Estimation of serum total cholesterol (TC) [241]

Enzymatic cholesterol oxidase peroxidase method (CHOD PAP) was employed for estimating total cholesterol in the serum by using commercial kits (Tranasia Bio-medicals Ltd, ERBA Diagnostics, Mannheim GmBH).[241]

Assay principle

Enzyme cholesterol-esterase breaks down cholesterol esters into free fatty acids and free cholesterol. Subsequently, enzyme cholesterol oxidase causes oxidation of free cholesterol to produce cholestenone and dihydrogen dioxide. The dihydrogen dioxide reacts with ampyron and phenols to produce a chromophore dye quinoneimine which was quantified at a wavelength of 505 nm spectrophotometrically.

Reagents

Ready to use liquids provided in the assay kit R1 and R2.

Table 4.13.1 shows assay procedure for estimation of serum total cholesterol (TC).

	Blank	Standard	Test
Sample (µl)	--	--	10
Standard (µl)	--	10	--
Reagent (µl)	1000	1000	1000
Distilled water (µl)	10	--	--
Incubation for 10 minutes at 37^0C.			

The absorbance of the test sample and the standard against the blank at 505 nm by UV-VIS visible spectrophotometer (Shimadzu, Model: UV 1800) was measured. The following equation was used to express serum cholesterol level in terms of mg/dL;

Calculation

$$\text{Total Cholesterol (mg / dl)} = \frac{\text{ED of Test}}{\text{ED of Std}} \text{ X Conc. of Std}$$

4.13.2 Estimation of serum triglyceride (TGL) [242-244]

Serum triglycerides were estimated by enzymatic (Glycerol-3-phosphate oxidase) (GPO-PAP) modified Wako method using a commercially available kit (ERBA Diagnostics, Mannheim GmBH).

Assay principle

The enzyme lipase hydrolyzes triglycerides to release free fatty acids and glycerol.[244] Enzymatic phosphorylation of glycerol by glycerol-kinase gives rise to glycerol-3-phosphate, which subsequently gets oxidized to glycerone and hydrogen peroxide by glycerol-phosphate-oxidase.[243] The enzyme peroxidase catalyzes hydrogen peroxide with 4-Amino antipyrine and 3,5 dichloro 2-hydroxybenzene sulfonate. The amount of quinoneimine dye produced is directly proportional to the concentration of triglycerides of the sample when measured at 505 nm. [244]

$$[TGL] + [H_2O] \xrightarrow{LPL} [Glycerol] + [FFA]$$

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

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

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

Reagent Composition

The reagent bottle and AQUA-4 was dissolved and allowed to stand for 10 minutes at room temperature.

Table 4.13.2 shows assay procedure for estimation of serum triglycerides.[244]

	Blank	Standard	Test
Working reagent (µl)	1000	1000	1000
Distilled water (µl)	10	---	---
Standard (µl)	--	10	--
Sample (µl)	---	---	10
Incubation for 10 minutes at 37^0C.			

The absorbance of the test sample and the standard against the blank at 505 nm by UV-VIS visible spectrophotometer (Shimadzu, Model: UV 1800) was measured. The following equation was used to express serum triglycerides level in terms of mg/dL.

Calculation

$$\text{Triglycerides (mg/dl)} = \frac{\text{[Abs. of Test]}}{\text{[Abs. Of Std]}} \text{ X [Conc. of Std]}$$

4.13.3 Estimation of serum high density lipoprotein cholesterol (HDL-C)[245]

Serum HDL was measured using classic precipitation method of polyethylene glycol methyl ether (PEGME) and polyvinyl sulfonic acid (PVS) coupling.

Assay principle

The PVS/PEGME couple solubilizes LDL, VLDL and chylomicron, solubilizes and releases HDL making it available. The enzymes cholesterol oxidase (CHOD) and cholesterol esterase (CHER) interact with high-density lipoprotein (HDL) producing hydrogen peroxide, which is then detected using Trinder's reaction.

$$\text{HDL-C} \xrightarrow{\text{CHOD, CHER}} \text{Fatty acids} + H_2O_2$$

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

Reagents and preparation

The liquids R1 and R2 were readily usable. Calibrator was reconstituted in 1000 µl of D.W. at 20-25^{0}C.

Table 4.13.3 shows assay procedure for estimation of serum high density lipoprotein cholesterol (HDL-C).

	Reagent Blank	Sample/Calibrator
Reagent 1 (µl)	750	750
D.W. (µl)	10	10
Calibrator / Sample (µl)	---	10
Incubation for 5 minutes at 37^{0}C.		
Reagent 2 (µl)	250	250
Incubation at 37^{0}C for 5 minutes.		

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

Calculation

$$\text{HDL (mg/dl)} = \frac{\text{[Abs. of sample] ^ [Abs. of blank]}}{\text{[Abs. of cal.] ^ [Abs. of cal. blank]}} \text{ X [Conc of Calibrator]}$$

4.13.4 Estimation serum low density lipoprotein cholesterol (LDL-C) [227,246]

The Friedewald's equation [246] was employed to determine LDL in the serum as under; [227]

$$\text{LDL-C} = \text{TC - HDL-C - } \frac{\text{TGL}}{5}$$

4.14 Estimation of serum Liver function tests

4.14.1 Estimation of serum bilirubin [247]

Serum bilirubin total and direct was estimated by modified Pearlman & Lee method using a commercially available kit (ERBA Diagnostics, Mannheim GmBH).

Assay principle

Total bilirubin glucuronate in presence of solubilizing agent is directly coupled with 3,5dichlorophenyl sulphodiazonium and produces red colored dye azobilirubin, the colour intensity of which is directly proportional to the concentration of total bilirubin in the sample, measured at 540-550 nm spectrophotometrically.

Reagent preparation

Test	Working Reagent	Reagent-1	Reagent-2	Reagent-3
Bilirubin Total (ml)	25	25	---	0.5
Bilirubin Direct (ml)	25	---	25	0.25

Table 4.14.1 shows assay procedure for estimation of serum bilirubin.

	Reagent blank	Standard (Cal.)	Sample
Working reagent (ml)	0.500	0.500	0.500
Sample (ml)	---	---	0.025
Standard (Cal.) (ml)	---	0.025	---
D.W. (ml)	0.025	---	---
Incubation at 37 °C for 5 minutes.			

The absorbance of the test sample $A_{[Sam]}$ and the standard $A_{[Std]}$ against the reagent blank at 540 nm by UV-VIS visible spectrophotometer (Shimadzu, Model: UV 1800) was measured. The following equation was used to express serum bilirubin level in terms of mg/dL;

Calculation

$$\text{Total \& Direct Bilirubin (mg/dl) in serum} = \frac{\Delta A\ [Sam]}{\Delta A\ [Std]}\ \text{X Calibrator concentration}$$

4.14.2 Estimation of serum AST (aspartate transaminase) [248]

Serum AST was estimated by IFCC primary reference procedure method using a commercially available kit (ERBA Diagnostics, Mannheim GmBH).

Assay principle

Aspartate is catalyzed to α-ketoglutarate by Aspartate aminotransferase (AST) (glutamate oxaloacetate (GOT) producing glutamate and oxaloacetate. The malate dehydrogenase (MDH) and NADH (nicotinamide adenine dinucleotide hydrogenase) reduces oxaloacetate to malate as follows;

$$[\text{L Aspartate}] + [\text{2 oxoglutarate}] \xrightarrow{\text{AST (GOT)}} [\text{L Glutamate}] + [\text{Oxaloacetate}]$$

$$[\text{Oxaloacetate}] + [\text{NADH}] + [H^+] \xrightarrow{\text{Malate Dehydrogenae (MDH)}} [\text{Malate}] + [\text{NAD}]$$

$$[\text{Sample pyruvate}] + [\text{NADH}] + [H^+] \xrightarrow{\text{Lactate Dehydrogenae (LDH)}} [\text{L Lactate}] + [\text{NAD}]$$

Conversion of NADH to NAD by oxidation is measured at 340 nm of absorbance. The decreasing concentration of NADH is proportional to the catalytic concentration of AST present in the sample.

Table 4.14.2 shows assay procedure for estimation of serum AST (aspartate transaminase).

	Reagent blank
Reagent-1 (Buffer) (ml)	1.000
Sample (ml)	0.100
Incubation for 5 min. at 37°C.	
Reagent-2 (substrate) (ml)	0.250
Incubation at 37°C for 1 min.	

Absorbance of the calibrator and the sample against the reagent blank were measured initially. Followed by 1, 2 and 3 minutes absorbance change was measured and 1 minute absorbance change (ΔA/min) was calculated. The following equation was used to express the serum AST level in the sample;

Calculation

$$\text{AST / GOT (U/L)} = \frac{\Delta A \text{ Sam / min.}}{\Delta A \text{ Cal / min.}} \text{ X Concentration of calibrator}$$

One IU denotes the quantity of an enzyme required to converts 1 µmol of substrate in one minute, under standard conditions.

4.14.3 Estimation of serum ALT (alanine transaminase)[248]

Serum ALT was estimated by IFCC primary reference procedure method using a commercially available kit (ERBA Diagnostics, Mannheim GmBH).

Assay principle

Alanine aminotransferase (ALT) or Glutamate pyruvate transaminase (GPT) catalyzes the alanine to 2-oxoglutarate to form L-glutamate and pyruvate. Enzyme lactate dehydrogenase (LDH) reduces pyruvate to lactate together with NADH to NAD through oxidation. The rate of NADH oxidation is monitored by decreasing absorbance at 340 nm which is proportional to the concentration of ALT present in the sample.

L Glutamate + Pyruvate

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{Lactate Dehydrogenae (LDH)}} \text{L Lactate} + \text{NAD}$$

$$\text{Sample pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{Lactate Dehydrogenae (LDH)}} \text{L Lactate} + \text{NAD}$$

Table 4.14.3 shows assay procedure for estimation of serum ALT (alanine transaminase).

	Reagent blank
Reagent-1 (Buffer) (ml)	1.000
Sample (ml)	0.100
	Incubation at 37°C for 5 minutes.
Reagent-2 (substrate) (ml)	0.250 ml
	Incubation at 37°C for 1 minute.

Readings of initial absorbance of the calibrator and the sample were taken against the reagent blank. Followed by readings of absorbance change after at 1, 2 and 3 min was taken with an interval of one minute. Change in absorbance per minute (ΔA/min) was calculated. The following equation was used to express the serum ALT level in the sample;

Calculation

$$\text{ALT / GPT (U/L)} = \frac{\Delta A \text{ Sam / min.}}{\Delta A \text{ Cal / min.}} \text{ X Concentration of calibrator}$$

One IU denotes the quantity of an enzyme required to converts 1 µmol of substrate in one minute, under standard conditions.

4.14.4 Estimation of serum alkaline phosphatase [249]

Serum ALP was estimated by IFCC primary reference procedure method using a commercially available kit (ERBA Diagnostics, Mannheim GmBH).

Assay principle

In an alkaline pH, alkaline phosphatase catalyzes the hydrolysis of 4-nitrophenyl phosphate (substrate) to yellow colored 4-nitrophenol and phosphate. Rate of change in absorbance at 405-415 nm is monitored over a fixed time interval, which is directly proportional to the activity of the ALP, present in the sample.

$$[\text{AMP}] + [\text{4-NPP}] + [H_2O] \xrightarrow[\text{Mg2++ / Alkaline Ph}]{\text{ALP}} [\text{4-nitrophenol}] + [\text{Phosphate}]$$

Table 4.14.4 shows assay procedure for estimation of serum alkaline phosphatase.

	Reagent blank	Calibrator	Sample
Reagent-1 (ml)	0.800	0.800	0.800
Sample (ml)	---	---	0.020
Calibrator (ml)	---	0.020	---
Distilled water (ml)	0.020	---	---
Incubation at 37°C for 5 minutes.			
Reagent-2	0.200	0.200	0.200
Incubation at 37°C for 1 minute.			

Readings of initial absorbance of the calibrator and the sample were taken against the reagent blank. Followed by readings of absorbance change after at 1, 2 and 3 min was taken with an interval of one minute. Change in absorbance per minute (ΔA/min) was calculated. The following equation was used to express the serum ALP level in the sample;

Calculation

$$\text{ALP (U/l)} = \frac{\Delta A \text{ Sam / min.}}{\Delta A \text{ Cal / min.}} \text{ X Concentration of calibrator}$$

One IU denotes the quantity of an enzyme required to converts 1 µmol of substrate in one minute, under standard conditions.

4.14.5 Estimation of serum total proteins [250,251]

Serum TP was estimated by biuret reaction method using a commercially available kit (ERBA Diagnostics, Mannheim GmBH).

Assay principle

The protein peptides react with copper sulphate ions in alkaline solution sodium hydroxide forming a blue-violet ion complex which is proportional to the protein concentration in the sample at an absorbance of 546 nm.

4.14.5 Estimation of serum total proteins [250, 251]

	Reagent blank	Standard (Cal.)	Sample
Reagent (ml)	1.00	1.00 ml	1.00 ml
Distilled water (ml)	0.02	---	---
Sample (ml)	---	---	0.02
Standard (Calibrator) (ml)	---	0.02	---
Incubation in the dark for 5 minutes.			

Absorbance of the sample A_1 and the standard (calibrator) A_2 against the reagent blank is read in interval of 30 minutes.

Calculation

The following equation was used to express the total protein level in the sample;

$$\text{Total protein level (gm/dl)} = \frac{A\,1}{A\,2} \text{ X Concentration of Std}$$

4.14.6 Estimation of serum albumin [252]

Serum albumin was estimated by Bromo Cresol Green method using a commercially available kit (ERBA Diagnostics, Mannheim GmBH).

Assay principle

Albumin present in the sample binds specifically with Bromo Cresol Green (BCG) which is an ionic dye at pH 4.2. The reaction gives rise to a change of color from yellow green to green blue and a shift in absorbance of BCG dye, which is proportional to the concentration of albumin, when measured spectrophotometrically at absorbance of 578 nm.

Table 4.14.6 showing assay procedure for estimation of serum albumin [252]

	Reagent blank	Standard (Cal.)	Sample
Reagent-1 (ml)	1.0	1.0	1.0
Sample (ml)	---	---	0.01
Standard (Calibrator) (ml)	---	0.01	---
Distilled water (ml)	0.01 ml	---	---
Incubation at 37°C for 5 minutes.			
Reagent-2 (ml)	0.200	0.200	0.200
Incubation at 37 °C for 5 minutes.			

Absorbance of the A_{Sam} and the standard (calibrator) A_{Std} against the reagent blank is read.

Calculation

The following equation was used to express the albumin level in the sample;

$$\text{Albumin (g/dl)} = \frac{\Delta A\ \text{Sam}}{\Delta A\ \text{Std}} \text{ X Concentration of Std}$$

4.15 Oxidative stress assessment

4.15.1 Estimation of serum Malondialdehyde (MDA) [253]

Serum MDA was estimated by the method of Buege and Aust. Malondialdehyde (MDA) is one of the byproducts of lipid peroxidation and commonly utilized as an oxidative stress marker.

Assay principle

Degradation of polyunsaturated fatty acid (PUFA) by a free radical chain reaction generates Malondialdehyde. MDA reacts with thiobarbituric acid and forms pink colored MDA-TBA adduct which is read at 535 nm spectrophotometrically.

Reagents and preparation

TCA-TBA-HCl reagents, MDA standards and working standards and serum samples were prepared.

Table 4.15.1 shows standardization procedure for estimation of serum Malondialdehyde (MDA).

The standardization in a range of 2-10 µM/L was carried out referring to the table and all the reagents were added according to the values given below;

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
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	---	1	1
In hot water bath for 15 min.				

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

Estimation of MDA in the sample

Assay procedure

100 µl of serum was diluted with distilled water to prepare 500 µl solution. 1000 µl of TCA-TBA-HCl reagent was mixed with the diluted sample, kept in a hot water bath for 15 minutes, cooled down and then centrifuged. The pink colored supernatant was aspirated and the optical densities were read at 535 nm by UV-VIS visible spectrophotometer (Shimadzu, Model: UV 1800).

Calculation

The optical densities of pink colour of the sample showed a direct correlation with the concentration of MDA in the sample. The MDA values were determined by plotting a graph of optical densities against the standards multiplied by the respective dilution factors and expressed in µM/L.

4.15.2 Estimation of tissue lipid peroxide level (LPO)[254]

Assay principle[256]

Lipid peroxide concentration in the tissue samples was determined indirectly by means of derivatizing malondialdehyde (MDA) with thiobarbituric acid (TBA) at high temperature in an acidic condition. Thiobarbituric acid reacts with MDA to form a pink colored MDA-TBA adduct, which is measured spectrophotometrically at 532 nm.

Reagents preparation

Thiobarbituric acid (TBA), sodium dodecyl sulfate (SDS), and Glacial acetic acid (GAA) was prepared.

Tissue homogenate preparation[254]

Approximately 100 mg of wet tissue was mixed with 1mM EDTA buffer by Polytron homogenizer set at pH of 7.0 on 20 seconds for few minutes. The homogenate

was centrifuged for 20 min at 3000 xg at 40 ^{O}C and the supernatant was utilizes for assaying LPO. 500 µl of 10% tissue homogenate was taken.

Assay procedure [254]

500 µl of tissue homogenate was combined with 200 µl of SDS, 1500 µl of GAA, 1500 µl of TBA and 700 µl of distilled water in test tubes and kept in hot water bath for one hour and subsequently cooled. Next, 1000 µl of distilled water was combined with 5000 µl butanol:pyridine (in a ratio of 15:1), was mixed and centrifuged at 1000 rpm/10 minutes. The colored layer in the supernatant was measured against distilled water used as a blank at 532 nm. The measurement of lipid peroxidation was expressed in nanomoles of reactive substances produced by thiobarbituric acid per gram of tissue.

4.16 Nitrosative stress assessment

4.16.1 Estimation of serum Nitric Oxide concentration[255]

Serum NO was estimated by the method of Greiss Reaction.

Assay principle

Nitrate, the stable derivative of nitric oxide, was converted to nitrite using the cadmium reduction method following deproteination and subsequent coupling with N-naphthyl ethylene diamine. Vanadium chloride catalyzes the conversion of sodium nitrate to sodium nitrite which later combines with Greiss reagent, forming a colored complex that is quantified at 540 nm using a spectrophotometer.

Reagents preparation

Cadmium granules, Glycine-NaOH buffer, solutions of sulfanilamide, N-(1-Naphthyl) ethylene diamine dihydrochloride) (NED), standard sodium nitrite, stock standard and working standard were prepared.

Assay procedure

1. **Deproteinization**

500 µl of serum was mixed with 2000 µl of $ZnSO_4$ (75 mmol/L). To this solution, 2500 µl of NaOH (55 mmol/L) was added, mixed for 2 to 3 minutes and later centrifuged at 10,000 rpm for 10 minutes in a cooling centrifuge (REMI) machine. About 500 µl of protein-free supernatant was extracted and used for analysis.

2. **Activation of cadmium granules**

The stored cadmium granules were rinsed in distilled water and stirred in $CuSO_4$ (5 mmol/L) solution for 2 minutes. The copper-covered granules were activated by washing in the glycine-NaOH buffer solution and utilized within 10 minutes. After being used the granules were washed with distilled water and stored in H_2SO_4 (0.1 mmol/L) solution.

Table 4.16.1 shows assay procedure for estimation of serum Nitric Oxide.

Erlenmeyer flasks	Blank (B)	Test (T)	Standard (S)
Glycine-NaOH buffer (ml)	1	1	1
Deionized water (ml)	1	1	1
Deproteinized sample (ml)		1	
Working standard (ml)			1
Freshly activated cadmium granules (gm)	2.5-3	2.5-3	2.5-3
The contents of all the flasks were stirred to swirl the granules for 90 min.			
Distilled water dilution (ml)	4	4	4
2 ml of solution from each above flask were pipetted in 3 separate test tubes.			
Dry test tubes	Blank (B)	Test (T)	Standard (S)
Sulphanilamide (ml)	1	1	1
N-(1-Naphthyl) thylene diamine (ml)	1	1	1
All the three tubes were shaken well			

After 20 min the OD of Sample & Standard was read against the blank at 540 nm on a spectrophotometer.

Calculation

The following equation was used to calculate the nitric oxide levels in the sample and was expressed in µmol/L.

$$\text{Nitric Oxide (NO) (µmol/L)} = \frac{\text{OD of sample}}{\text{OD of Standard}} \text{ X Conc. of Std. X D.F.}$$

4.17 Antioxidants assessment

4.17.1 Estimation of serum superoxide dismutase (SOD) level [256]

Serum SOD was estimated by the method of Paoletti & Mocali.

Assay principle

The oxidation of NADPH is utilized as an indicator of superoxide breakdown. Molecular oxygen produces superoxide in sequence of reaction which includes mercaptoethanol, $MnCl_2$ and EDTA. Addition of SOD to the assay mixture prevents the oxidation of the nucleotide. Thus, in high enzyme concentrations, the absorbance at 340 nm remains constant, whereas in the control without addition of SOD the absorbance decreases. The inhibition of NADPH oxidation was observed at 340 nm.

Assay procedure

1. The assay solution contained 800 µL of triethanolamine-diethanolamine-HCl buffer (100mM), 40 µL of NADPH, 25 µL of $MnCl_2$, kept at room temperature for 5 minutes.
2. The control sample was of an equal volume of the phosphate buffered saline.
3. The 100µL of mercaptoethanol (10 mM) was added and mixed well.

Absorbance at 340 nm was measured at every 5 minutes for 20 minutes in a UV-VIS spectrophotometer, to track the decrease in values for samples with SOD activity. One unit of SOD activity inhibits NADPH oxidation by 50%.

4.17.2 Estimation of serum ascorbic acid (Vitamin-C)[257]

Serum ascorbic acid was estimated by the method of Roe and Koether.

Assay principle [257]

In presence of H_2SO_4, ascorbic acid is oxidized to 2,3-Diketo-L-gulonate which then reacts with 2,4-Dinitrophenylhydrazine (DNPH) to form diphenyl-hydrazone (DPH) giving rise to red color which is measured spectrophotometrically at 505 nm.

Reagents preparation

Trichloro acetic acid (TCA), 2,4, dinitrophenyl hydrazine (2,4, DNPH), thiourea, $CuSO_4$, combined color reagent, 85% H_2SO_4, stock solution of vitamin C and working standard were prepared.

Table 4.17.2 shows assay procedure for standardization of serum ascorbic acid (Vitamin-C).

S No.	B	S1	S2	S3	S4	S5
Conc. of Vit C (mg/dl)	0.0	0.2	0.4	0.6	0.8	1.0
Vol. of working standard (ml)	0.0	0.1	0.2	0.3	0.4	0.5
DW (ml)	0.5	0.4	03	0.2	0.1	0.0
TCA (ml)	0.5	0.5	0.5	0.5	0.5	0.5
Color reagent (ml)	0.4	0.4	0.4	0.4	0.4	0.4
Mixed well and kept in a warm water bath for 5 min.						
Cooled in an ice bath for 5 min.						
85% H_2SO_4 (ml)	2.0	2.0	2.0	2.0	2.0	2.0

20 minutes after adding 2 ml of chilled 85% H_2SO_4, the reading was taken at 505 nm by UV-VIS 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

500 µl of the serum sample was added to 500 µl of 10% TCA and mixed well for 10-15 seconds. Centrifuged for 10 min at 3500 rpm and supernatant was taken.

Assay procedure

500 µl of supernatant was added with 400 µl of a color reagent and placed in water bath at 56^{O}C for 1 hour. Then cooled and 2000 µl of chilled 85% H_2SO_4 was added and after 20 minutes the readings were taken at 505 nm spectrophotometrically (Shimadzu, Model: UV 1800).

Calculations

$$\text{Vitamin-C (mg/dl)} = \frac{\text{OD of test}}{\text{OD of Standard}} \text{ X } \frac{\text{Conc. of Standard}}{\text{Volume of test}} \text{ X } 100$$

4.17.3 Estimation of serum Vitamin D [258]

1,25-OH_2D_3 is the main form of Vitamin D in the circulation and accurately reflect the overall vitamin D status in the body.

Assay principle [258]

The method is based on competitive binding technique of solid phase enzyme-linked immunoassay, leading to formation of blue color; the absorbance is measured at 450 nm. The concentration of 1,25-OH_2D_3 in the sample is inversely proportional to the color intensity.

Reagent and preparation

Standards, 51X Biotin conjugate and 1X Wash Buffer were prepared.

Table 4.17.3 showing assay procedure for estimation of serum Vitamin D.

	Standards	Controls	Samples
Working solution of biotinylated 1,25-OH_2D_3 reagents (µl)	10	10	10
Mixing the contents in the wells for 20 seconds at 200-400 RPM			
Covering the plate			
Incubation for 90 minutes at room temp.			
Draining out the contents of the wells.			
1X Wash Buffer (µl)	300	300	300
Draining out the 1X Wash Buffer (3 times).			
Enzyme conjugate (Streptavidin-HRP) (µl)	200	200	200
Incubation for 30 minutes, at room temp.			
Draining out the contents of the wells.			
1X Wash Buffer (µl)	300	300	300

Draining out the 1X Wash Buffer (3 times).			
TMB Substrate (µl)	200	200	200
Incubation for 30 minutes at room temp.			
Stop Solution (µl)	50	50	50
Mixing plate contents for 30 seconds.			

The absorbance was measured at 450 nm using ELISA microplate reader at 450 nm within 10 minutes of adding the stop solution. (Model: Merilyzer EIAQUAN, Meril Diagnostics Pvt. Ltd.).

4.17.4 Estimation of serum Vitamin E (α-tocopherol) [259]

Vitamin E was estimated by the modified method of Jargar *et al*.

Assay principle

Serum α-tocopherol was measured through reduction of ferric to ferrous ions, resulting in formation of a red complex with α-α′ dipyridyl.

Reagents and preparation

Stock standard, 2,2'-Bipyridyl, Ferric Chloride and working standards were prepared.

Table 4.17.4.1 shows standardization procedure for serum Vitamin E (α-tocopherol).

		B	S1	S2	S3	S4	S5
Working standard	α-tocopherol (µl)	0	150	300	450	600	750
	Conc. (µg/ml)	0	4	8	12	14	16
Ethanol (µl)		750	600	450	300	150	0
Distilled Water		750	750	750	750	750	750
Xylene (µl)		750	750	750	750	750	750
Centrifugation for 30 min. at 3000 rpm.							
500 µl of xylene layer							
2,2'-Bipyridyl (µl)		500	500	500	500	500	500
$FeCl_3$ (µl)		100	100	100	100	100	100
Addition of 2 ml of chilled 85% H_2SO_4							

The absorbance 200 µl of all the solutions and blank were measured on non-antibody-coated microplates at 492 nm using ELISA reader. A graph was drawn by

plotting absorbance against the concentration of α-tocopherol (µg/ml). After 2 minutes interval, the OD was measured at 492 nm. The curve was plotted.

Analysis of serum α-tocopherol

Sample preparation

Serum was protected from sunlight and undue agitation. α-tocopherol darkens on exposure to light and slowly gets oxidized by atmospheric oxygen.

Table 4.17.4.2 shows assay procedure for serum Vitamin E (α-tocopherol). [260]

Centrifuge tubes	Sample (S)	Blank (B)
Absolute ethanol (µl)	750	750
Addition of serum slowly to obtain a fine protein precipitate (µl)	750	750
Distilled water (µl)		750
Stoppering and shaking the tubes for 30 seconds.		
n-heptane (µl)	750	750
Stoppering and shaking the tubes for 30 seconds.		
Centrifugation of all the tubes at 3000 rpm for 10 minutes.		
Transfer of 500 µl of supernatant xylene layer into test tubes.		
2, 2'-bipyridyl(µl)	500	500
$FeCl_3$ (µl)	100	100
Waiting for 2 min.		
Transfer of 200 µl of solution from these tubes to plain microplate respectively.		

Readings were taken in ELISA reader at 492 nm within 4 min.

$$\text{Slope} = \frac{abs.\ of\ Std\ [2] - abs.\ of\ Std\ [1]}{abs.\ of\ Std\ [2] - abs.\ of\ Std\ [1]}$$

Calculation

$$\text{Vitamin-E (µg/dl)} = \frac{\text{OD of test - OD. of Blank}}{\text{Slope}} \text{ X Dilution factor}$$

4.18 Estimation of serum vascular marker

4.18.1 Estimation of serum VEGF gene expression [236, 261]

Serum vascular endothelial growth factor (VEGF) was estimated by using Rat VEGF ELISA Kit (Chongqing Biospes Co. Ltd, China).

Assay principle

The technique is based on sandwich method of rat VEGF specific antibody coated on well plate in ELISA. Standards and samples were transferred by pipetting into the wells where VEGF attaches to the wells via immobilized antibody. The wells were drained out and treated with biotinylated anti-rat VEGF antibody. Then drained out excessive biotinylated antibody, HRP-conjugated streptavidin was added to the wells. Once more the wells were drained out and a TMB substrate solution was pipetted into the wells. Addition of stop solution turns blue color to yellow; the intensity of which is detected at 450 nm, indicating quantity of the VEGF in the sample.

Preparation of sample and reagents

Serum samples, wash buffer, standards, biotin-conjugated anti-rat VEGF antibody, Avidin-Biotin-Peroxidase Complex (ABC) working solution were prepared.

Table 4.18.1 shows assay procedure for estimation of serum VEGF gene expression.

	Standards (pg/ml)								Control (0)	Test sample
	1000	500	250	125	62.5	31.2	15.6	0		
Standard solutions	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	---	---
Diluents buffer (ml)	---	---	---	---	---	---	---	---	0.1	---
diluted sample (ml)	---	---	---	---	---	---	---	---	---	0.1
Incubation at 37^0C for 90 minutes sealed with a cover.										
Discarding of plate contents.										
Biotin-conjugated anti-rat VEGF antibody working solution	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Incubation at 37^0C for 60 minutes sealed with a cover.										
Washing of plate was washed with wash buffer (3 washes).										

ABC working solution	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Incubation at 37^0C for 30 minutes sealed with a cover.										
Washing of plate was washed with wash buffer (5 washes).										
TMB substrate (µl)	90	90	90	90	90	90	90	90	90	90
Incubation at 37^0C for 30 minutes sealed with a cover.										
Stop solution (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Change of color from blue to yellow.										

The absorbance at 450 nm was measured using microplate reader (Model: Merilyzer EIAQUAN, Meril Diagnostics Pvt. Ltd.).

Calculation

Relative O.D. 450 was calculated by subtracting the O.D. 450 of the zero well from the O.D. 450 of each well. The standard curve was drawn by plotting the relative O.D. 450 of each standard solution (Y) against the corresponding concentration of the standard solutions (X). The concentration of Rat VEGF in the samples was calculated using the standard curve interpolation.

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

4.19 Serum chromium concentration [262]

Atomic Absorption Spectrometry is a method of analysis utilized in measuring quantity of metal atoms / ions present in a sample.

Assay principle

When atoms are exposed to light at specific wavelengths, they absorb the energy and excite electrons. This allows them to be measured, with the amount absorbed directly proportional to the concentration of the absorbing ions or atoms.

Instrumentation

Integrated platform from PerkinElmer Instruments, (Norwalk, CT USA) was used.

1) Analyst 700 atomic absorption spectrometer.
2) Radiation source Cr hollow cathode lamp.
3) Pyrocoated graphite tubes with L'vov platforms.
4) Background corrector GF 3000.
5) Graphite furnace HGA-400.
6) Autosampler AS-800.

Preparation of reagents and standard solutions

Working standard solutions were prepared by dilution of stock 1 g/L solution of chromic acid in acid washed plastic polypropylene vessels, making concentrations of 0, 1, 2, 4, 6, 8 and 10 µg / L and refrigerated at -20ºC storage in plastic tubes.

Sample: The **s**amples and calibrating standards were diluted (1/5) with Triton X-100 in nitric acid, then mixed with sodium borohydride ($NaBH_4$). 20 µL of the diluted solution, followed by 5 µL of the matrix modifier solution were injected into the furnace in Argon gas.

Assay procedure

Atomization system by Hydride-generating atomizers*:* A metal hydride was produced and passed to the atomization chamber using an inert gas. Next, it was placed in a hollow graphite tube which was heated until the sample was completely vaporized.

Continuum light source: Free atoms exposed to deuterium lamps, causing electronic transitions from the ground to excited electronic states. The emitted light helps to identify the element being tested.

Monochromator: A monochromator was placed between the sample and the detector to reduce the background interference (other wavelengths) and improve the detection limits (same wavelength of light).

Detection system: The light signals are converted into an electrical signal by the detector, which measures the light beam in proportion to its intensity and transforms it

into absorption data.

Interpretation: The electrical output is directly proportional to concentration, calibrated using known solutions. A curve of radiation (absorbance) versus concentration is used to determine the value of uunknown concentration of Cr(VI).

Calculations

The equation used for the calibration curve was:

$$y = (7 \times 10^{-5} \pm 6.0 \times 10^{-5})\,(\text{Cr-VI}) + (5.0 \times 10^{-3} \pm 8.0 \times 10^{-4})$$

Where, ***y*** is the integrated absorbance, and Cr(VI) is the mass of Cr-VI deposited in the furnace in nanograms (ng).

4.20 The sacrifice of animals and collection of tissues

The animals were sacrificed on day-22 after completion of intervention by an overdose of ketamine (150 mg/kg, ip) in the morning hours to avoid the effects of circadian rhythms on the rats. [227] After collection of blood samples, the rats were carefully dissected. Heart, lungs and liver were separated, trimmed of fat and wiped clean. The individual organs were immediately weighed. The organosomatic index was calculated for each organ.

$$\text{Organo-somatic index} = \frac{\text{Organ wieght}}{\text{Body weight}} \text{ X } 100$$

Part of the tissues was stored in 10% neutral buffered formalin for further histopathological examination (HPE) and the remaining was kept in tissue container and stored at -20^{o} C for biochemical analysis of tissue homogenate preparation.

4.21 Histopathological examination of tissues [227,263]

Following the sacrifice of the rats, their lungs, heart and aorta were dissected for histopathologic examination.

***Collection and fixation of tissue*:** The tissues were washed in normal saline, wiped the excess fluid, weighed and then each specimen was kept for fixation in 10% neutral

buffered formalin solution for a period of 24 hours.

Dehydration: The tissues were kept for dehydration using ethanol in increasing concentrations of 50%, 70%, 90%, 95% and absolute alcohol. Samples were kept immersed in each alcohol for 1 hour.

Clearing: The tissue bits were rinsed in a solution containing xylol and alcohol (in 1:1 ratio) twice for one hour. Next, tissues were kept and changed twice in xylol.

Impregnation: Paraffin wax having a melting point 58-60°C was employed to impregnate the tissue bits. The wax was first melted and strained before being utilized. Next, the liquefied wax was transferred to an embedding oven with a temperature that was 2°C higher than the melting point. The tissues were immersed in two consecutive baths of molten paraffin wax for duration of 2 hours.

Embedding: The Leuckhart's L pieces arranged in desired size on a flat hard surface and a small quantity of freshly molten wax was poured into the Leuckhart's L pieces. Next, tissue bits were removed from the melted paraffin and firmly pressed with the cutting surface facing downward. The blocks were then cooled by placing in cold water to avoid crystallization. Once solidified, the tissue blocks were taken off from the removed and labeled.

Section cutting: A rotary microtome was utilized for sections cutting. 4-5 micron tissue sections were cut and placed on a water bath kept at 43-47°C. The sections were then transferred onto slides coated with albumin and excess water was wiped off.

Staining: Haematoxylin and eosin stains were used to stain the tissue sections. Initial steps involved deparaffinizing the slides using xylene for duration of 1-2 minutes. Following that, they were rehydrated by immersion for 1 minute in increasingly diluted alcohol solutions beginning with pure alcohol, then 90%, and finally 70% alcohol. Afterwards, the slides were rinsed in water for 60 seconds and then treated with

hematoxylin staining for 30 to 60 seconds. Any excess of stain was rinsed off with water before being counterstaining with eosin for another 10 seconds. The slides were subsequently placed in jar containing xylene for one minute, secured with DPX mountant and labelled. The slides were subjected for histopathology examination by using a light microscope at both 100x and 400x magnification, assessed, and captured in photographs by using Olympus CH-20i with Magnus digital color camera, Model No. SDC-242.

4.22 Normalized Wall Index [264, 265]

The cardiac vasculature (coronary artery and elastic artery) was evaluated for changes in the architecture to assess the effect of vitamin D supplementation on heavy metal Cr(VI)-induced cardiovascular remodeling. The histological images of the coronary artery were analyzed using *Image J* software (https://imagej.nih.gov/ij/). The coronary artery's Lumen area (LA) and total vessel area (TVA) were manually outlined and measurements were acquired through the digimizer software. Wall area was determined by utilizing the formula given below; [265]

Wall area (WA) = Total vessel area - Lumen area.

$$\text{Normalized wall index (NWI)} = \frac{\text{Wall area}}{\text{Total vessel area}}$$

4.23 STATISTICAL ANALYSIS:

The entire data collected from each experimental group were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, through SPSS software 15.0 on windows. The *p* value <0.05 was deemed to be statistically significant.

Chapter-5

Results

5.0 RESULTS:

The results of all the parameters are discussed under the following headings:

5.1. Gravimetry.

5.1.1 Change in body weight percentage.

5.1.2 Change in organ somatic index.

5.2 Evaluation of cardiac electrophysiology.

5.2.1 Heart Rate (HR).

5.2.2 Measurement of Blood Pressure (SBP, DBP & MAP).

5.2.3 Analysis of Heart Rate Variability (HRV-LF, HF & LF/HF).

5.3 Hematological analysis - Complete blood counts.

5.4 Biochemical analysis of glucose homeostasis.

5.4.1 Estimation of fasting blood glucose after acute exposure.

5.4.2 Estimation oral glucose tolerance test (OGTT).

5.4.3 Estimation of plasma insulin.

5.4.4 Analysis of insulinogenic index.

5.4.5 Estimation of liver glycogen estimation.

5.5 Biochemical analysis of serum lipid profile.

5.5.1 Estimation of serum total cholesterol (TC).

5.5.2 Estimation of serum triglyceride (TGL).

5.5.3 Estimation of serum high density lipoprotein cholesterol (HDL-C)

5.5.4 Estimation of serum low density lipoprotein cholesterol (LDL-C).

5.6 Biochemical analysis of serum liver function tests.

5.6.1 Estimation of serum bilirubin.

5.6.2 Estimation of serum aspartate transaminase (AST).

5.6.3 Estimation of serum alanine transaminase (ALT).

5.6.4 Estimation of serum alkaline phosphatase.

5.6.5 Estimation of serum total proteins.

5.6.6 Estimation of serum albumin.

5.7 Analysis serum oxidative stress.

5.7.1 Estimation of serum Malondialdehyde (MDA).

5.8 Analysis of tissue oxidative stress.

5.8.1 Estimation of tissue lipid peroxide (LPO) of heart.

5.8.2 Estimation of tissue lipid peroxide (LPO) of aorta.

5.8.3 Estimation of tissue lipid peroxide (LPO) of lungs.

5.9 Analysis of serum nitrosative stress:

5.9.1 Estimation of serum nitric oxide.

5.10 Analysis of antioxidants status:

5.10.1 Estimation of serum superoxide dismutase (SOD).

5.10.2 Estimation of serum ascorbic acid (vitamin C).

5.10.3 Estimation of serum Vitamin D_3.

5.10.4 Estimation of serum α-tocopherol (vitamin E).

5.11 Analysis of molecular markers.

5.11.1 Estimation of serum VEGF gene expression.

5.12 Analysis of serum chromium concentration.

5.13 Evaluation of histopathology of tissues (Heart, aorta and lungs).

5.14. Assessment of Normalized Wall Index.

The results of each of the above parameters are expressed in the following order:

1. Tables contain mean $\pm$ standard deviation of observations of six rats in each group. In each row, values with different superscripts (a, b, c & d) indicate statistically significant difference from each other ($p \leq 0.05$). Post-hoc Tukey's-test analysis was performed to test for differences among the means when one-way analysis of variance (ANOVA) test indicated a significant *p* value ($p \leq 0.05$).

2. Figures (bar graph) denote mean $\pm$ standard deviation (SD) of six observations in each group. Post-hoc Tukey's test analysis was performed to test for differences among the means when one-way analysis of variance (ANOVA) test indicated a significant *p* value ($p \leq 0.05$).

5.1 GRAVIMETRY

5.1.1 Gravimetry

The body weights of rats were measured both before and after the treatment in each group. **Table 5.1.1** shows comparative values of experimental groups with initial body weight (day-1), final body weight (day-22) and percentage changes in body weight. Initial body weights of all the groups were in similar range and there was no statistical significant difference between them.

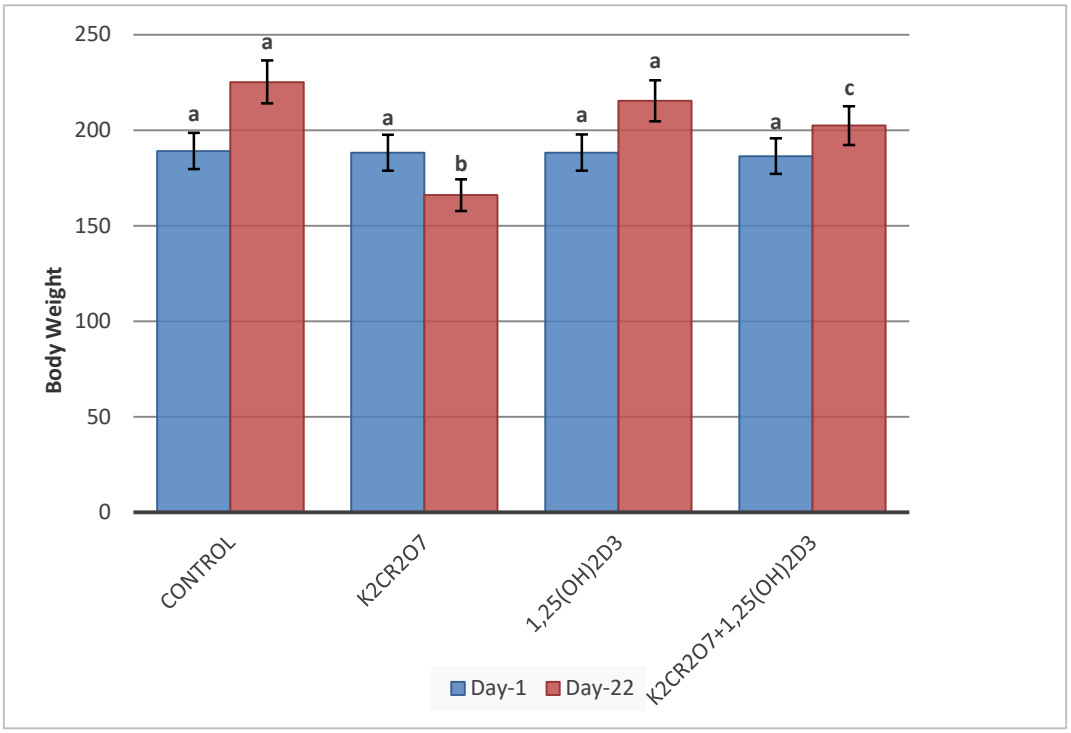
After 21 days of respective treatments, the values of final body weights were significantly different between the groups. Group-2 ($K_2Cr_2O_7$) had significant % body weight loss of 11.76%, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which had a weight gain of 19.11% and 14.49% respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had a lower weight gain of 8.59%, than the Group-1 (control) and Group-3 ($1,25(OH)_2D_3$), but it was still significantly higher than that of Group-2 ($K_2Cr_2O_7$).

Table 5.1.1: shows percentage body weight changes of experimental groups (n=6).

Groups	Initial Body Weight (Day-1)	Final Body Weight (Day-22)	% body weight change
Group-1 (Control)	189.11 ± 6.43[a]	225.25 ± 12.52[a]	19.05 ± 3.84[a]
Group-2 ($K_2Cr_2O_7$)	188.23 ± 4.20[a]	166.08 ± 4.43[b]	- 11.76 ± 2.69[b]
Group-3 ($1,25(OH)_2D_3$)	188.30 ± 5.59[a]	215.41 ± 7.15[a]	14.49 ± 5.49[a]
Group-4 ($K_2Cr_2O_7$+ $1,25(OH)_2D_3$)	186.46 ± 4.39[a]	202.46 ± 5.87[c]	8.59 ± 3.08[c]
F-value	0.273	17.922	16.036
p-value	0.844	0.000*	0.000*

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.1.1: shows percentage body weight changes of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.1.2. Organ somatic index

After the sacrifice of the animals, the organ somatic index of the lungs and heart of all the groups of rats were measured. **Table 5.1.2** shows comparative values of experimental groups with organ somatic index.

Group-2 ($K_2Cr_2O_7$) had a significantly increased cardio-somatic index of 0.815, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were of 0.678 and 0.638 respectively. Even though Group-4 ($K_2Cr_2O_7$+$1,25(OH)_2D_3$) had an increase of 0.723, than Group-1 (control) and Group-3 ($1,25(OH)_2D_3$), but it was still significantly lower than 0.815 of Group-2 ($K_2Cr_2O_7$).

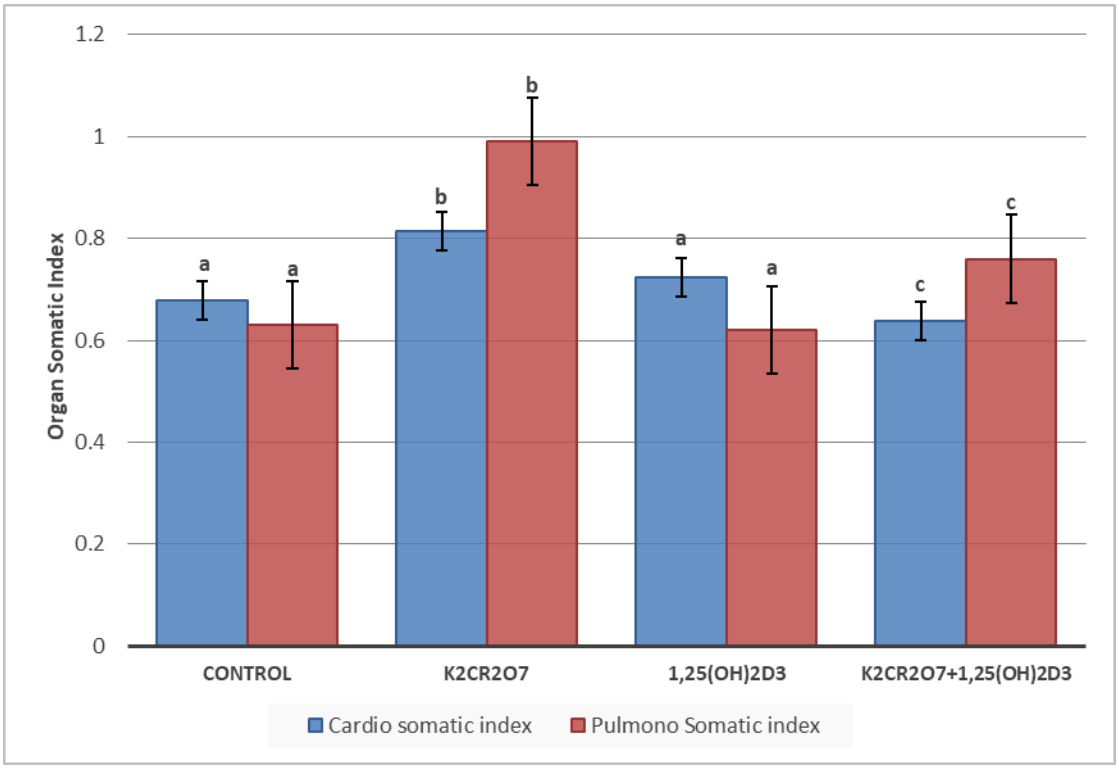
Group-2 ($K_2Cr_2O_7$) had a significantly increased pulmono-somatic index of 0.099, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were of 0.063 and 0.062 respectively. Even though Group-4 ($K_2Cr_2O_7$+$1,25(OH)_2D_3$) had an increase of 0.062, than Group-1 (control) and Group-3 ($1,25(OH)_2D_3$), but it was still significantly lower than 0.099 of Group-2 ($K_2Cr_2O_7$).

Table 5.1.2 shows organ somatic index of experimental groups (n=6).

Organ-somatic index	Cardio-somatic index	Pulmono-somatic index
Group-1 (Control)	0.678 ± 0.072^a	0.063 ± 0.008^a
Group-2 ($K_2Cr_2O_7$)	0.815 ± 0.064^b	0.099 ± 0.025^b
Group-3 ($1,25(OH)_2D_3$)	0.638 ± 0.073^a	0.062 ± 0.013^a
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	0.723 ± 0.042^c	0.076 ± 0.002^c
F-value	8.534	8.034
p-value	0.000*	0.000*

Each value is Mean$\pm$SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.1.2 shows organ somatic index of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.2 CARDIAC ELECTROPHYSIOLOGY

5.2.1 Heart Rate

The pre-intervention heart rates of rats of all groups were within the similar range and there was no statistically significant difference between them. After 21 days of different treatments, the heart rate showed significant variations among the groups. **Table 5.2.1** shows comparative values of heart rates of experimental groups.

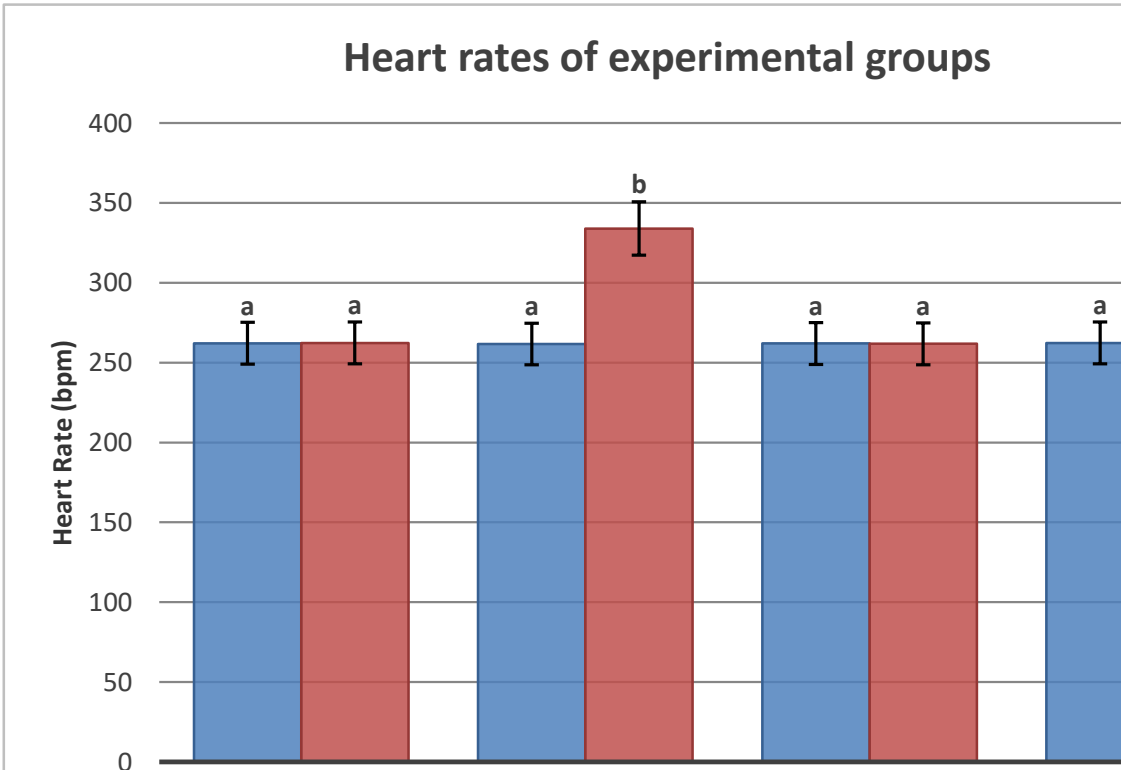
Group-2 ($K_2Cr_2O_7$) had a significantly increased heart rate of 334.00 bpm, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 262.33 bpm and 261.83 bpm respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increase of 294.16 bpm, than Group-1 (control) and Group-3 ($1,25(OH)_2D_3$), but it was still significantly lower than 334.00 bpm of Group-2 ($K_2Cr_2O_7$).

Table 5.2.1 shows the heart rates of experimental groups (n=6).

Groups	Heart Rate (bpm)		t-value	p-value
	Pre-treatment (Day-1)	Post-treatment (Day-22)		
Group-1 (Control)	262.16 ± 1.47[a]	262.33 ± 1.63[a]	0.222	0.833
Group-2 ($K_2Cr_2O_7$)	261.66 ± 0.81[a]	334.00 ± 4.04[b]	50.588	0.000*
Group-3 ($1,25(OH)_2D_3$)	262.00 ± 1.41[a]	261.83 ± 1.47[a]	0.415	0.695
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	262.33 ± 1.86[a]	294.16 ± 2.99[c]	18.930	0.000*
F-value	0.234	926.483		
p-value	0.871	0.000*		

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.2.1 shows the heart rates of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.2.2 Measurement of Blood Pressure

The Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP) and Mean Blood Pressure (MAP) of rats in all the groups were measured before and after the treatment.

Table 5.2.2.1 shows comparative values of pre and post -intervention SBP of experimental groups. Pre-intervention SBP of all the groups were in similar range and there was no statistically significant difference amongst them. After 21 days of respective treatments, SBP was significantly different between the groups.

Group-2 ($K_2Cr_2O_7$) had a significantly increased SBP of 164.8 mmHg in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 119.6 mmHg and 120.0 mmHg respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had a higher SBP of 137.1 mmHg, it was still significantly lower than 164.8 mmHg of Group-2 ($K_2Cr_2O_7$).

Table 5.2.2.1 shows Systolic Blood Pressure of experimental groups (n=6).

Groups	**SBP** (mmHg)		**t-value**	**p-value**
	Pre-treatment (Day-1)	**Post-treatment** (Day-22)		
Group-1 (Control)	119.5 ± 1.41^a	119.6 ± 2.58^a	0.113	0.914
Group-2 ($K_2Cr_2O_7$)	119.6 ± 0.51^a	164.8 ± 17.93^b	6.104	0.002*
Group-3 ($1,25(OH)_2D_3$)	119.1 ± 1.60^a	120.0 ± 16.00^a	1.722	0.146
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	118.5 ± 4.18^a	137.1 ± 14.38^c	4.032	0.010*
F-value	0.268	13.696		
p-value	0.847	0.000*		

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Table 5.2.2.2 shows comparative values of pre and post -intervention DBP of experimental groups. Pre-intervention DBP of all the groups were in similar range and there was no statistically significant difference amongst them. After 21 days of respective treatments, DBP was significantly different between the groups.

Group-2 ($K_2Cr_2O_7$) had a significantly increased DBP of 91.03 mmHg in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 77.50 mmHg and 78.32 mmHg respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had a higher DBP of 85.31 mmHg, it was still significantly lower than 91.03 mmHg of Group-2 ($K_2Cr_2O_7$).

Table 5.2.2.2 shows Diastolic Blood Pressure of experimental groups (n=6).

Groups	**DBP** (mmHg)		**t-value**	**p-value**
	Pre-treatment (Day-1)	**Post-treatment** (Day-22)		
Group-1 (Control)	78.50 $\pm$ 2.34[a]	77.50 $\pm$ 2.81[a]	0.612	0.567
Group-2 ($K_2Cr_2O_7$)	80.30 $\pm$ 0.51[a]	91.03 $\pm$ 4.09[b]	4.049	0.010*
Group-3 ($1,25(OH)_2D_3$)	79.72 $\pm$ 5.13[a]	78.32 $\pm$ 5.39[a]	1.455	0.176
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	80.62 $\pm$ 5.16[a]	85.31 $\pm$ 5.39[c]	6.048	0.002
F-value	0.3569	14.614		
p-value	0.7847	0.000*		

Each value is Mean$\pm$SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Table 5.2.2.3 shows comparative values of pre and post -intervention MAP of experimental groups. Pre-intervention MAP of all the groups was in similar range and there was no statistically significant difference amongst them. After 21 days of respective treatments, SBP was significantly different between the groups.

Group-2 ($K_2Cr_2O_7$) had a significantly increased MAP of 117.88 mmHg in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 91.55 and 91.54

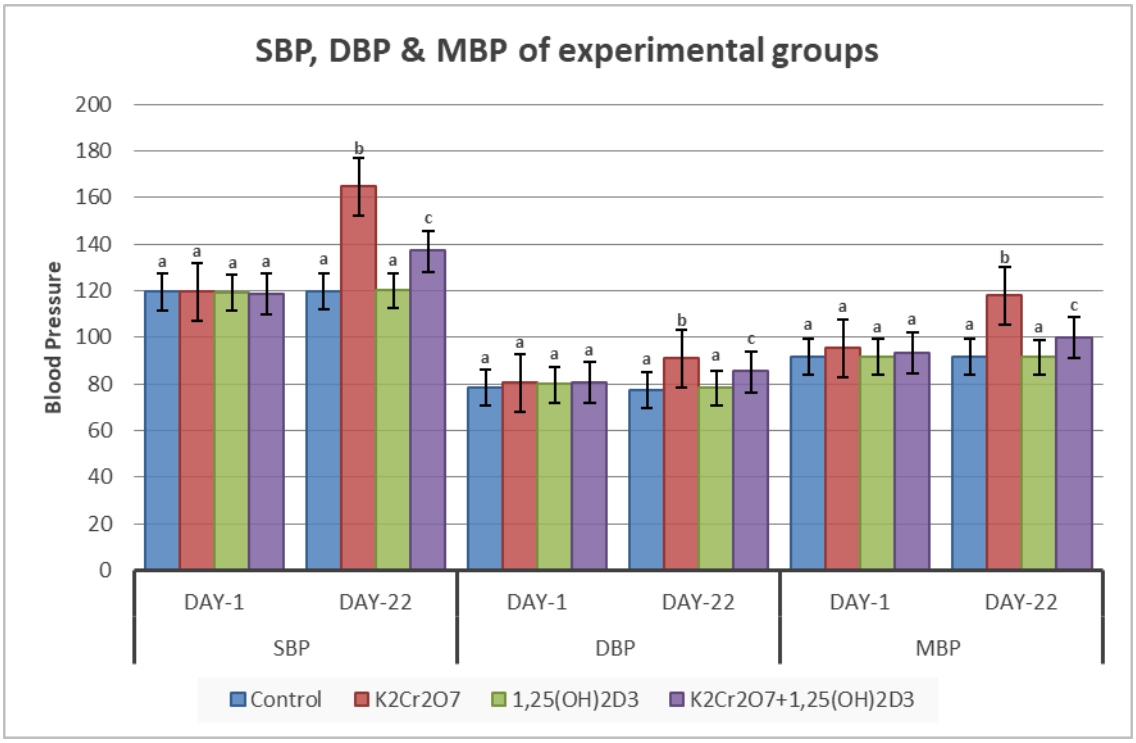
mmHg respectively. Even though Group-4 (K_2Cr_2O7+$1,25(OH)_2D_3$) had a higher MAP of 99.72, it was still significantly lower than 117.88 mmHg of Group-2 ($K_2Cr_2O_7$).

Table 5.2.2.3 shows Mean Blood Pressure of experimental groups (n=6).

Groups	**MAP** (mmHg)		t-value	p-value
	Pre-treatment (Day-1)	**Post-treatment** (Day-22)		
Group-1 (Control)	91.58 ± 1.93[a]	91.55 ± 1.48[a]	0.028	0.979
Group-2 ($K_2Cr_2O_7$)	95.50 ± 3.26[a]	117.88 ± 6.01[b]	8.150	0.000*
Group-3 ($1,25(OH)_2D_3$)	91.58 ± 1.93[a]	91.54± 1.43	0.028	0.979
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	93.38 ± 5.35a	99.72 ± 2.38[c]	2.652	0.024*
F-value	1.786	80.422		
p-value	0.1822	0.000*		

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.2.2 shows SBP, DBP & MBP of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.2.3 Heart Rate Variability analysis (HRV)

Heart rate variability analysis of rats in all the groups was done to assess the level of sympathetic activity (low frequency component-LF), parasympathetic activity (high frequency component-HF) and sympathovagal balance (low frequency by high frequency ratio-LF/HF), before and after the treatment.

Table 5.2.3.1 shows comparative values of LF of experimental groups on pre-intervention (day-1) and post-intervention (day-22). Pre-intervention LF of all the groups were in similar range and there was no statistically significant difference amongst them. After 21 days of respective treatments, the post-intervention LF values were significantly different between the groups.

Group-2 ($K_2Cr_2O_7$) had a significantly increased LF (69.55 nu), in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which was LF (42.00 nu) and LF (37.46 nu) respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) showed a higher LF (59.52 nu), than Group-1 (control) and Group-3 ($1,25(OH)_2D_3$), but it was still lower than LF (69.55 nu) of Group-2 ($K_2Cr_2O_7$).

Table 5.2.3.1 shows LF of experimental groups (n=6).

Groups	**LF** (nu)		**t-value**	**p-value**
	Pre-treatment (Day-1)	**Post-treatment** (Day-22)		
Group-1 (Control)	41.91 ± 6.29^a	42.00 ± 4.01^a	2.240	0.075
Group-2 ($K_2Cr_2O_7$)	38.81 ± 7.34^a	69.55 ± 1.52^b	12.154	0.000*
Group-3 ($1,25(OH)_2D_3$)	37.10 ± 1.93^a	37.46 ± 3.78^a	0.207	0.839
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	39.36 ± 1.97^a	59.52 ± 3.38^c	10.716	0.000*
F-value	0.9429	122.60		
p-value	0.4386	0.000*		

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Table 5.2.3.2 shows comparative values of experimental groups with HF on pre-intervention (day-1) and post-intervention (day-22). Pre-intervention HF of all the groups were in similar range and there was no statistically significant difference amongst them. After 21 days of respective treatments, the post-intervention HF values were significantly different between the groups.

Group-2 ($K_2Cr_2O_7$) had a significantly decreased HF (33.83 nu) in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which was HF (51.91 nu) and HF (52.09 nu) respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had a lower HF (41.09 nu) than Group-1 (control) and Group-3 ($1,25(OH)_2D_3$), but it was still significantly higher than HF (33.83 nu) of Group-2 ($K_2Cr_2O_7$).

Table 5.2.3.2 shows HF of experimental groups (n=6).

HRV (nu)	**HF** (nu)		**t-value**	**p-value**
Groups	**Pre-treatment** (Day-1)	**Post-treatment** (Day-22)		
Group-1 (Control)	52.06 ± 6.29[a]	51.91 ± 3.93[a]	2.234	0.076
Group-2 ($K_2Cr_2O_7$)	54.73 ± 7.3[a]	33.83 ± 1.51[b]	12.864	0.000*
Group-3 ($1,25(OH)_2D_3$)	52.46 ± 3.78[a]	52.09 ± 5.53[a]	0.00	0.999
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	53.46 ± 3.38[a]	41.09 ± 5.53[c]	7.780	0.001
F-value	0.2870	24.114		
p-value	0.8342	0.000*		

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Table 5.2.3.3 shows comparative values of experimental groups with LF/HF on pre-intervention (day-1) and post-intervention (day-22). Pre-intervention LF/HF of all the groups were in similar range and there was no statistically significant difference amongst them. After 21 days of respective treatments, the post-intervention LF/HF ratio was significantly different between the groups.

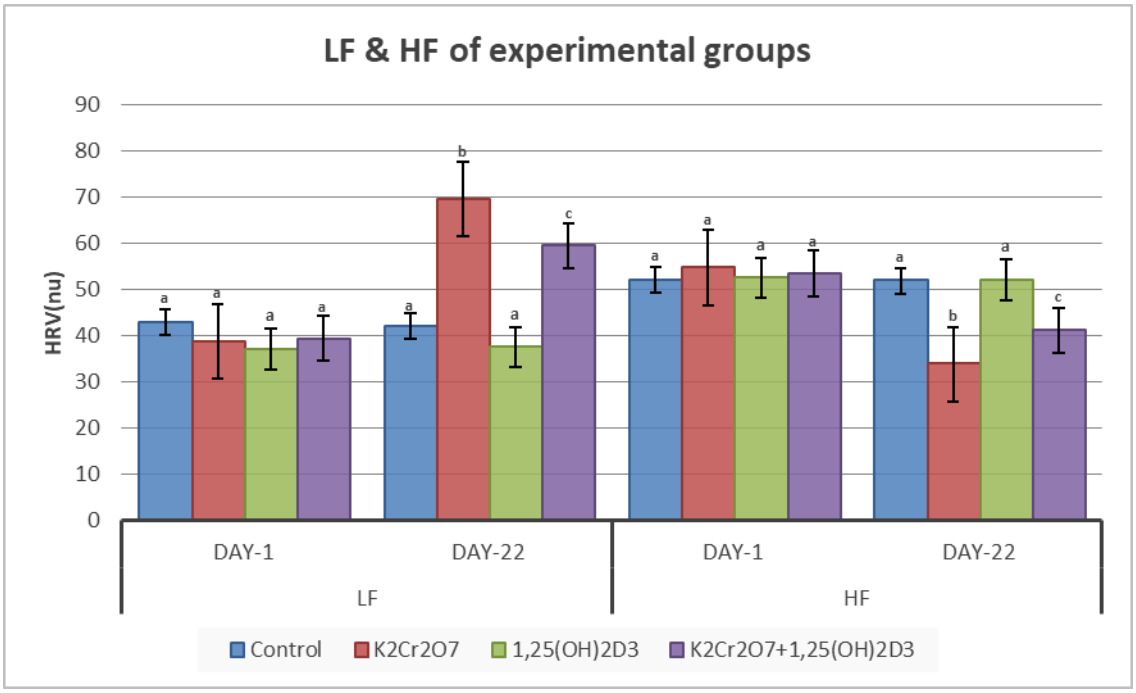
Group-2 ($K_2Cr_2O_7$) had a significantly increased LF/HF ratio of 2.05, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which was LF/HF ratio of 0.86 and LF/HF ratio of 0.71 respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased LF/HF ratio of 1.44, than Group-1 (control) and Group-3 ($1,25(OH)_2D_3$), but it was still significantly lower than LF/HF ratio of 2.05 of Group-2 ($K_2Cr_2O_7$).

Table 5.2.3.3 shows LF/HF ratio of experimental groups (n=6).

Groups	**LH/HF Ratio**		**t-value**	**p-value**
	Pre-treatment (Day-1)	**Post-treatment** (Day-22)		
Group-1 (Control)	0.82 ± 0.19^a	0.86 ± 0.14^a	2.244	0.075
Group-2 ($K_2Cr_2O_7$)	0.70 ± 0.17^a	2.05 ± 0.21^b	47.122	0.000*
Group-3 ($1,25(OH)_2D_3$)	0.68 ± 0.05^a	0.71 ± 0.04^a	1.148	0.278
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	0.73 ± 0.07^a	1.44 ± 0.01^c	33.396	0.000*
F-value	1.6268	127.5		
p-value	0.3123	0.000*		

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.2.3 shows LF and HF of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.3 HEMATOLOGICAL PARAMETERS - COMPLETE BLOOD COUNTS

Complete blood counts of rats in all the groups were done as shown in **table 5.3**. CBC values were significantly different between the groups.

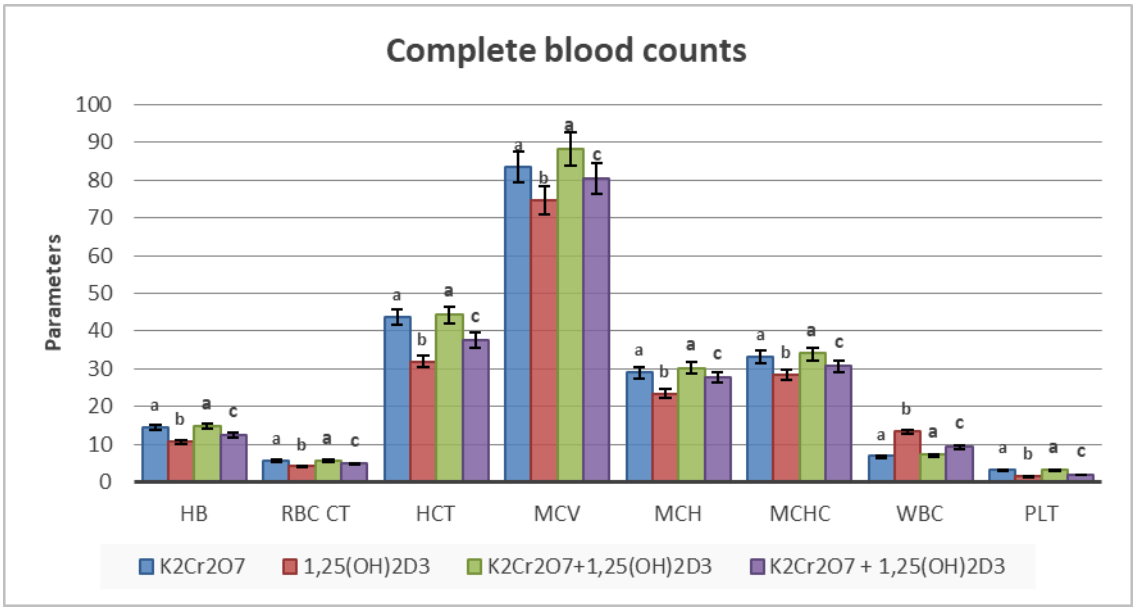
Group-2 ($K_2Cr_2O_7$) had a significantly decreased Hb (10.63 gm/dL), platelets (1.63 x 10^3 cells/µL) and an increased WBC (13.31 x 10^3 cells/µL) counts, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which had [Hb (14.56 gm/dL), platelets (3.11 x 10^3 cells/µL) and WBC (6.81 x 10^3 cells/µL)] and [Hb (14.78 gm/dL), platelets (3.12 x 10^3 cells/µL) and WBC (7.15 x 10^3 cells/µL)] respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) showed Hb 12.53 gm/dl, platelets 2.00 x 10^3 cells/µL and WBC 9.36 x 10^3 cells/µL, but the values were much better than those of Group-2 ($K_2Cr_2O_7$).

Table 5.3 shows CBC of experimental groups (n=6).

Groups	Hb (gm/dL)	RBC Ct (10^6/µL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (gm/dL)	WBC (10^3/µL)	Plt (10^3/µL)
Group-1 (Control)	14.56 ± 1.13^a	5.60 ± 0.41^a	43.70 ± 3.41^a	83.4 ± 3.51^a	29.0 ± 2.15^a	33.21 ± 1.38^a	6.81 ± 0.64^a	3.11 ± 0.46^a
Group-2 ($K_2Cr_2O_7$)	10.63 ± 0.85^b	4.08 ± 0.31^b	31.9 ± 2.55^b	74.61 ± 2.75^b	23.41 ± 1.39^b	28.41 ± 2.55^b	13.31 ± 2.85^b	1.49 ± 0.31^b
Group-3 (Vit-D_3)	14.78 ± 0.97^a	5.61 ± 0.33^a	44.35 ± 2.92^a	88.28 ± 3.73^a	30.18 ± 1.83^a	34.0 ± 1.02^a	7.15 ± 1.02^c	3.12 ± 0.52^a
Group-4 (Cr(VI) + Vit-D_3)	12.53 ± 0.44^c	4.83 ± 0.17^c	37.6 ± 1.32^c	80.4 ± 0.84^c	27.83 ± 1.13^c	30.78 ± 0.82^c	9.36 ± 0.90^c	2.00 ± 0.22^c
F-value	28.776	32.477	28.776	22.694	18.689	15.067	20.622	25.211
p-value	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.3 shows CBC of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.4 GLUCOSE HOMEOSTASIS

5.4.1 Fasting blood glucose after acute exposure

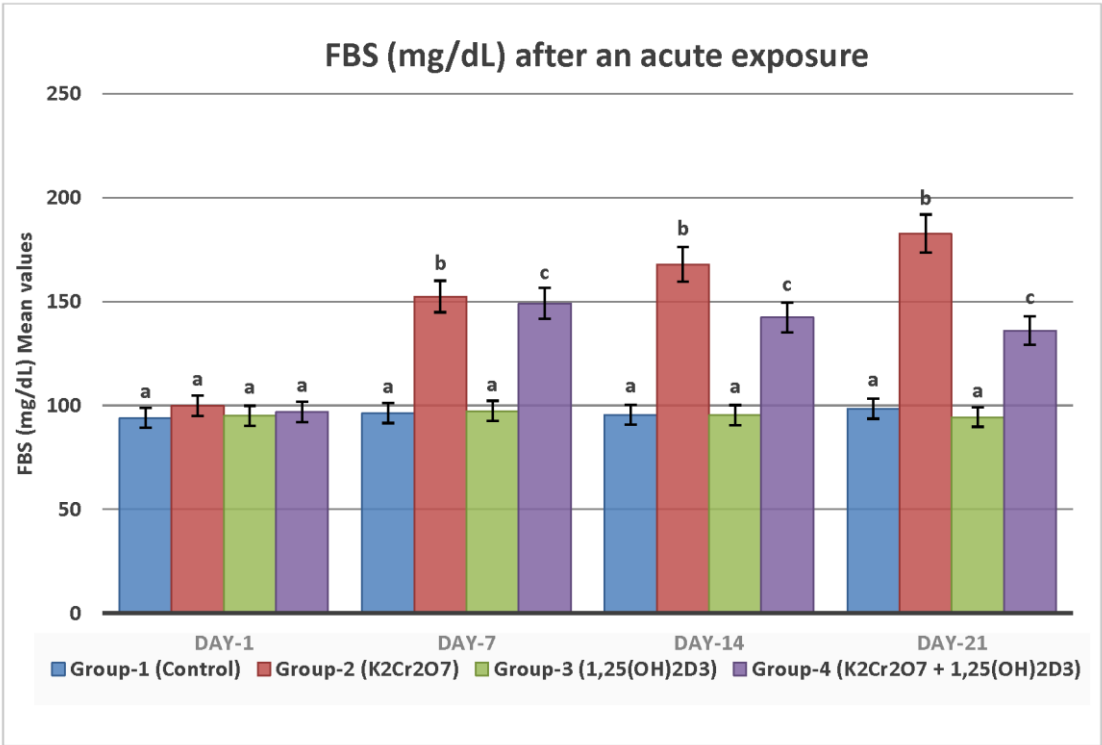
Table 5.4.1 shows comparative values of FBS in rats of all the groups. Group-2 ($K_2Cr_2O_7$) had a significantly progressive increased fasting blood glucose level on day-7, 14 & 21, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which remained within the lower range throughout the course of 21 days. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased FBS, but started to decline significantly after 14 days.

Table 5.4.1 shows FBS of experimental groups (n=6).

Groups	**FBS** (mg/dL)			
	Day-1	**Day-7**	**Day-14**	**Day-21**
Group-1 (Control)	94.00 ± 3.89[a]	96.34 ± 3.14	95.47 ± 3.07	98.50 ± 4.50[a]
Group-2 ($K_2Cr_2O_7$)	99.83 ± 2.89[a]	152.41 ± 4.49	167.91 ± 4.92	182.66 ± 22.78[b]
Group-3 ($1,25(OH)_2D_3$)	95.00 ± 4.24[a]	97.33 ± 2.92	95.28 ± 3.21	94.33 ± 13.70[a]
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	96.83 ± 4.35[a]	149.07 ± 3.47	142.33 ± 3.81	136.00 ± 6.13[c]
F-value	2.611	308.28	565.07	55.370
p-value	0.080	0.000*	0.000*	0.000*

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.4.1 shows FBS of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.4.2 Oral glucose tolerance test (OGTT)

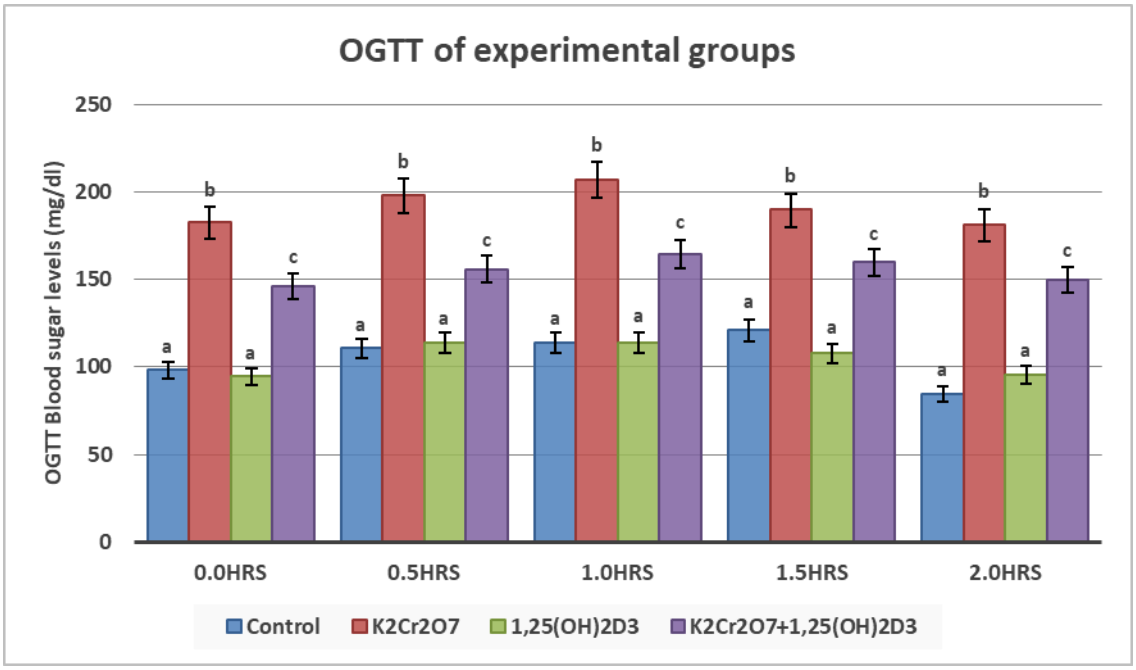
Table 5.4.2 shows comparative values of OGTT of experimental groups. The OGTT in Group-1 (control) rats was normal i.e. gradual increase of glucose level till 1.0hr and followed by decrease of blood glucose levels. At 2.0hrs the value became near normal level. OGTT of Group-2 ($K_2Cr_2O_7$) showed blood glucose levels from 0.0hr to till 2.0hrs remained higher than baseline value. All the values of Group-2 ($K_2Cr_2O_7$) were also found to be significantly higher than Group-1 (control) from baseline FBG to till 2.0hrs at any given duration during OGTT. Group-4 ($K_2Cr_2O_7$+1,25$(OH)_2D_3$) showed significantly decrease in blood glucose level at every interval till 2.0hrs, even though it never reached to similar baseline value at 2.0hrs, in contrast to Group-2 ($K_2Cr_2O_7$) the values of Group-4 ($K_2Cr_2O_7$+1,25$(OH)_2D_3$) at any interval were significantly lower.

Table 5.4.2 shows OGTT of experimental groups (n=6).

OGTT (mg/dL)	**0.0hrs**	**0.5hrs**	**1.0hrs**	**1.5hrs**	**2.0hrs**
Group-1 (Control)	98.00 ± 4.50$^{a, x}$	110.54 ± 10.56$^{b, x}$	113.76 ± 10.65^{b,x}	120.76 ± 10.97^{c,x}	84.45 ± 10.43^{a,x}
Group-2 ($K_2Cr_2O_7$)	182.66 ± 22.78$^{a, y}$	197.76 ± 15.43$^{b, y}$	206.76 ± 12.45$^{b, y}$	189.56 ± 12.65^{c,y}	180.75 ± 11.24^{c,y}
Group-3 ($1,25(OH)_2D_3$)	94.33 ± 13.70$^{a, x}$	113.76 ± 10.65^{b,x}	113.75 ± 15.75^{b,x}	107.76 ± 16.50^{b,x}	95.56 ± 16.34^{c,x}
Group-4 ($K_2Cr_2O_7$+$1,25(OH)_2D_3$)	146.00 ± 6.13$^{a, z}$	155.75 ± 12.50$^{b, z}$	164.56 ± 10.87^{c,z}	159.76 ± 10.97^{b,z}	149.65 ± 9.56^{b,z}
F-value	55.370	18.436	19.337	39.496	19.834
p-value	0.000*	0.000*	0.000*	0.000*	0.000*

Group-1, control; Group-2, K2Cr2O7; Group-3, 1,25(OH)2D3; Group-4, K2Cr2O7 + 1,25(OH)2D3. n=6 rats in each group. In each row, values with different superscripts (a, b, c) are significantly different from each other ($p < 0.05$). Vertical columns indicate variation of blood glucose level among four different groups at different time interval till 2 hrs. In each column, values with different superscripts (x, y, z) are significantly different from each other ($p<0.05$).

Figure 5.4.2 shows OGTT of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.4.3 Plasma insulin

Table 5.4.3 shows comparative values of plasma insulin of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased fasting glucose level with significant decrease of plasma insulin level of 0.54 µg/L, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which remained within the normal range of 1.07 µg/L and 1.11 µg/L respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) lowere plasma insulin of 0.75 µg/L, but it was significantly higher than 0.54 µg/L of Group-2 ($K_2Cr_2O_7$).

5.4.4 Insulinogenic index

Table 5.4.3 shows comparative values of insulinogenic index of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased insulinogenic index of 0.0058, compared to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 0.0034 and 0.0031 respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased insulinogenic index of 0.0052, but it was significantly lower than 0.0058 of Group-2 ($K_2Cr_2O_7$).

5.4.5 Liver glycogen estimation

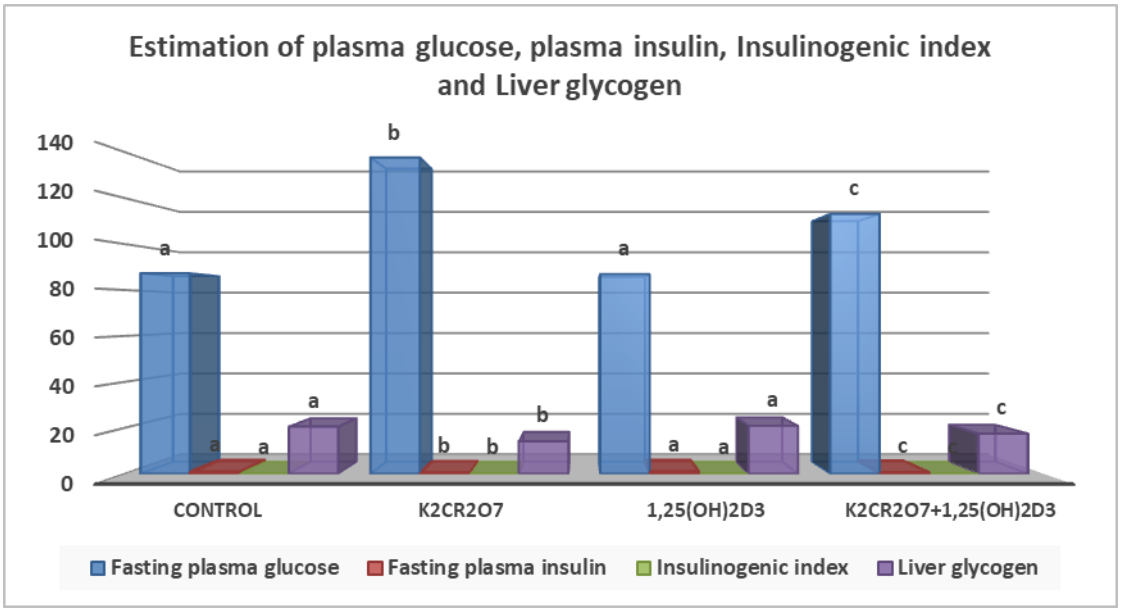
Table 5.4.3 shows comparative values of liver glycogen of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly decreased liver glycogen 14.14 mg/g, compared to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 20.56 mg/g and 20.93 mg/g respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had decreased 17.53 mg/g, but it was significantly higher than 14.14 mg/g of Group-2 ($K_2Cr_2O_7$).

Table 5.4.3 shows fasting plasma glucose, fasting plasma insulin, insulinogenic index and liver glycogen of experimental groups (n=6).

Groups	Fasting plasma glucose (mg/dL)	Fasting plasma insulin (µg/L)	Insulinogenic index	Liver glycogen (mg/g)
Group-1 (Control)	87.27 ± 3.85[a]	1.07 ± 0.17[a]	0.0034 ± 0.00052[a]	20.56 ± 1.49[a]
Group-2 ($K_2Cr_2O_7$)	137.65 ± 12.29[b]	0.54 ± 0.09[b]	0.0058 ± 0.00064[b]	14.14 ± 1.18[b]
Group-3 ($1,25(OH)_2D_3$)	86.91 ± 3.65[a]	1.11 ± 0.14[a]	0.0031 ± 0.00029[a]	20.93 ± 1.55[a]
Group-4 ($K_2Cr_2O_7$+$1,25(OH)_2D_3$)	113.08 ± 3.42[c]	0.75 ± 0.22[c]	0.0052 ± 0.00035[c]	17.53 ± 2.11[c]
F-value	74.05	16.829	47.710	22.870
p-value	0.000*	0.000*	0.000*	0.000*

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.4.3 shows fasting plasma glucose, fasting plasma insulin and liver glycogen of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.5 SERUM LIPID PROFILE

5.5.1 Serum total cholesterol (TC)

Table 5.5 shows comparative values of TC of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased TC of 108.58 mg/dl, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were TC of 73.58 and 62.7 mg/dl respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased TC of 84.9 mg/dl, but was significantly lower than 108.58 mg/dl of Group-2 ($K_2Cr_2O_7$).

5.5.2 Estimation of serum triglyceride (TGL)

Table 5.5 shows comparative values of TGL of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased TGL of 132.9 mg/dl, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 83.05 and 71.65 mg/dl respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased TGL of 91.28 mg/dl, but was significantly lower than compared to 132.9 mg/dl of Group-2 ($K_2Cr_2O_7$).

5.5.3 Serum high density lipoprotein cholesterol (HDL-C)

Table 5.5 shows comparative values of HDL of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly decreased HDL of 18.08 mg/dl, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were HDL of 36.18 mg/dl and HDL of 40.16 mg/dl respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had a decreased HDL of 29.16 mg/dl, but it was significantly higher than to HDL of 18.08 of Group-2 ($K_2Cr_2O_7$).

5.5.4 Serum low density lipoprotein cholesterol (LDL-C)

Table 5.5 shows comparative values of LDL of experimental groups. Group-2 ($K_2Cr_2O_7$) showed a significantly increased LDL of 63.8 mg/dl, compared to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were LDL of 20.73 mg/dl and LDL of 15.36 mg/dl respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an

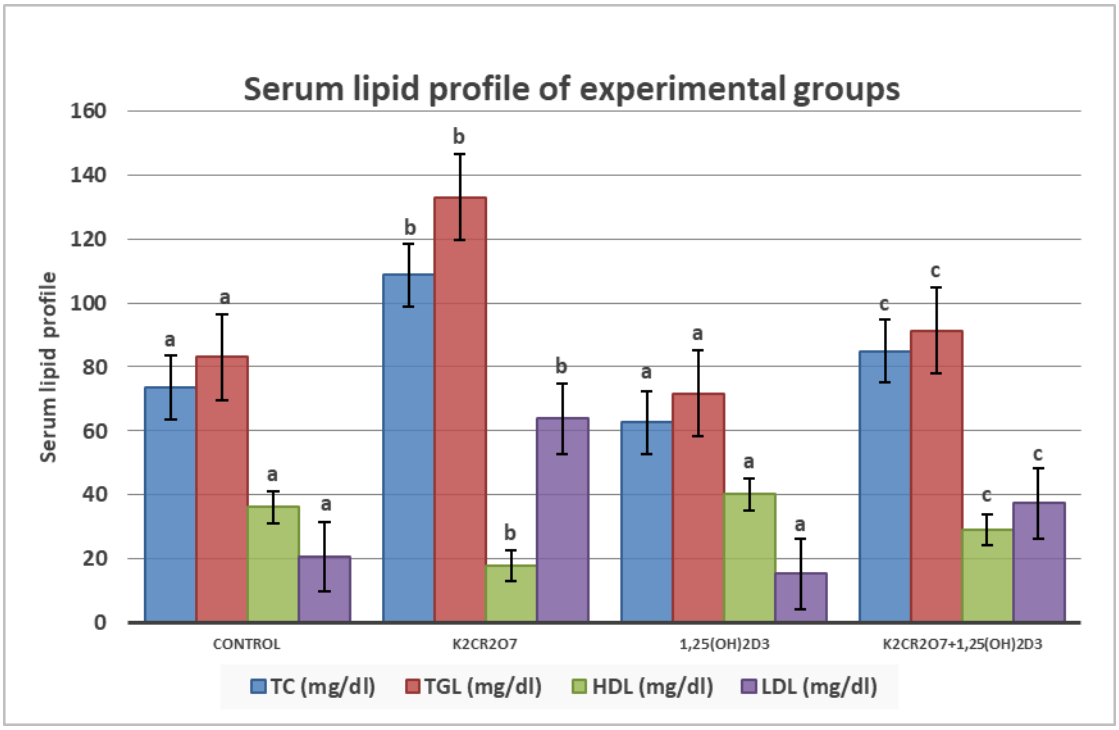
increased LDL of 37.36 mg/dl, but it was significantly lower than to TGL of 132.9 mg/dl of Group-2 ($K_2Cr_2O_7$).

Table 5.5 shows serum lipid profile of experimental groups (n=6).

Groups	TC (mg/dl)	TGL (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Group-1 (Control)	73.58 ± 7.36^a	83.05 ± 6.57^a	36.18 ± 5.28^a	20.73 ± 7.02^a
Group-2 ($K_2Cr_2O_7$)	108.58 ± 8.56^b	132.9 ± 11.34^b	18.08 ± 2.23^b	63.8 ± 9.53^b
Group-3 ($1,25(OH)_2D_3$)	62.7 ± 3.75^a	71.65 ± 5.73^a	40.16 ± 5.1^a	15.36 ± 6.86^a
Group-4 ($K_2Cr_2O_7$+$1,25(OH)_2D_3$)	84.9 ± 6.03^c	91.28 ± 6.25^c	29.16 ± 4.14^c	37.36 ± 7.47^c
F-value	52.922	70.126	29.353	79.011
p-value	0.000*	0.000*	0.000*	0.000*

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.5 shows serum lipid profile of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.6 SERUM LIVER FUNCTION TESTS

5.6.1 Serum bilirubin

Table 5.6 shows comparative values of serum bilirubin of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased serum total bilirubin of 1.13 mg/dl, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 0.87 mg/dl and 0.83 mg/dl respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased total bilirubin of 1.02 mg/dl, but it was significantly lower than to bilirubin of 1.13 mg/dl of Group-2 ($K_2Cr_2O_7$).

5.6.2 Serum SGOT/ALT (aspartate transaminase)

Table 5.6 shows comparative values of serum SGOT/ALT of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased serum SGOT of 99.00 IU/dl, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 24.98 IU/dl and 22.61 IU/dl respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased serum SGOT of 58.15 IU/dl, but it was significantly lower than serum SGOT of 99.0 IU/dl of Group-2 ($K_2Cr_2O_7$).

5.6.3 Estimation of serum SGPT/AST (alanine transaminase)

Table 5.6 shows comparative values of serum SGPT/AST of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased serum SGPT/AST of 142.0 IU/dl, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 85.0 IU/dl and 82.06 IU/dl respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased serum SGPT/AST of 119.20 IU/dl, but it was significantly lower than serum SGPT/AST of 142.0 IU/dl of Group-2 ($K_2Cr_2O_7$).

5.6.4 Serum alkaline phosphatase

Table 5.6 shows comparative values of serum ALP of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased serum ALP of 157.35 IU/dl, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 61.21 IU/dl and 60.25 IU/dl respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased serum ALP of 84.36 IU/dl, but it was significantly lower than serum ALP of 157.35 IU/dl of Group-2 ($K_2Cr_2O_7$).

5.6.5 Serum total proteins

Table 5.6 shows comparative values of serum total proteins of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly decreased serum total proteins of 5.26 gm/dl, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 10.41 gm/dl and 10.51 gm/dl respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had a decreased serum total proteins of 7.21 gm/dl, but it was significantly higher than serum total proteins of 5.26 gm/dl of Group-2 ($K_2Cr_2O_7$).

5.6.6 Serum albumin

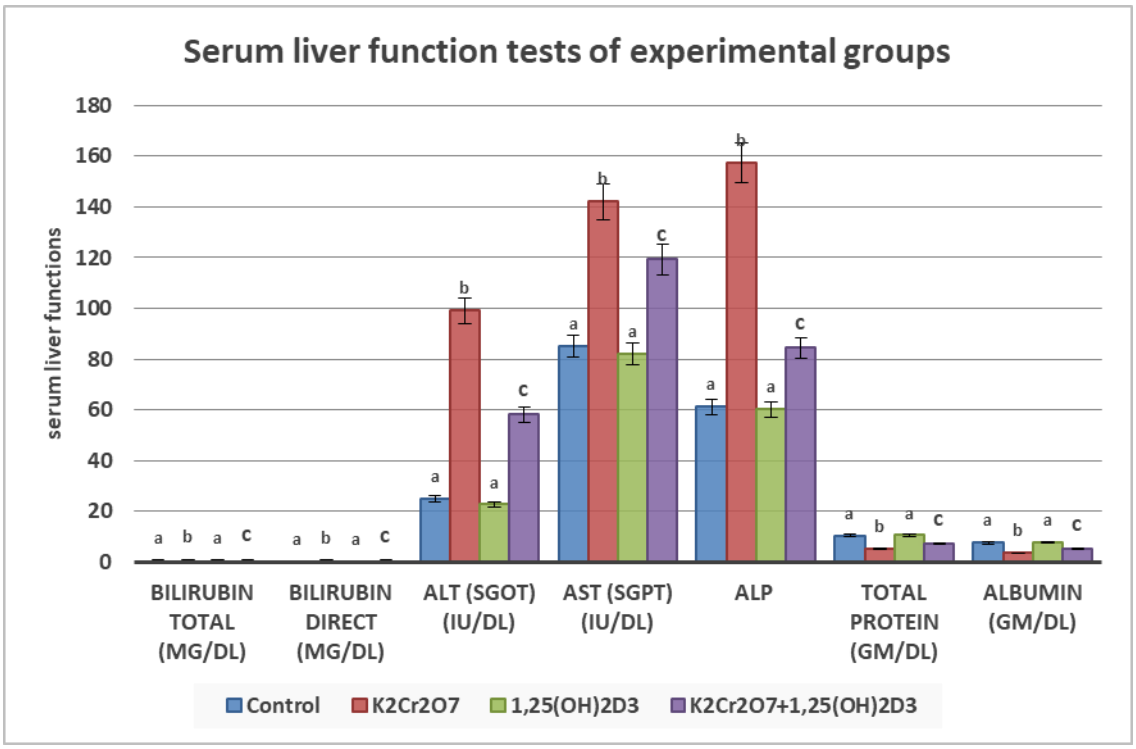
Table 5.6 shows comparative values of serum albumin of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly decreased serum albumin of 3.50 gm/dl, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 7.61 gm/dl and 7.81 gm/dl respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had decreased serum albumin of 5.11 gm/dl, but it was significantly higher than serum albumin of 3.50 gm/dl of Group-2 ($K_2Cr_2O_7$).

Table 5.6 shows serum liver function tests of experimental groups (n=9).

Groups	Bilirubin Total (mg/dl)	Bilirubin Direct (mg/dl)	ALT (SGOT) (IU/dL)	AST (SGPT) (IU/dL)	ALP (IU/dL)	Total protein (gm/dL)	Albumin (gm/dL)
Group-1 (Control)	0.87 ± 0.05^{a}	0.25 ± 0.06^{a}	24.98 ± 5.61^{a}	85.0 ± 4.52^{a}	61.21 ± 6.28^{a}	10.41 ± 1.24^{a}	7.61 ± 0.51^{a}
Group-2 ($K_2Cr_2O_7$)	1.13 ± 0.05^{b}	0.90 ± 1.41^{b}	99.00 ± 11.38^{b}	142.03 ± 5.76^{b}	157.35 ± 10.47^{b}	5.26 ± 0.45^{b}	3.50 ± 1.72^{b}
Group-3 ($1,25(OH)_2D_3$)	0.83 ± 0.04^{a}	0.26 ± 0.05^{a}	22.61 ± 5.54^{a}	82.06 ± 2.23^{a}	60.25 ± 6.09^{a}	10.51 ± 1.23^{a}	7.81 ± 0.70^{a}
Group-4 ($K_2Cr_2O_7$+1, $25(OH)_2D_3$)	1.02 ± 0.04^{c}	0.81 ± 0.03^{c}	58.15 ± 8.35^{c}	119.20 ± 7.73^{c}	84.36 ± 6.76^{c}	7.21 ± 0.61^{c}	5.11 ± 0.47^{c}
F-value	48.272	52.592	0.743	117.295	216.365	43.188	25.985
p-value	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.6 shows serum liver function tests of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.7 SERUM OXIDATIVE STRESS

5.7.1 Serum Malondialdehyde (MDA)

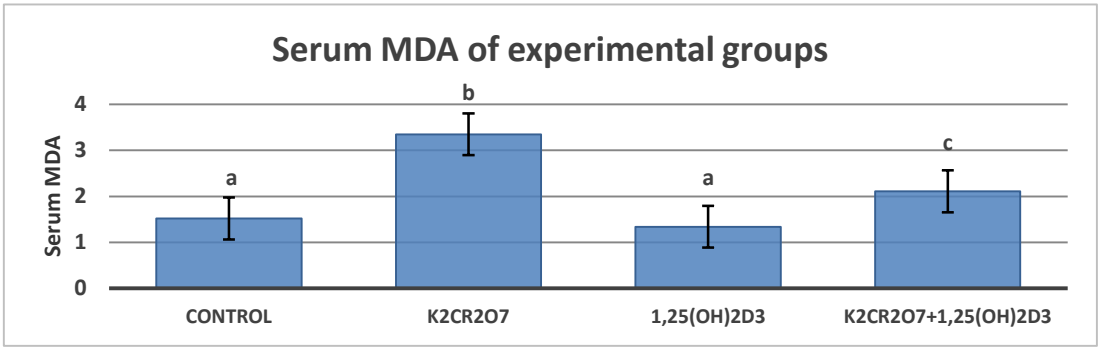
Table 5.7 shows comparative values of serum MDA of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased serum MDA of 3.35 µmol/L, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 1.52 µmol/L and 1.34 µmol/L respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased serum MDA of 2.11 µmol/L, but it was significantly lower than serum MDA of 3.35 µmol/L of Group-2 ($K_2Cr_2O_7$).

Table 5.7 shows serum MDA of experimental groups (n=9).

Groups	**Serum MDA** (µmol/L)
Group-1 (Control)	1.52 ± 0.10 [a]
Group-2 ($K_2Cr_2O_7$)	3.35 ± 0.49 [b]
Group-3 ($1,25(OH)_2D_3$)	1.34 ± 0.07 [a]
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	2.11 ± 0.32 [c]
F-value	53.114
p value	0.000*

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.7 shows serum MDA of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.8 TISSUE OXIDATIVE STRESS

5.8.1 Tissue LPO levels of heart

Table 5.8 shows comparative values of heart LPO of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased heart LPO of 10.53 Mm/gm, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 2.7 Mm/gm and 2.79 Mm/gm respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased heart LPO of 5.87 Mm/gm, but it was still significantly lower than heart LPO of 3.35 μmol/L of Group-2 ($K_2Cr_2O_7$).

5.8.2 Tissue LPO levels of Aorta

Table 5.8 shows comparative values of aortic LPO of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased aortic LPO of 2.02 Mm/gm, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 1.08 Mm/gm and 1.01 Mm/gm respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased aortic LPO of 1.36 Mm/gm, but it was still significantly lower than aortic LPO of 2.02 μmol/L of Group-2 ($K_2Cr_2O_7$).

5.8.3 Tissue LPO levels of lungs

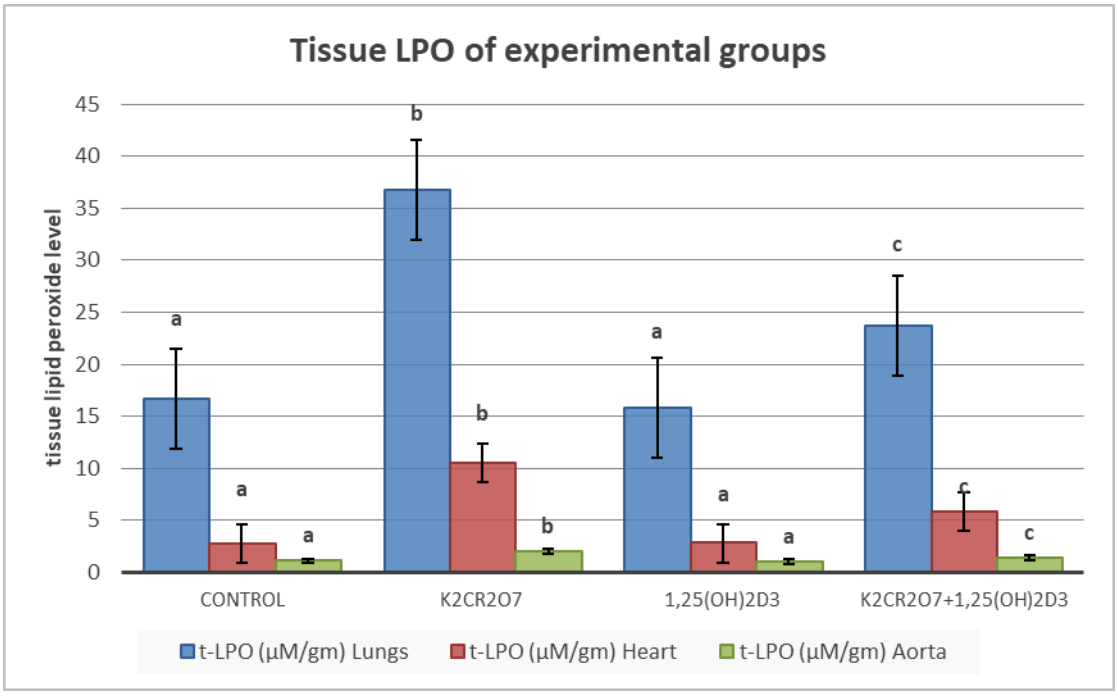
Table 5.8 shows comparative values of lungs LPO of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significanty increased lung LPO of 36.74 Mm/gm, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 16.68 Mm/gm and 15.84 Mm/gm respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) showed increased lung LPO of 23.68 Mm/gm, but it was still significantly lower than lung LPO of 36.74 μM/gmL of Group-2 ($K_2Cr_2O_7$).

Table 5.8 shows tissue LPO of experimental groups (n=6).

Groups	tissue-LPO (μM/gm)		
	Heart	**Aorta**	**Lungs**
Group-1 (Control)	2.7 ± 0.39 [a]	1.08 ± 0.12 [a]	16.68 ± 0.12 [a]
Group-2 ($K_2Cr_2O_7$)	10.53 ± 1.01 [b]	2.02 ± 0.58 [b]	36.74 ± 3.49 [b]
Group-3 ($1,25(OH)_2D_3$)	2.79 ± 0.37 [a]	1.01 ± 0.08 [a]	15.84 ± 0.78 [a]
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	5.87 ± 0.66 [c]	1.36 ± 0.27 [c]	23.68 ± 1.7 [c]
F-value	182.618	11.693	134.742
p-value	0.000*	0.000*	0.000*

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.8 shows tissue LPO of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.9 SERUM NITROSATIVE STRESS

5.9.1 Serum Nitric Oxide (NO) concentration

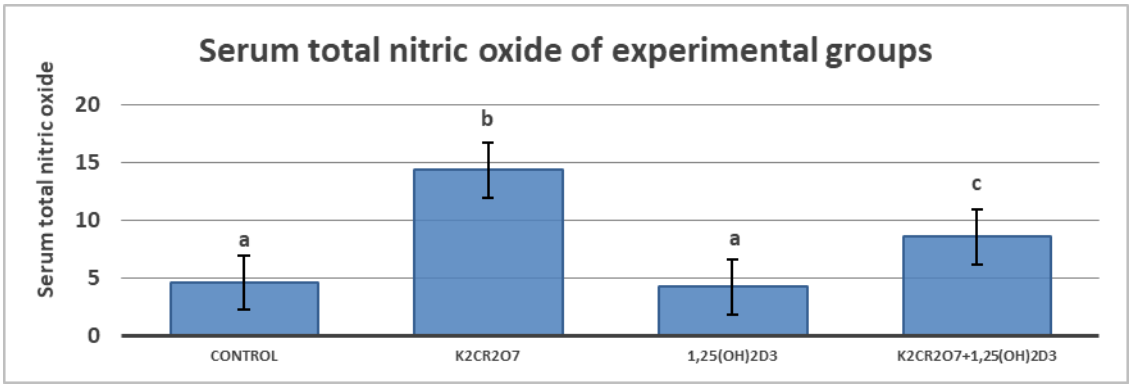
Table 5.9 shows comparative values of serum NO of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased serum NO of 14.33 µmol/L, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 4.58 µmol/L and 4.19 µmol/L respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased serum NO of 8.56 µmol/L, but it was still significantly lower than serum NO of 14.33 µmol/L of Group-2 ($K_2Cr_2O_7$).

Table 5.9 shows serum total nitric oxide of experimental groups (n-9).

Groups	**Serum Total nitric oxide** (µmol/L)
Group-1 (Control)	4.58 ± 0.43 [a]
Group-2 ($K_2Cr_2O_7$)	14.33 ± 1.87 [b]
Group-3 ($1,25(OH)_2D_3$)	4.19 ± 0.33 [a]
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	8.56 ± 0.89 [c]
F-value	114.987
p-value	0.000*

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.9 shows serum total nitric oxide of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.10 SERUM ANTIOXIDANTS

5.10.1 Serum superoxide dismutase (SOD)

Table 5.10 shows comparative values of serum SOD of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly decreased serum SOD of 19.01 U/ml, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 37.93 U/ml and 36.56 U/ml respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had a decreased serum SOD of 25.60 U/ml, but it was still significantly higher than serum SOD of 14.33 μmol/L of Group-2 ($K_2Cr_2O_7$).

5.10.2 Serum ascorbic acid (vitamin C)

Table 5.10 shows comparative values of serum ascorbic acid of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly decreased serum ascorbic acid of 0.61 mg/dL, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 1.11 mg/dL and 1.01 mg/dL respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had a decreased serum ascorbic acid of 0.84 mg/dL, but it was still significantly higher than serum ascorbic acid of 0.61 mg/dL of Group-2 ($K_2Cr_2O_7$).

5.10.3 Serum $1,25(OH)_2D_3$ (Vitamin D_3)

Table 5.10 shows comparative values of serum $1,25(OH)_2D_3$ of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly decreased serum $1,25(OH)_2D_3$ of 19.60 ng/ml, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 37.16 ng/ml and 70.72 ng/ml respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had a decreased serum $1,25(OH)_2D_3$ of 30.79 ng/ml, but it was still significantly higher than serum $1,25(OH)_2D_3$ of 19.60 ng/ml of Group-2 ($K_2Cr_2O_7$).

5.10.4 Serum α-tocopherol (vitamin E)

Table 5.10 shows comparative values of serum $1,25(OH)_2D_3$ of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly decreased serum α-tocopherol of 1.48

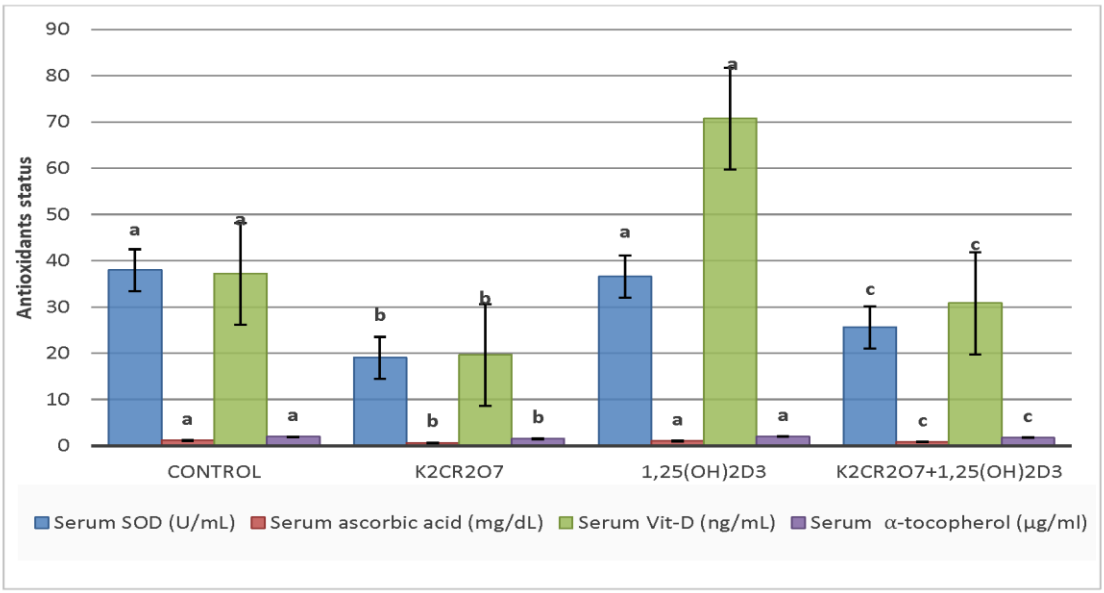
µg/ml, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 1.91 µg/ml and 1.98 µg/ml respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had a decreased serum α-tocopherol of 1.73 µg/ml, but it was still significantly higher than serum α-tocopherol of 1.48 µg/ml of Group-2 ($K_2Cr_2O_7$).

Table 5.10 shows serum SOD, vit C, vit D and vit E of experimental groups (n=9).

Groups	**Serum SOD** (U/mL)	**Serum ascorbic acid** (mg/dL)	**Serum Vit-D_3** (ng/mL)	**Serum α-tocopherol** (µg/ml)
Group-1 (Control)	37.93 ± 4.42^a	1.11 ± 0.16^a	37.16 ± 3.80^a	1.91 ± 0.08^a
Group-2 ($K_2Cr_2O_7$)	19.01 ± 2.08^b	0.61 ± 0.05^b	19.60 ± 2.75^b	1.48 ± 0.12^b
Group-3 ($1,25(OH)_2D_3$)	36.56 ± 3.37^a	1.01 ± 0.06^a	70.72 ± 5.30^a	1.98 ± 0.05^a
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	25.60 ± 1.65^c	0.84 ± 0.09^c	30.79 ± 2.99^c	1.73 ± 0.05^c
F-value	51.674	26.022	169.092	40.316
p-value	<0.05	<0.05	<0.05	<0.05

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.10 shows serum SOD, vit C, vit D and vit E of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.11 MOLECULAR MARKER

5.11.1 Serum VEGF gene expression

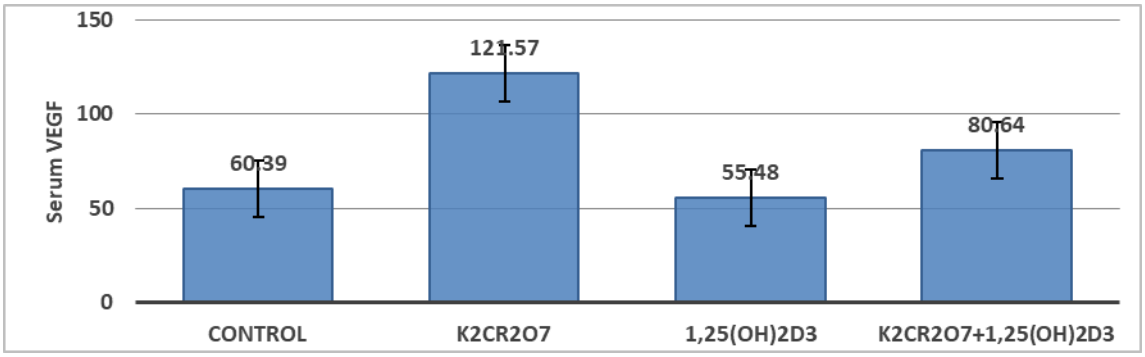
Table 5.11 shows comparative values of serum VEGF of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased serum VEGF of 121.57 pg/ml, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 60.39 pg/ml and 55.48 pg/ml respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased serum VEGF of 80.64 pg/ml, but it was still significantly lower than serum VEGF of 121.57 pg/ml of Group-2 ($K_2Cr_2O_7$).

Table 5.11 shows serum VEGF of experimental groups (n=9).

Groups	**Serum VEGF** (pg/ml)
Group-1 (Control)	60.39 ± 3.29 [a]
Group-2 ($K_2Cr_2O_7$)	121.57 ± 9.05 [b]
Group-3 ($1,25(OH)_2D_3$)	55.48 ± 5.33 [a]
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	80.64 ± 6.29 [c]
F-value	134.926
p-value	0.000*

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.11 shows serum VEGF of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.12 SERUM CHROMIUM CONCENTRATION

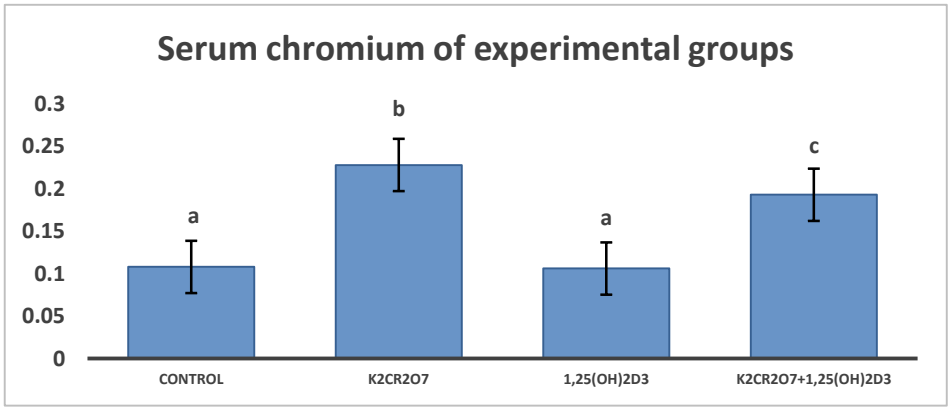
Table 5.12 shows comparative values of serum Cr(VI) of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased serum Cr(VI) of 0.228 mg/dl, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 0.108 mg/dl and 0.106 mg/dl respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased serum Cr(VI) of 0.193 mg/dl, but it was still significantly lower than serum Cr(VI) of 0.228 mg/dl of Group-2 ($K_2Cr_2O_7$).

Table 5.12 shows serum chromium of experimental groups (n=9).

Groups	**Serum Cr(IV)** (mg/dL)
Group-1 (Control)	0.108 ± 0.034 [a]
Group-2 ($K_2Cr_2O_7$)	0.228 ± 0.074 [b]
Group-3 ($1,25(OH)_2D_3$)	0.106 ± 0.05 [a]
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	0.193 ± 0.062 [c]
F-value	6.911
p value	0.000*

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.12 shows serum chromium of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

5.13 HISTOPATHOLOGIC EVALUATION OF THE TISSUES

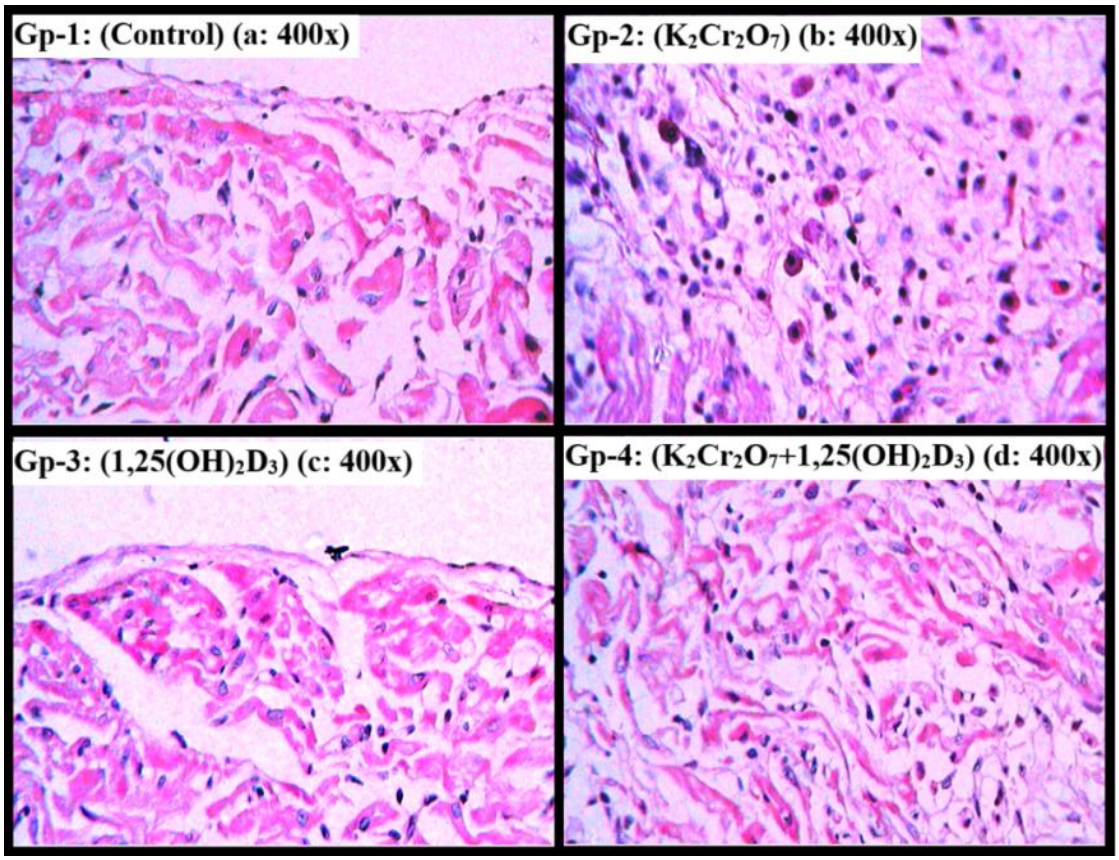
5.13.1 Histopathology of heart

Table 5.13.1 shows histopathological changes in heart of experimental animals.

Sl.No	Histopathological findings	Group-1 (n=6) (Control)	Group-2 (n=6) ($K_2Cr_2O_7$)	Group-3 (n=6) ($1,25(OH)_2D_3$)	Group-4 (n=6) ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)
	Denotations: Absent (-), Present (Mild=+, Moderate= ++, Severe=+++ & N=Normal)				
1	Myocardial architectural distortion	-	+++	-	+
2	Endocardial distortion	-	+	-	-
3	Myocardial distortion	-	+++	-	+
4	Pericardial distortion	-	-	-	-
5	Myocardial cellularity	N	↑↑↑	N	↑
6	Myocytic disorganization	-	+++	-	+
7	Myocytic hypertrophy and atrophy	-	+++	-	+
8	Myocytic degeneration / necrosis	-	+++	-	+
9	Myocardial inflammation	-	+++	-	+
10	Interstitial fibrosis	-	+++	-	+

Table 5.13.1 shows comparative values of histopathological changes in the heart. Group-2 ($K_2Cr_2O_7$) showed diffuse distortion in cardiac parenchymal architecture which included endocardial inflammation (Fig.5.13.1.1), myocardial injury such as myocytic hypertrophic and/or atrophic degeneration, splitting (Fig.5.13.1.3) and interstitial mononuclear inflammation, vascular congestion and fibrosis. These changes were significantly of moderate to severe range especially in the left ventricle, in contrast to group-4 (Cr(VI)+Vit-D_3 treated) which were of mild affected. Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) did not show any significant histomorphologic changes in the heart.

Fig.5.13.1.1 showing photomicrographs of endocardium (H&E stain under 400x).Gp-1,Gp-2, Gp-3 & Gp-4



13.1.1 (Table 5.13.1) showing photomicrographs of endocardium of rats (H&E stain under 400x). Gp-1, (Control): Normal, Gp-2 ($K_2Cr_2O_7$): Endocardial thickening edema, inflammatory cells, macrophages & fibrosis. Gp-3 ($1,25(OH)_2D_3$):Normal, Gp-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$): Mild changes.

Fig.5.13.1.2 showing photomicrographs of myocardium (H&E stain under 400x).Gp-1,Gp-2, Gp-3 & Gp-4.

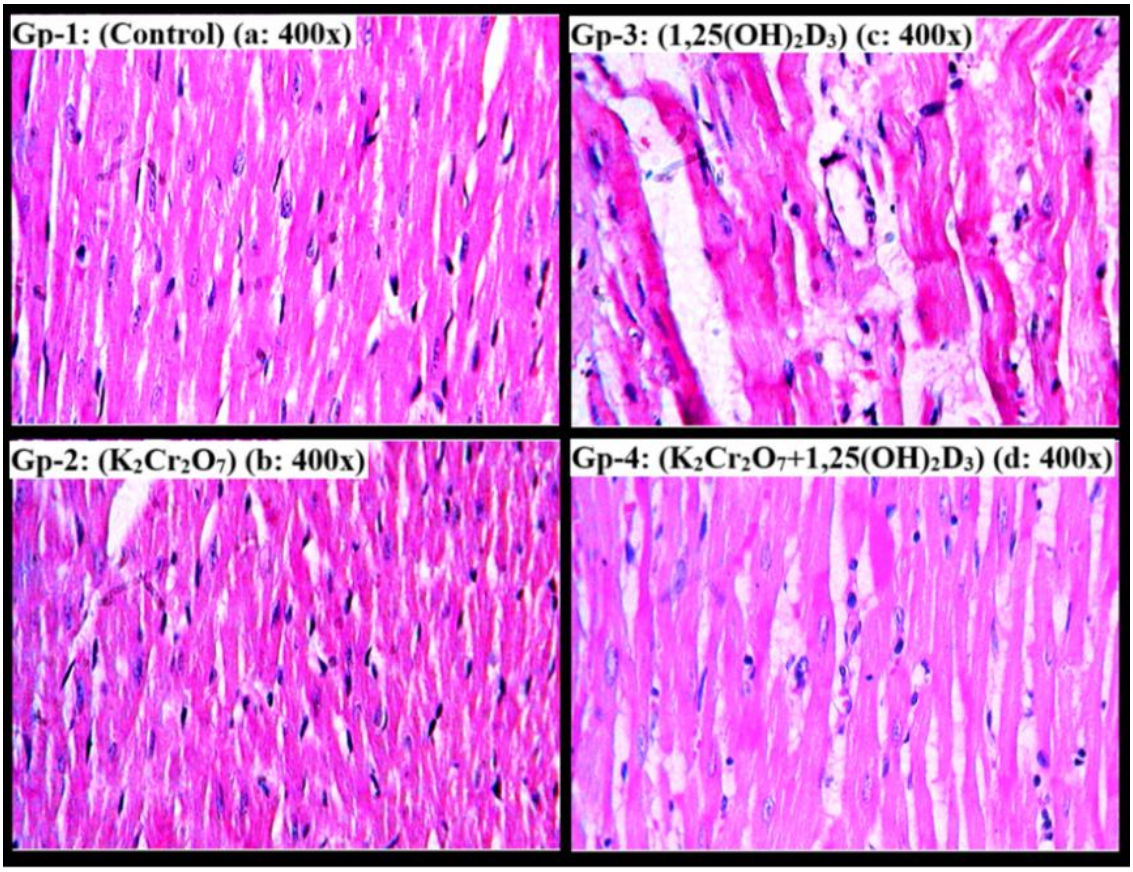
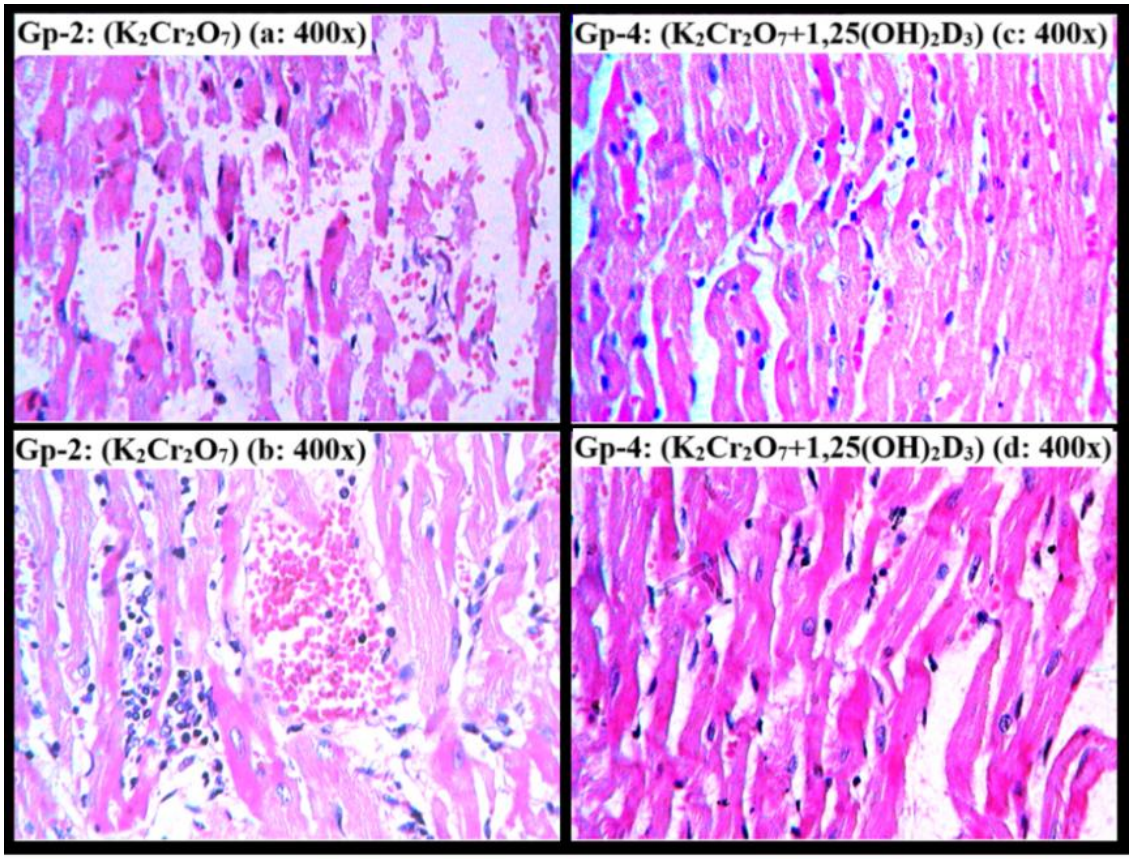


Fig.5.13.1.2 (Table 5.13.1) showing photomicrographs of myocardium of rats (H&E stain under 400x). Gp-1, (Control): Normal, Gp-2 ($K_2Cr_2O_7$): Myoocardial degeneration, interstitial edema, inflammatory cells & fibrosis, Gp-3 ($1,25(OH)_2D_3$):Normal, Gp-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$): Mild changes.

Fig.5.13.1.3 showing photomicrographs of myocardium (H&E stain under 400x). Gp-2 (a&b) & Gp-4.



.5.13.1.3 (Table 5.13.1) showing photomicrographs of myocardium of rats (H&E stain under 400x). Gp-2 ($K_2Cr_2O_7$) (a&b): Myocardial degeneration, interstitial edema, congestion, inflammatory cells & fibrosis. Gp-4 ($K_2Cr_2O_7$ + 1,25$(OH)_2D_3$) (c&d): with mild changes.

5.13.2 Histopathology of coronary artery

Table 5.13.2 shows histopathological changes in coronary vessels of experimental animals.

Sl.No	Histopathological findings	Group-1 (n=6) (Control)	Group-2 (n=6) ($K_2Cr_2O_7$)	Group-3 (n=6) ($1,25(OH)_2D_3$)	Group-4 (n=6) ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)
	Denotations: Absent (-), Present (Mild=+, Moderate= ++, Severe=+++ & N=Normal)				
1	Coronary intimal hyperplasia	-	+++	-	-
2	Coronary medial hypertrophy	-	+++	-	+
3	Coronary atheromatous plaques	-	-	-	-
4	Coronary lumen	N	↓↓↓	N	↓
5	Vascular capillary thrombosis	-	-	-	-

Table 5.13.2 shows comparative values of histopathological changes in the coronary vessels. Group-2 ($K_2Cr_2O_7$) showed diffuse distortion and thickening of intimal layer, diffuse hyperplasia of smooth muscle cells in the media with fibrosis in the adventitial layer and perivascular mononuclear inflammatory cell infiltrate, resulting in thickening of the arterial wall with luminal narrowing causing obvious arteriosclerotic changes (Fig.5.13.2.2). These changes were significantly of moderate to severe range, in contrast to group-4 (Cr(VI)+Vit-D_3 treated) which were of mild affected. Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) did not show any significant histomorphologic changes in the coronary vessels.

Fig.5.13.2.1 showing photomicrographs of coronary artery (H&E stain under 400x). Gp-1, Gp-2, Gp-3 & Gp-4.

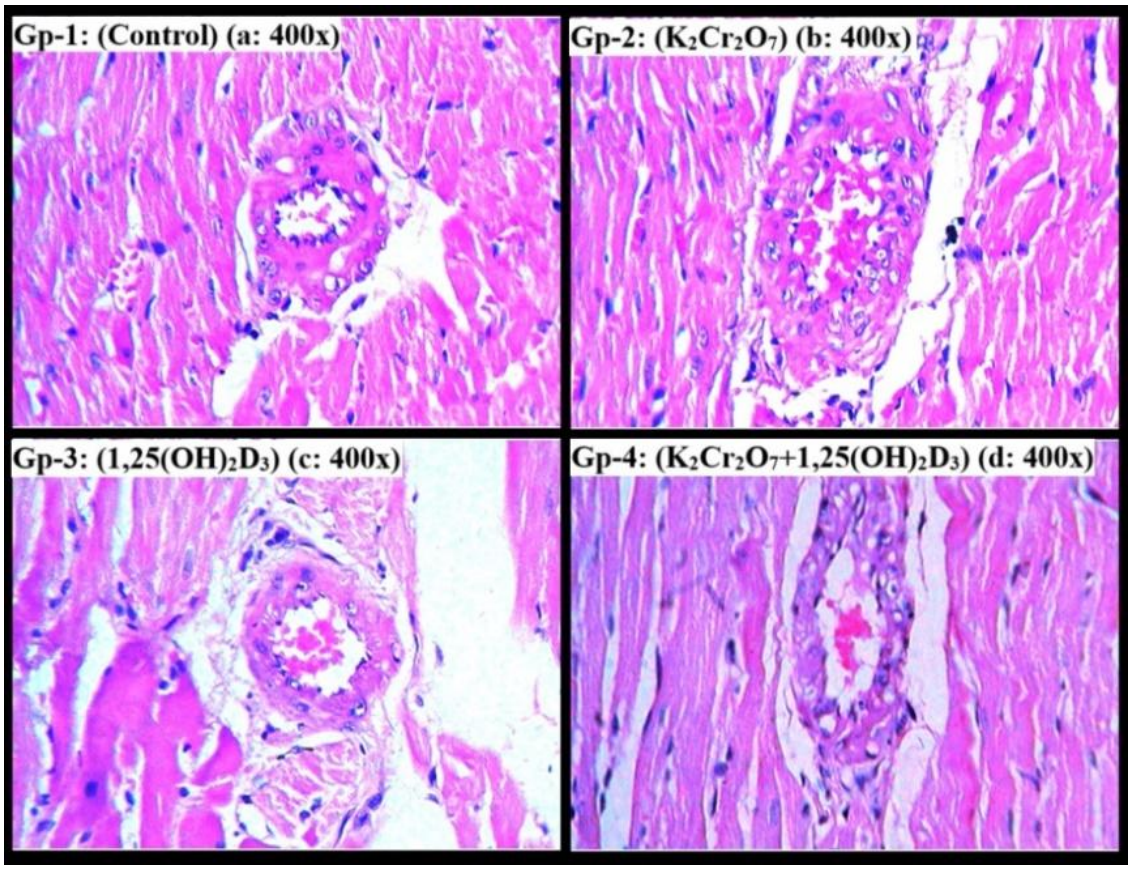


Fig.5.13.2.1 (Table 5.13.2) showing photomicrographs of coronary artery of rats (H&E stain under 400x). Gp-1, (Control): Normal, Gp-2 ($K_2Cr_2O_7$): Intimal thickening, medial hyperplasia, perivascular inflammation, and narrowing of lumen, Gp-3 ($1,25(OH)_2D_3$): Normal, Gp-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$): with mild changes.

Fig.5.13.2.2 showing photomicrographs of coronary artery (H&E stain under 400x). Gp-1, Gp-2, Gp-3 & Gp-4.

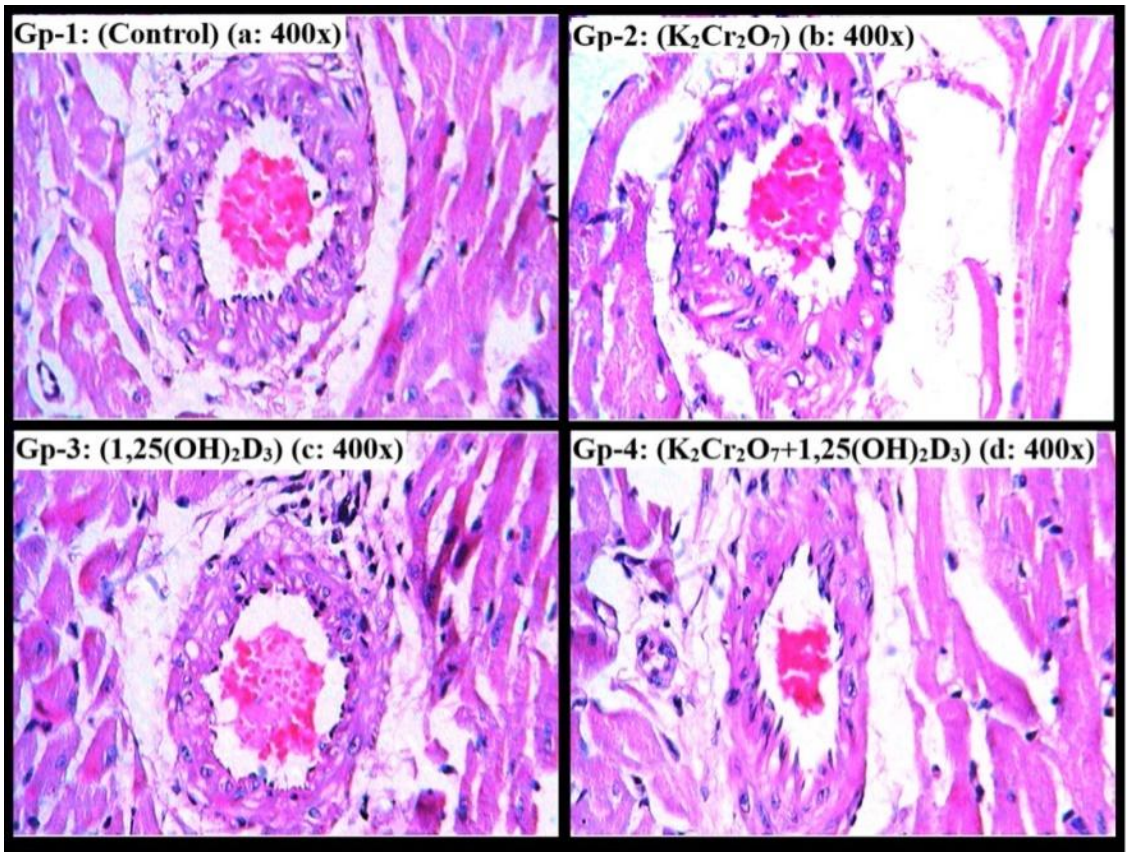


Fig.5.13.2.2 (Table 5.13.2) showing photomicrographs of coronary artery of rats (H&E stain under 400x). Gp-1, (Control): Normal, Gp-2 ($K_2Cr_2O_7$): Intimal thickening, medial hyperplasia, perivascular inflammation, and narrowing of lumen, Gp-3 ($1,25(OH)_2D_3$): Normal, Gp-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$): with mild changes.

5.13.3 Histopathology of aorta

Table 5.13.3 shows histopathological changes in aorta of experimental animals.

Sl.No	Histopathological findings	Group-1 (n=6) (Control)	Group-2 (n=6) ($K_2Cr_2O_7$)	Group-3 (n=6) ($1,25(OH)_2D_3$)	Group-4 (n=6) ($K_2Cr_2O_7$ + $1,25(OH)2D_3$)
	Denotations: Absent (-), Present (Mild=+, Moderate= ++, Severe=+++ & N=Normal)				
1	Intimal thickness/cellularity	N	+++	N	+
2	Medial thickness / cellularity / hyperplasia	-	+++	-	+
3	Atherosclerotic changes	-	-	-	-
4	Smooth muscle cell morphology and arrangement	N	+++	N	+
5	Adventitial thickness and cellularity	-	-	-	-
6	Inflammation	-	+	-	-
7	Fibrosis	-	+++	-	+
8	Vascular thrombosis	-	-	-	-
9	Aneurysmal changes	-	+	-	-

Table 5.13.3 shows comparative values of histopathological changes in the aorta. Group-2 ($K_2Cr_2O_7$) showed diffuse distortion and thickening of intimal layer, diffuse hyperplasia of smooth muscle cells in the media with fibrosis in the adventitial layer and perivascular mononuclear inflammatory cell infiltrate, resulting in thickening of the arterial wall with luminal narrowing causing obvious arteriosclerotic changes (Fig.5.13.3.1). There were foci of early plexiform aneurysmal changes. These changes were significantly of moderate to severe range, in contrast to group-4 (Cr(VI)+Vit-D_3 treated) which were of mildly affected. Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) did not show any significant histomorphologic changes in the coronary vessels.

Fig.5.13.3.1 showing photomicrographs of aorta (H&E stain under 100x). Gp-1, Gp-2, Gp-3 & Gp-4.

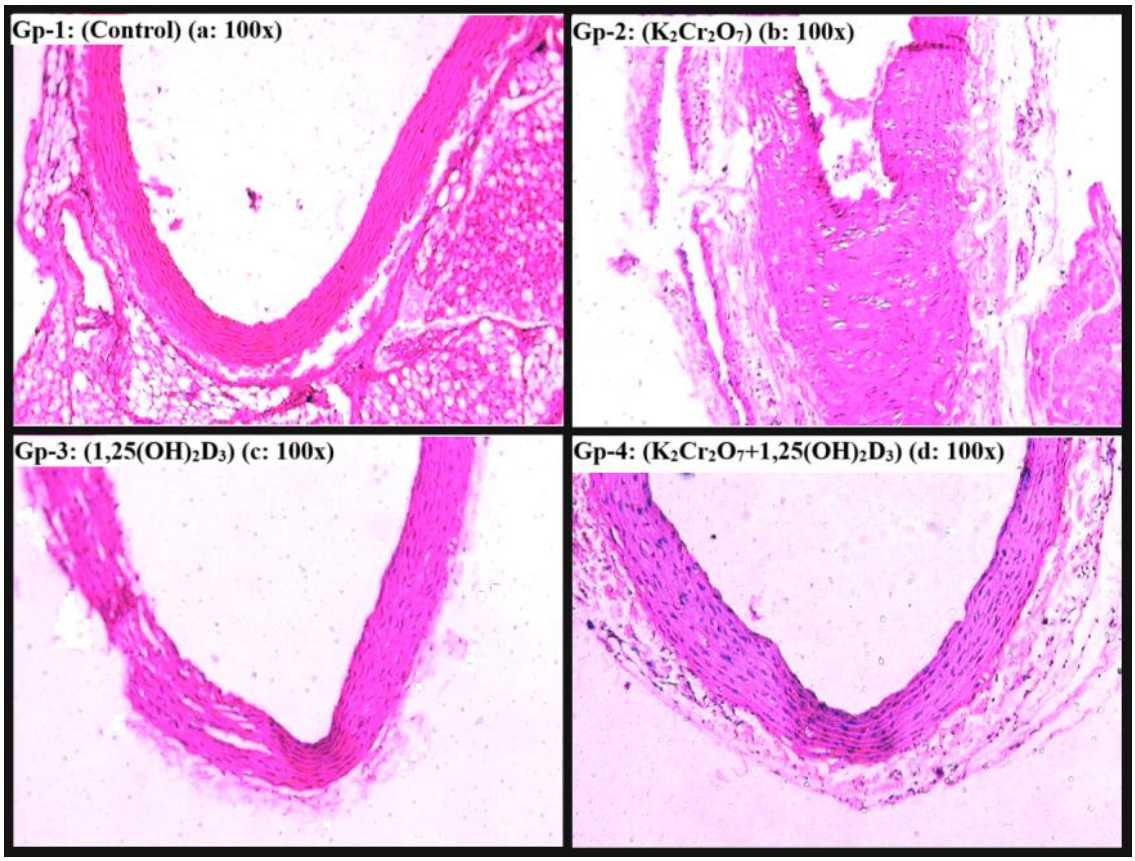


Fig.5.13.3.1 (Table 5.13.3) showing photomicrographs of arch of aorta of rats (H&E stain under 100x). Gp-1, (Control): Normal, Gp-2 ($K_2Cr_2O_7$): Intimal thickening, hyperplasia of smooth muscle cells causing thickening of tunica media, perivascular inflammation, fibrosis, Gp-3 ($1,25(OH)_2D_3$): Normal, Gp-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$): with mild changes.

Fig.5.13.3.2 Photomicrographs of aorta (H&E stain under 400x). Gp-1, Gp-2, Gp-3 & Gp-4.

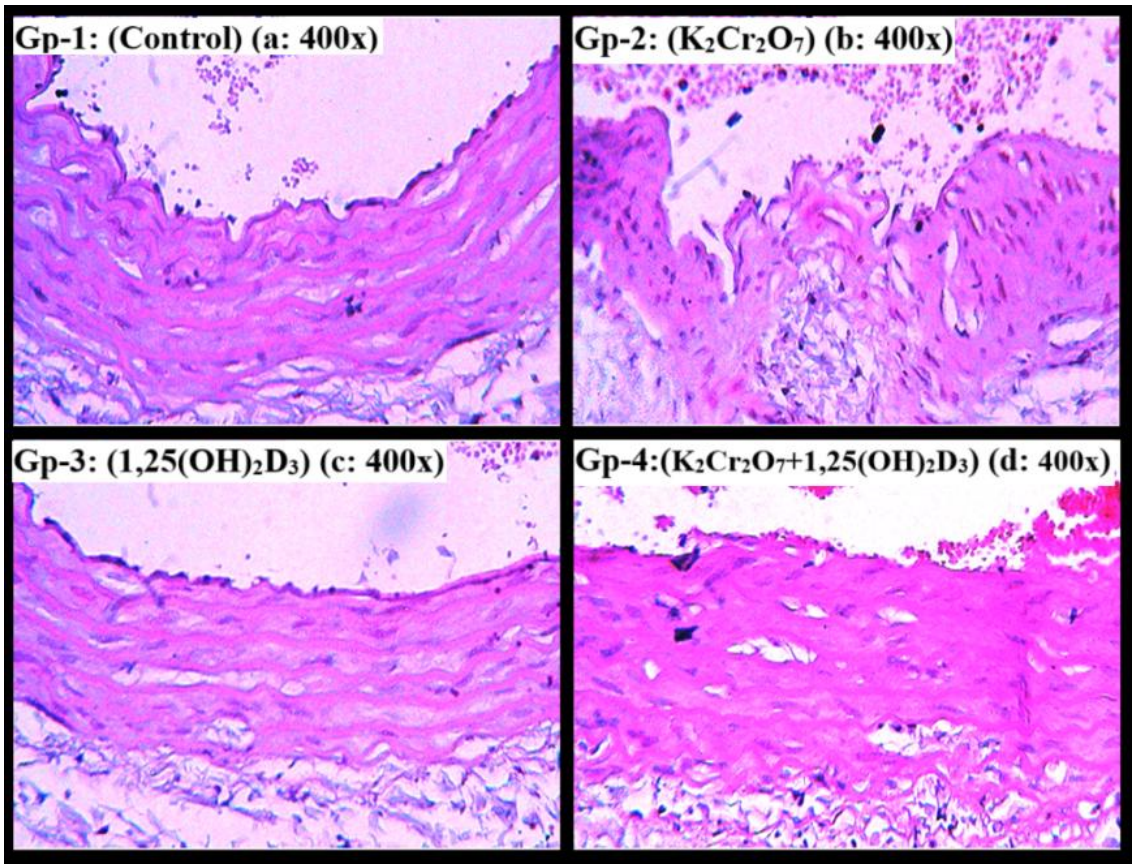


Fig.5.13.3.2 (Table 5.13.3) showing photomicrographs of arch of aorta of rats (H&E stain under 400x). Gp-1, (Control): Normal, Gp-2 ($K_2Cr_2O_7$): Intimal thickening, hyperplasia of smooth muscle cells causing thickening of tunica media and mild aneurysmal changes. Gp-3 ($1,25(OH)_2D_3$): Normal, Gp-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$): with mild changes.

Fig.5.13.3.3 showing photomicrographs of aorta of rats (H&E stain under 400x). Gp-2 & Gp-4.

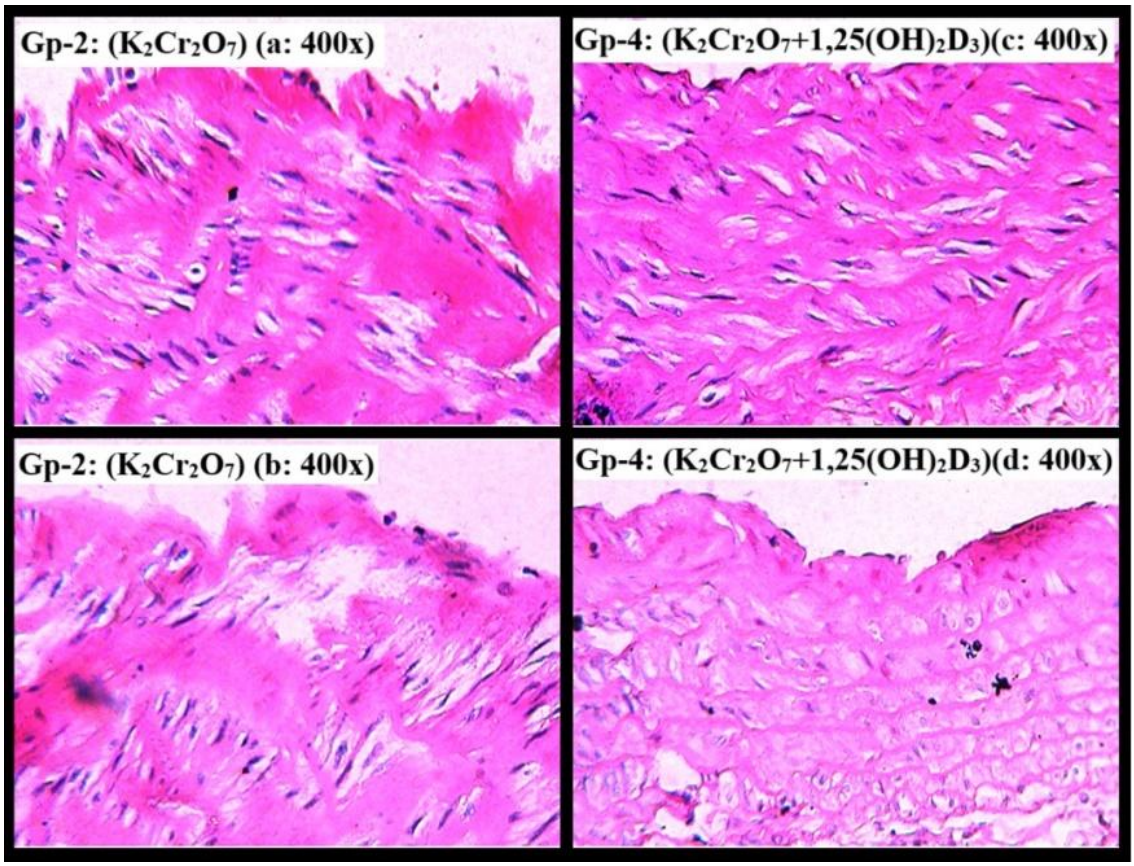


Fig.5.13.3.3 (Table 5.13.3) showing photomicrographs of arch of aorta of rats (H&E stain under 400x). Gp-2 ($K_2Cr_2O_7$)(a&b): Intimal thickening, hyperplasia of smooth muscle cells causing thickening of tunica media and fibrosis. Gp-4 ($K_2Cr_2O_7$ + 1,25$(OH)_2D_3$)(c&d): with mild changes.

5.13.4 Histopathology of lungs

Table 5.13.4 shows histopathological changes in lungs of experimental animals.

Sl.No	Histopathological findings	Group-1 (n=6) (Control)	Group-2 (n=6) (K2Cr2O7)	Group-3 (n=6) (1,25(OH)2D3)	Group-4 (n=6) (K2Cr2O7 + 1,25(OH)2D3)
	Denotations: Absent (-), Present (Mild=+, Moderate= ++, Severe=+++ & N=Normal)				
1	Pulmonary architecture distortion	-	+++	-	+
2	Alveolar space dilatation	-	+++	-	
3	Alveolar space patency	+	-	+	+/-
4	Alveolar space contents	Empty	Edema	Empty	Mixed
5	Alveolar wall thickness	-	+++	-	+
6	Alveolar walls cellularity	-	+++	-	+
7	Alveolar walls basement membrane	Intact	Thickened	Intact	Mixed
8	Alveolar epithelium	Flat	Plumped	Flat	Mixed
9	Alveolar macrophages	-	+++	-	+
10	Hyaline membranes	-	+	-	-
11	Interstitial thickness	Thin	Thick	Thin	Mixed
12	Interstitial inflammation	-	+++	-	+
13	Interstitial fibrosis	-	+++	-	+
14	Multinucleated giant cells	-	+++	-	+
15	Vascular medial hypertrophy	-	+++	-	+
16	Vascular hyalinization	-	+++	-	+
17	Vascular capillary proliferation	-	+++	-	+
18	Vascular capillary thrombosis	-	+	-	-
19	Bronchiolar wall thickness/cellularity	N	+++	N	+
20	Bronchial mucousal regeneration	-	-	-	+
21	Bronchial metaplastic changes	-	+++	-	+
22	Bronchial dysplastic changes	-	-	-	-
23	Bronchial inflammation	-	+++	-	+
24	Bronchial mucosal changes	-	+++	-	+
25	Bronchial goblet cell hyperplasia	-	+++	-	+
26	Infectious agents	-	-	-	-
27	Mucous plugging	-	+	-	-
28	Neoplastic changes	-	-	-	-
29	Other features (Cysts and cavities)	-	-	-	-
30	Dystrophic calcification	-	+	-	-

Table 5.13.4 shows comparative histopathological changes in the lungs. Group-2 ($K_2Cr_2O_7$) showed diffuse distortion in pulmonary parenchymal architecture which included alveolar spaces being ephased by thick edematous interstitial wall containing diffuse mononuclear cell inflammation, macrophages with foci of multinucleated giant cells and fibrosis. The proliferative blood vessels within the interstitium appeared congested. The medium sized blood vessels contained hypercellular medial layer showing hyalinized and sclerotic changes resulting in luminal narrowing (Fig.5.13.4.4). Bronchiolar mucosal changes included degeneration, regenerative, metaplastic changes with goblet cell hyperplasia. The wall was thickened mucous with smooth cell hyperplasia with diffuse peribrochiolar inflammatory cell infiltrate. (Fig.5.13.4.5) These changes were significantly of moderate to severe range, in contrast to group-4 (Cr(VI)+Vit-D_3 treated) which were of mildly affected. Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) did not show any significant histomorphologic changes in the heart.

Fig.5.13.4.1 showing photomicrographs of lung (H&E stain under 100x). Gp-1, Gp-2, Gp-3 & Gp-4.

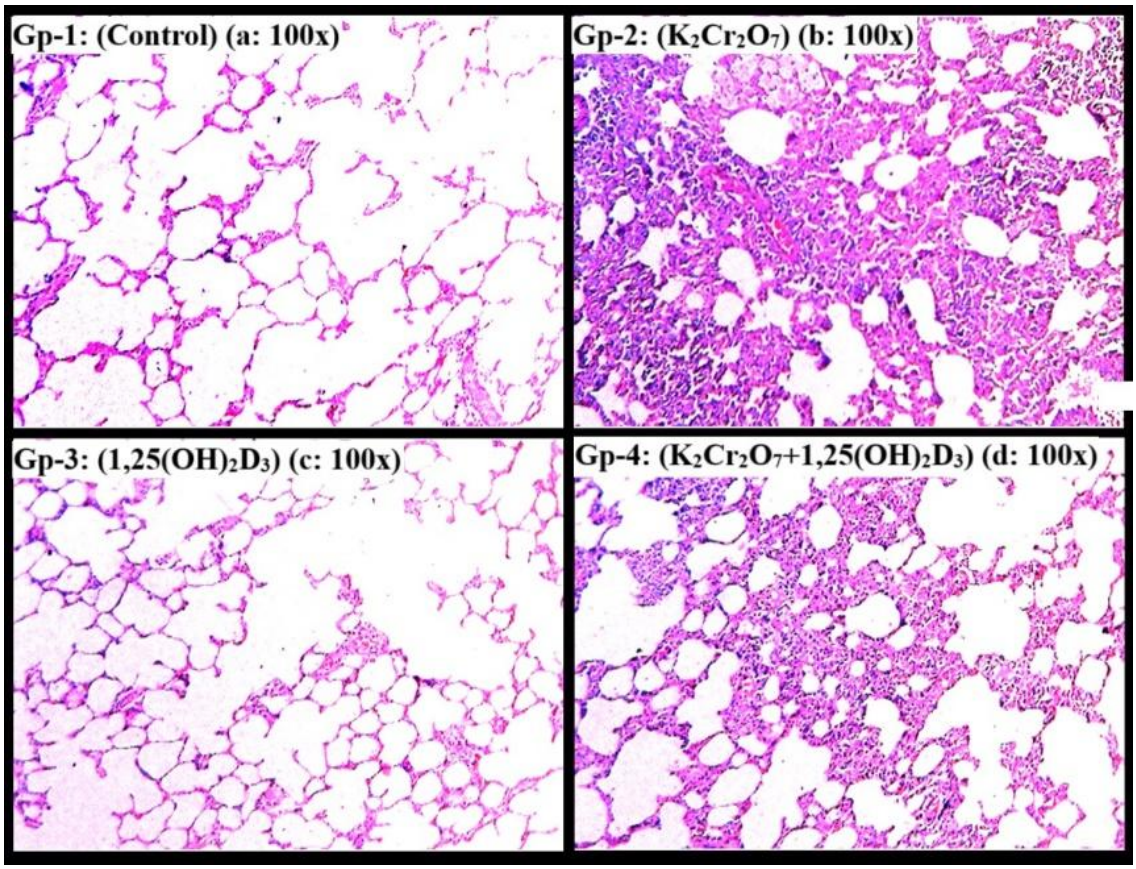


Fig.5.13.4.1 (Table 5.13.4) showing photomicrographs of lung rats (H&E stain under 100x). Gp-1, (Control): Normal, Gp-2 ($K_2Cr_2O_7$): distorted alveolar architecture with alveolar wall thickening, interstitial edema and inflammation and reduced alveolar spaces, Gp-3 ($1,25(OH)_2D_3$): Normal, Gp-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$): with mild changes.

Fig.5.13.4.2 showing photomicrographs of lung (H&E stain under 100x). Gp-1, Gp-2, Gp-3 & Gp-4.

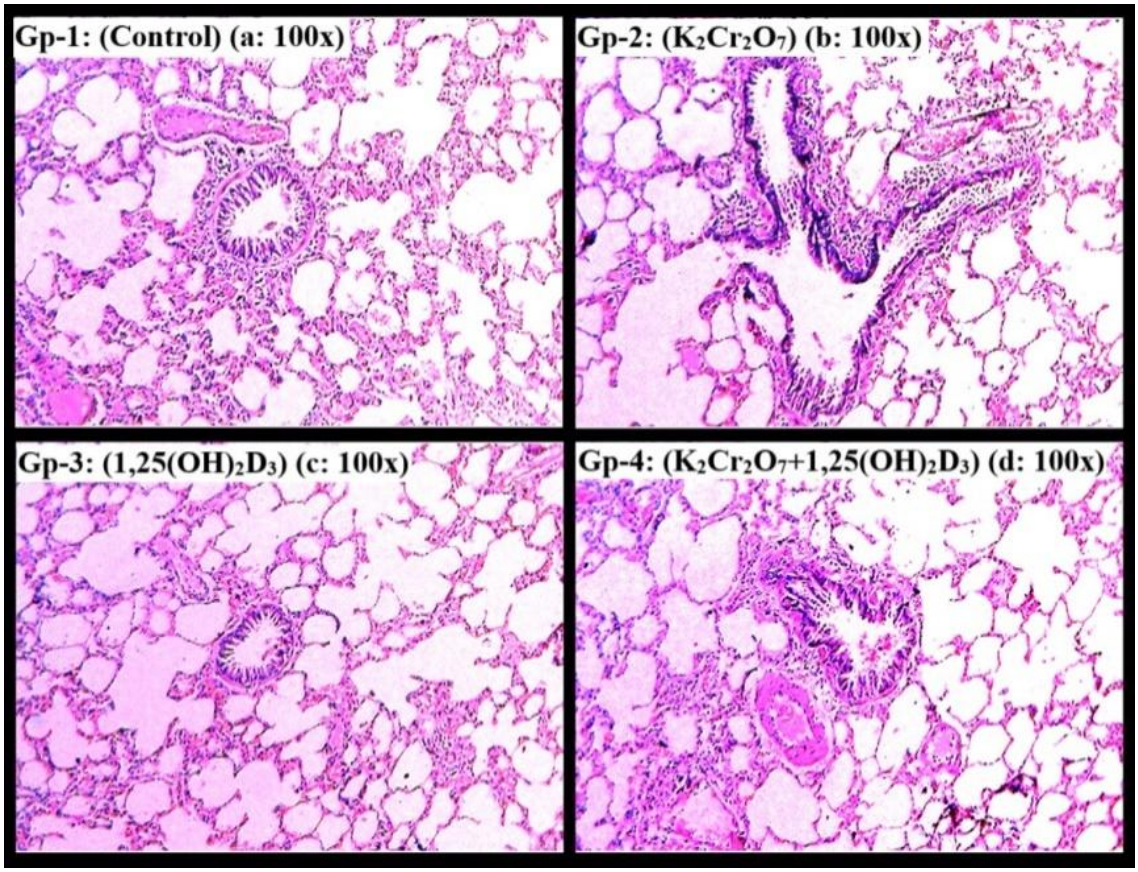


Fig.5.13.4.2 (Table 5.13.4) showing photomicrographs of lung rats (H&E stain under 100x). Gp-1, (Control): Normal, Gp-2 ($K_2Cr_2O_7$): distorted bronchiolar wall thickening, congestion, peribrochiolar edema and inflammation, Gp-3 ($1,25(OH)_2D_3$): Normal,
Gp-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$): with mild changes.

Fig.5.13.4.3 showing photomicrographs of lung (H&E stain under 100x). Gp-1, Gp-2, Gp-3 & Gp-4.

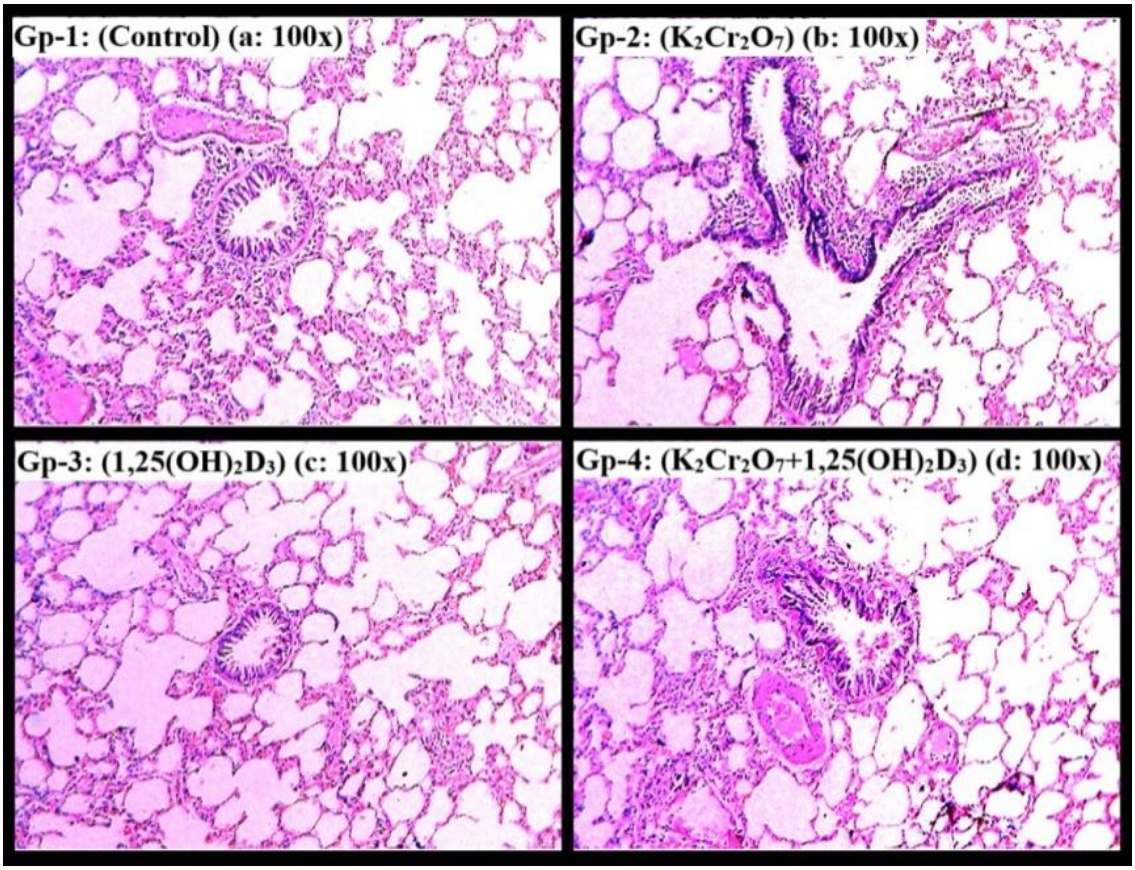


Fig.5.13.4.3 (Table 5.13.4) showing photomicrographs of lung rats (H&E stain under 100x).
Gp-1, (Control): Normal, Gp-2 ($K_2Cr_2O_7$): distorted bronchiolar wall thickening, congestion, peribrochiolar edema and inflammation, Gp-3 ($1,25(OH)_2D_3$): Normal, Gp-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$): with mild changes.

Fig.5.13.4.4 showing photomicrographs of lung (H&E stain under 400x). Gp-1, Gp-2, Gp-3 & Gp-4.

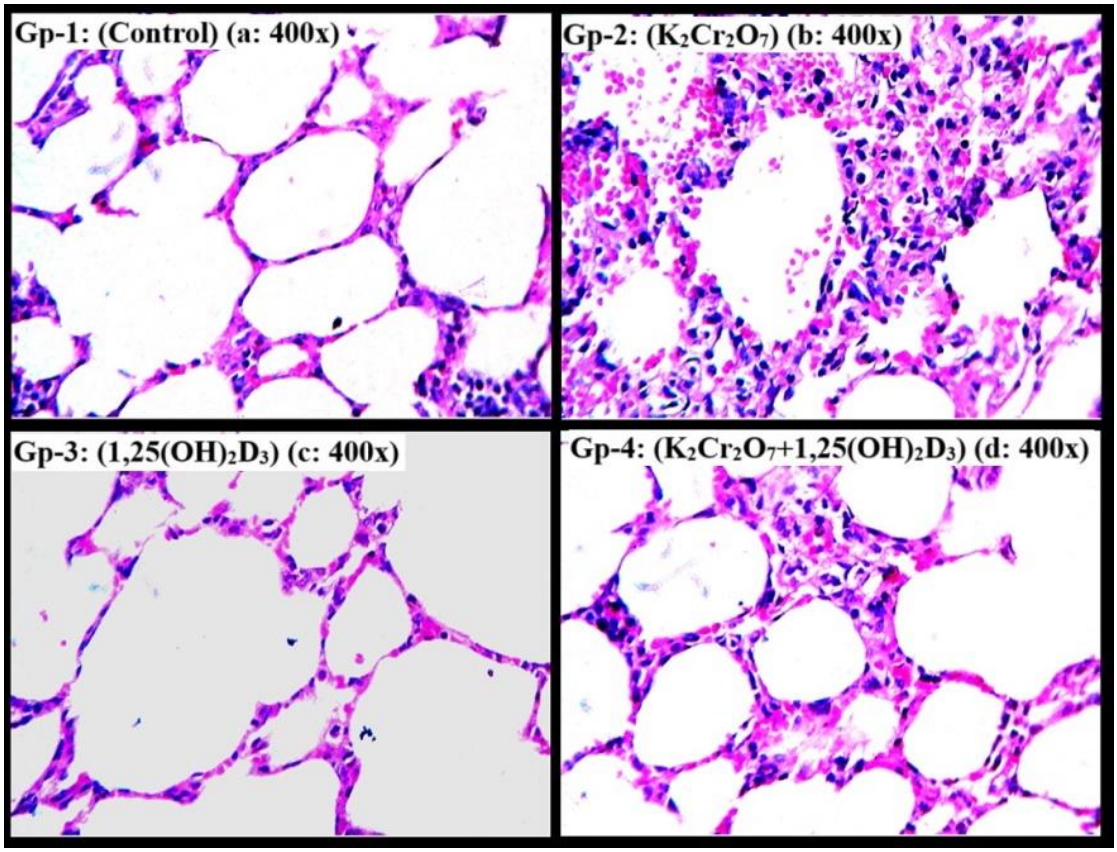


Fig.5.13.4.4 (Table 5.13.4) showing photomicrographs of lung rats (H&E stain under 400x).

Gp-1, (Control): Normal, Gp-2 ($K_2Cr_2O_7$): distorted alveolar architecture with alveolar wall thickening, interstitial edema, congestion and inflammation and reduced alveolar spaces.

Gp-3 ($1,25(OH)_2D_3$): Normal, Gp-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$): with mild changes.

Fig.5.13.4.5 showing photomicrographs of lung (H&E stain under 400x). Gp-1, Gp-2, Gp-3 & Gp-4.

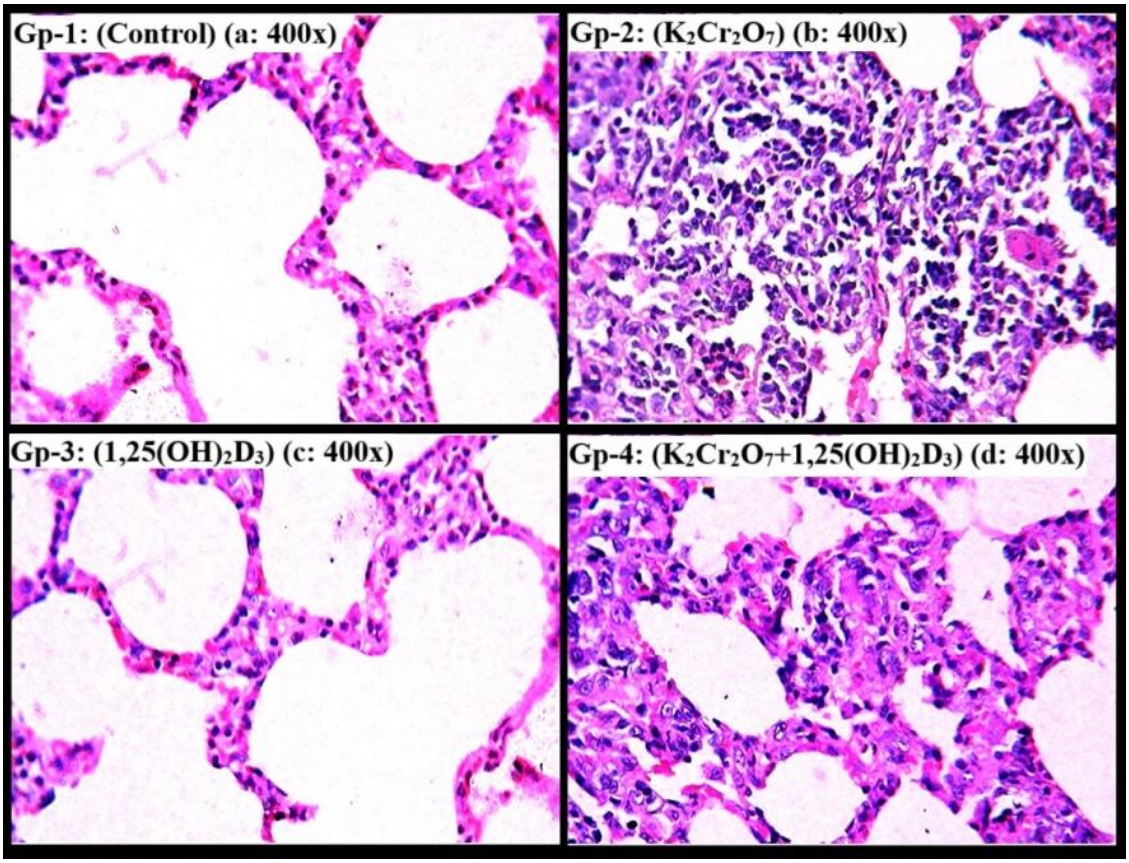


Fig.5.13.4.5 (Table 5.13.4) showing photomicrographs of lung rats (H&E stain under 400x).

Gp-1, (Control): Normal, Gp-2 ($K_2Cr_2O_7$): obliterated alveolar spaces with alveolar wall thickening, interstitial edema, congestion and inflammation, Gp-3 ($1,25(OH)_2D_3$): Normal, Gp-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$): with mild changes.

Fig.5.13.4.6 showing photomicrographs of lung (H&E stain under 100x). Gp-2 & Gp-4.

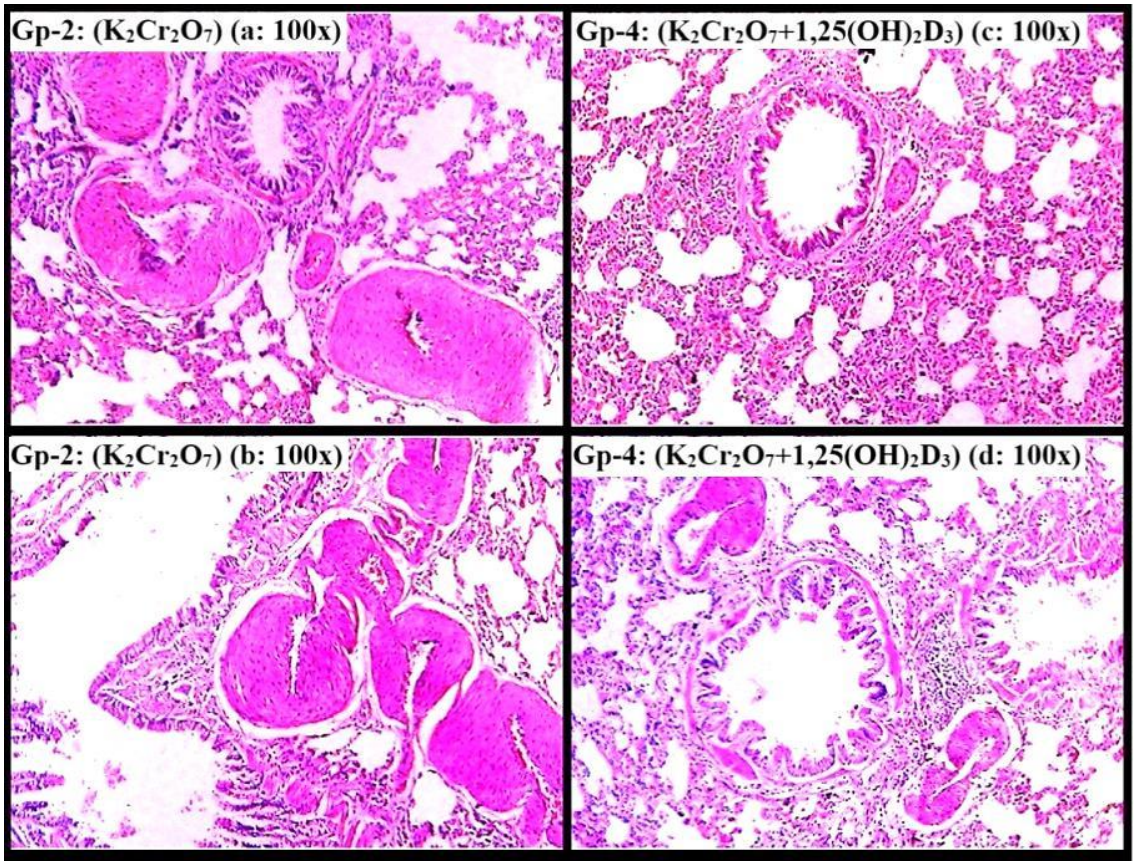


Fig.5.13.4.6 (Table 5.13.4) showing photomicrographs of lung rats (H&E stain under 100x). Gp-2 ($K_2Cr_2O_7$): Vascular intimal thickening, medial hypertrophy with hyalinization, congestion and perivascular inflammation, Gp-4 ($K_2Cr_2O_7$ + 1,25$(OH)_2D_3$): with mild changes.

Fig.5.13.4.7 showing photomicrographs of lung rats (H&E stain under 400x). Gp-2 & Gp-4.

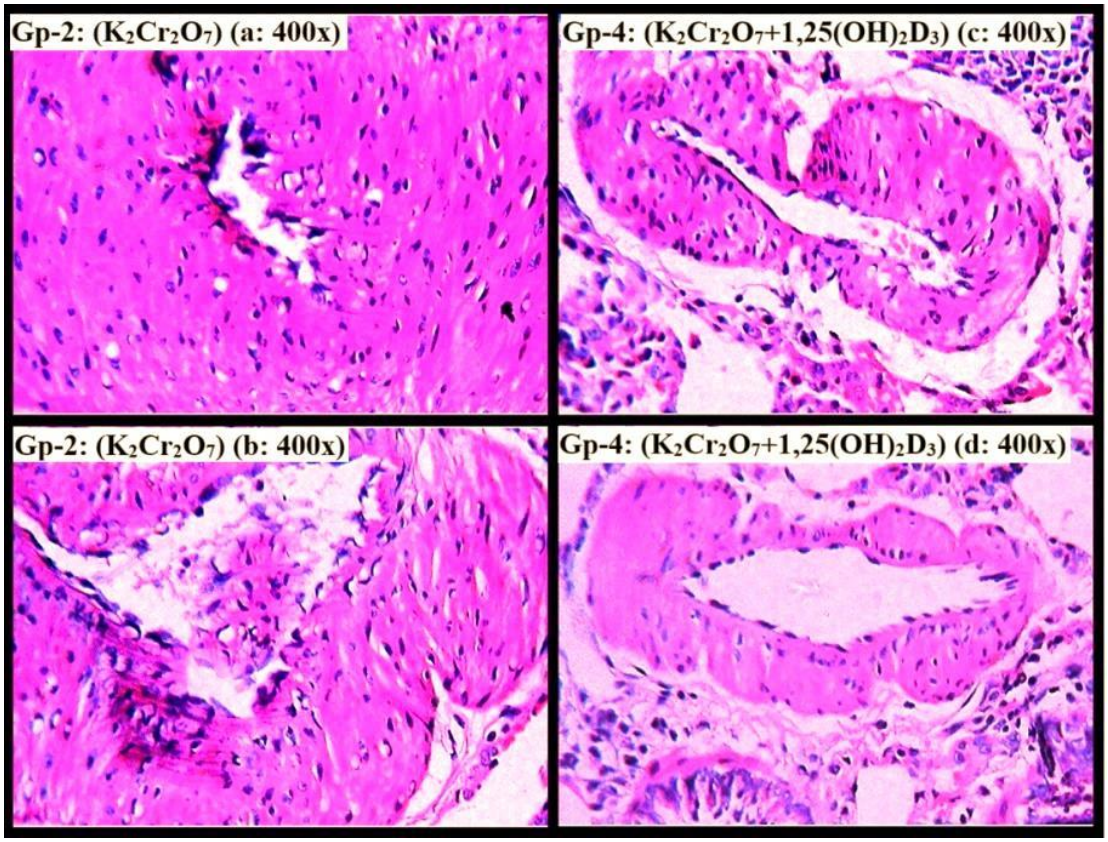


Fig.5.13.4.7 (Table 5.13.4) showing photomicrographs of lung rats (H&E stain under 400x). Gp-2 ($K_2Cr_2O_7$): Vascular intimal thickening, medial hypertrophy with hyalinization, congestion and perivascular inflammation. Gp-4 ($K_2Cr_2O_7$ + 1,25$(OH)_2D_3$): with mild changes.

Fig.5.13.4.8 showing photomicrographs of lung (H&E stain under 400x). Gp-2 & Gp-4.

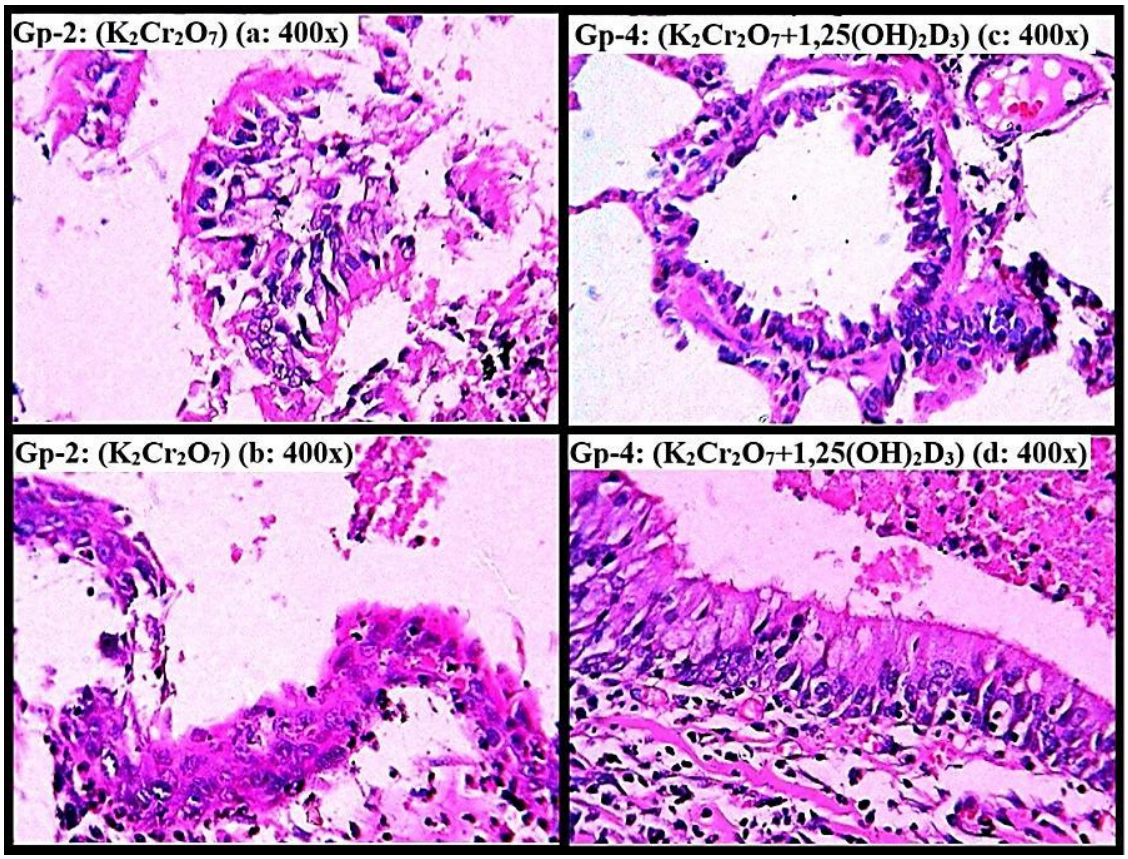


Fig.5.13.4.8 (Table 5.13.4) showing photomicrographs of lung rats (H&E stain under 400x). Gp-2 ($K_2Cr_2O_7$): Bronchiolar thickening, metaplastic chages and gobbler cell hyperplasia and peribronciolar inflammation. Gp-4 ($K_2Cr_2O_7$ + 1,25$(OH)_2D_3$): with mild changes.

Fig.5.13.5 showing gross photographs of lungs of Gp-2 & Gp-4.

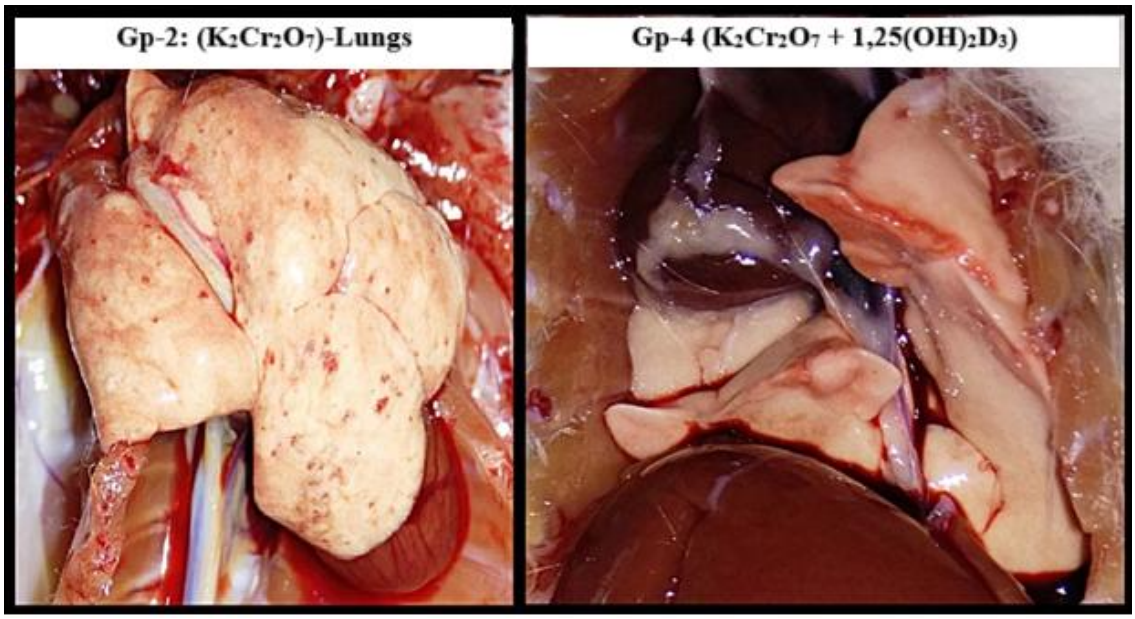


Fig.5.13.5 (Table 5.13.4) showing photographs of lung of rats. Gp-2 ($K_2Cr_2O_7$): Increased size and weight, edematous with petechial hemorrhages. Gp-4 ($K_2Cr_2O_7$ + 1,25$(OH)_2D_3$): with mild changes.

5.14. NORMALIZED WALL INDEX

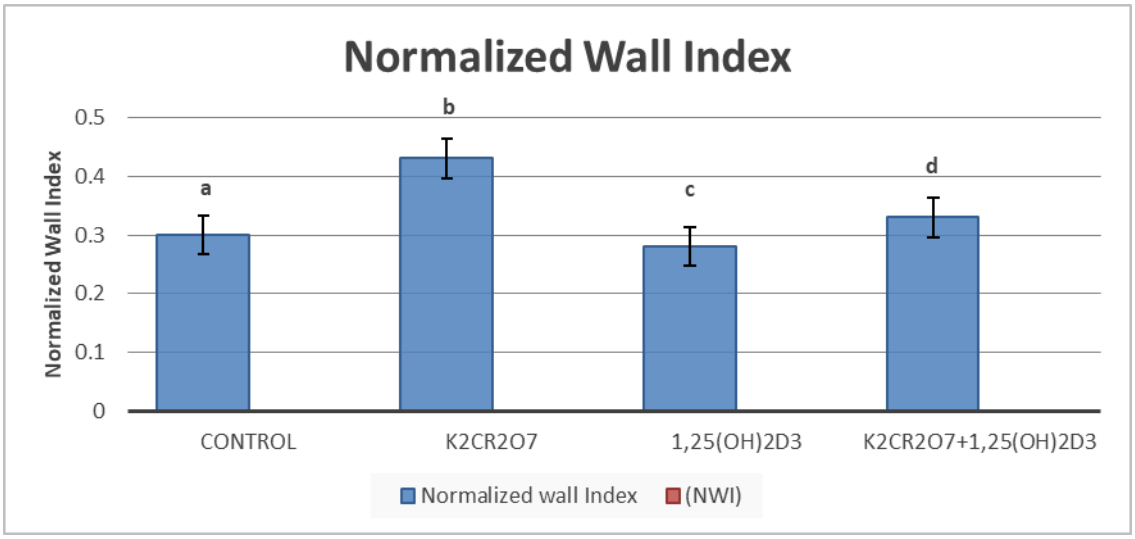
Table 5.14 shows comparative values of NWI of experimental groups. Group-2 ($K_2Cr_2O_7$) had a significantly increased NWI of 0.43, in contrast to Group-1 (control) and Group-3 ($1,25(OH)_2D_3$) which were 0.30 and 0.28 respectively. Even though Group-4 ($K_2Cr_2O7+1,25(OH)_2D_3$) had an increased NWI of 0.33, but it was still significantly lower than NWI of 0.43 of Group-2 ($K_2Cr_2O_7$).

Table 5.14 shows NWI of experimental groups (n=6).

Groups	NWI
Group-1 (Control)	0.30 ± 0.02 [a]
Group-2 ($K_2Cr_2O_7$)	0.43 ± 0.01 [b]
Group-3 ($1,25(OH)_2D_3$)	0.28 ± 0.03 [a]
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	0.33 ± 0.01 [c]
F-value	70.933
p-value	0.000*

Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Figure 5.14 shows NWI of experimental groups.



Each value is Mean±SD of six observations in each group. One way ANOVA followed by Post Hoc Tukey's multiple comparison tests in each row, values with different superscripts (a, b, c & d) indicate statistically significant difference between the groups. *p <0.05 was considered statistically significant.

Chapter-6

Discussion

6.0 DISCUSSION

6.1 GRAVIMETRY

6.1.1 Change in body weight percentage

Gravimetry was measured to evaluate the effects of $1,25(OH)_2D_3$) on $K_2Cr_2O_7$-exposed rats. In our study, $K_2Cr_2O_7$-exposure had a notable weight loss aligning with previous research on Cr(VI) causing significant weight loss in both humans[266] and animals.[267]

Our findings show that Cr(VI) negatively impacts the body weight gain of rats by reducing water and food intake, disrupting glucose and lipid homeostasis resulting in loss of appetite, gastritis and malabsorption, consequently causing low calorie intake and weight loss. Enhanced gluconeogenesis from the body proteins culminates in tissue breakdown and body wasting.[268] In addition, increased sympathetic activity reduces food intake.[269] Cr(VI) irritates the gastrointestinal tract, causing anorexia, nausea, vomiting, diarrhoea, leading to weight loss. The severity of weight loss depends on the exposure level and duration. Chronic exposure causes gradual weight loss, while acute exposure results in rapid and severe effects. Weight loss may be reversible after stopping chromium exposure.[1]

In our study supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats led a significant weight gain, which may be ascribed to regulation of insulin, PTH and calcium improving skeletal mass. Additionally, it helped mitigate oxidative stress, inflammation, reverse metabolic disruptions from Cr(VI) toxicity, and support better weight management.[270]

6.1.2 Organo-somatic index

Organo-somatic index of the heart and lungs was measured to evaluate the effects of $1,25(OH)_2D_3$) on cardiorespiratory remodeling of $K_2Cr_2O_7$-exposed rats. In

our study, $K_2Cr_2O_7$-exposure increased organo-somatic index of both heart and lungs, regardless of reduction in body weight percentage.

Chromium-induced cardiotoxicity results in oxidative stress, imbalance in calcium levels, and interference with heart function, ultimately leading to ventricular dysfunction due to inflammation mediated by IL-1β/NF-kB.[271] Similar observations were found in previous studies.[272,273] $K_2Cr_2O_7$ can result in sympathetic overdriven hypertension causing ventricular cardiac hypertrophy. This over-activity also promotes the growth of ventricular smooth muscle cells, leading to an increase in cardiac mass as shown in histopathology.

In the same way, Cr(VI) is a toxic substance that affects the respiratory system and is linked to cancer.[66] Additionally, Cr(VI) is genotoxic that causes various types of damage at the cellular and molecular levels, such as Cr-DNA adducts, DNA breaks, chromosomal abnormalities and apoptosis[122] The initial reaction to chromate is a sudden inflammatory reaction which progresses to a widespread lung inflammation, with an increase in IL-6 and GRO-α levels seen 2 hours after exposure.[132] Neutrophils and lymphocytes are recruited at 6 and 8 hours and alveolar inflammation occurs at 48 hours after exposure to Cr(VI) causing hypersensitivity reactions such as allergic/asthma-like responses.[17]

Continuous Cr(VI)-exposure amplify the inflammatory reaction by producing cytokines that generate ROS and NOS, leading to more DNA damage in cells and causing pneumonitis and initiating carcinogenesis events involving hexavalent chromium.[124] Additionally, the damage and inflammation caused by Cr(VI) promotes survival signaling and the growth of epithelial cells.[274]

In our study, supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-induced rats reduced organo-somatic index of the heart and lungs significantly. Recent studies have

demonstrated that $1,25(OH)_2D_3$ ameliorates pressure induced cardiac hypertrophy and its remodelling by inhibiting multiple signalling pathways.[275] $1,25(OH)_2D_3$ exerts its protective effect in respiratory diseases by inhibiting PARP-1, the MAPK, the Wnt/ß-catenin, ROS, cytokines , PG's and the NOS-signaling systems.[276]

6.2 CARDIAC ELECTROPHYSIOLOGY

Electrophysiological parameters were analyzed to evaluate the effects of $1,25(OH)_2D_3$) on cardiovascular remodeling of $K_2Cr_2O_7$-exposed rats.

6.2.1 Blood Pressure

In our study, $K_2Cr_2O_7$-exposure increased the Mean Arterial Pressure significantly which is consistent with previous findings. [8, 273] The etiopathogenesis involves increased sympathetic drive, reactive oxygen species, disrupted oxidant-antioxidant balance, and excessive cyclooxygenase 2 (COX2) dependent endothelium contracting factors (EDCFs) by endothelial cells.[272, 273, 277]

Nitric oxide is a vasodilator and crucial in the maintenance of arterial blood pressure by counteracting the vasoconstrictor effects of endothelin, angiotensin II and renal sympathetic nerve activity. High NO levels in $K_2Cr_2O_7$-exposed group led to increased MAP values, potentially due to reduced bioavailability causing endothelial dysfunction.[278] $K_2Cr_2O_7$-exposure increased MAP and renal vascular resistance while lowering renal blood flow, attributed to decreased NO availability and elevated 3-nitrotyrosine levels. Chromium can induce vasoconstriction by raising intracellular calcium and vascular endothelial growth factor levels, increasing arterial tone and blood pressure.[279,280]

In our study, supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-induced rats lowered blood pressure significantly by reducing oxidative stress and suppressing endothelial cell apoptosis by cytokines and tumour necrosis factor-α (TNF-α) and angiotensin II.

[272] Research suggests that $1,25(OH)_2D_3$ can inhibit RAAS activity in both animals and humans.[281] The VDR is present in various vascular tissues, directly influences calcium influx, muscle relaxation and diastolic function.[282,283] $1,25(OH)_2D_3$ may also impact vascular stiffness and tone by reducing calcium influx. The enzyme 1α-hydrolase, involved in converting 25(OH)D to calcitriol,[284] is activated by inflammatory molecules in endothelial and vascular smooth muscle cells.[285-287]

6.2.2 Heart Rate Variability (HRV)

HRV analysis is a clinically approved indicator of cardiac autonomic balance.[288] In our study, $K_2Cr_2O_7$-exposure caused a significant sympatho-vagal imbalance consistent with previous study.[272] The imbalance may be caused by sympathetic over-drive with concomitant under-drive of parasympathetic activity, resulting in increased heart rate. This abnormality may be triggered by chemoreceptor reflex, due to $K_2Cr_2O_7$-induced oxidative stress, inflammatory response, and reduced antioxidants.[277] Besides, $K_2Cr_2O_7$ is neurotoxic and inhibits acetyl choline esterase (AChE) and cholinergic signalling transmission in sympathetic and parasympathetic nerves of the autonomic nervous system, by excessive ROS production.[289]

In our study, supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-induced rats improved sympatho-vagal balance significantly, indicating an antioxidative modulation of cardiac autonomic function.[272,277] Studies suggest that $1,25(OH)_2D_3$ crosses the blood brain barrier, regulates the autonomic nervous system, modulates parasympathetic activity, affects adrenal medullary function, and attenuates autonomic dysfunction.[290,291] Vitamin D's relationship with AIx may be mediated through plasma metanephrine, baroreflex sensitivity and heart rate variability.[291,292]

6.3 HEMATOLOGICAL PROFILE

Multiple studies on humans and animals have shown that prolonged exposure to Cr(VI) results in marked hematological changes. In our study, $K_2Cr_2O_7$-exposed rats had a significant lower total erythrocyte counts (TEC)- Hb concentration, RBC count, PCV, MCV, MCH & MCHC, correlating with previous research[3,293] and may be due to various etiopathogenetic factors.

Since Cr(VI) has similar size and charge with that of iron, it competes with Fe^{+3} for binding sites on transferrin affecting, iron metabolism and potentially leading to insulin resistance due to compromised nutrient bioavailability.[293]

$K_2Cr_2O_7$-induced oxidative stress disrupts oxidative phosphorylation in mitochondria of erythroid progenitor cells in the bone marrow. It causes inhibition of key enzymes like δ-aminolevulinic acid dehydratase (ALA-D), succinyl-CoA and glycine pool.[146] Over and above that ascorbic acid, glutathione and cysteine which are consumed excessively for Cr(VI) reduction, makes them unavailable for heme synthesis, producing micronucleated erythrocytes and thereby causing microcytic hypochromic anemia.[294]

Cr(VI) in the erythrocytes forms Cr-Hgb complexes[295, 296] inhibiting enzymes for haem biosynthesis and causing hemoglobin oxidation and membrane peroxidation.[297] This promotes oxidation of GSH, inhibition of glutathione reductase and methemoglobin reductase and transforms normocytes into echinocytes making their membrane fragile leading to eryptosis (the programmed death of erythrocytes) and resulting in hemolytic anemia and decrease in erythrocyte count.[298]

High doses or prolonged exposure to chromium can inhibit EPO production, affecting kidney function and complicating EPO dynamics through modulatory effects

on cellular signaling pathways.[299] The study reveals that chromium not only disrupts hemoglobin synthesis and dyserythtropoiesis, but also contributes to hemolysis.

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats significantly increased Hb concentration, TEC and its indices. This was due to the activation of erythropoietin and Erythropoietin receptor (EpoR) expression, which in turn induced antiapoptotic signals, enhanced erythropoiesis, and increased hemoglobin, red blood cell count, and indices.[300]

In our study, $K_2Cr_2O_7$-exposed rats showed a significant increase in total leucocyte counts (TLC), similar to previous research.[301] However, TLC in rats exposed to Cr(VI) compounds showed mixed results, with 14.4% showing leucocytosis and 19.6% showing leucopenia.[1] Cr(VI)-exposure causes oxidative stress, impaired leucocytic function, inflammatory responses, and cell turnover alterations, leading to leucocytosis. It also enhances immune pathways and increases the production of cytokines and chemokines that recruit leucocytes, further elevating leucocyte counts.[302] Studies show that Cr(VI) has myelosuppressive effects, reducing blood cell counts and causing rebound leucocytosis, which could be considered chromium toxicity indicators in individuals exposed to chromium-related compounds.[1]

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats significantly decreased TLC, which is a key inflammatory response. This is consistent with previous research that found that $1,25(OH)_2D_3$ supplementation inhibited ROS formation and reduced TLC. It also down-regulated pro-inflammatory signaling pathways such as Toll-like receptor (TLR4), NF-κB, and TNF-α and attenuated inflammation in a dose-dependent manner.[303, 304] $1,25(OH)_2D_3$ is a potent anti-oxidant and anti-inflammatory agent[188,189] that suppresses adhesion molecules (P-selectin, β1- and β2-integrin),[305] inflammatory cytokines (IL-6 and TNF-α),[306] free

radical production and endotoxemia.[307] Besides, it enhances the antioxidative pool (GSH, GPx, CAT and SOD), anti-inflammatory cytokine (IL-10) [194] and restores cellular Ca^{2+}-related molecules.[308]

In our study, $K_2Cr_2O_7$-exposed rats showed a significant decrease in platelet count, which is consistent with previous research.[3] Cr(VI)-induced thrombocytopenia involves complex mechanisms including suppressing bone marrow activity, reducing megakaryocyte proliferation and maturation,[309] depleting platelets in hemolytic anemia,[306] ROS-induced DNA damage platelet apoptosis, and increased consumption or destruction of platelets through disseminated intravascular coagulation or hypersplenism, leading to progressive thrombocytopenia.[310]

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats showed significant increase in platelet count. This might have been achieved by activation of VDR present on megakaryocytes, immunomodulation, calcium homeostasis, regulation of bone marrow microenvironment and bone marrow remodeling, for the differentiation and maturation of megakaryocytes by interaction with thrombopoietin.[311]

6.4 GLUCOSE HOMEOSTASIS

In our study, $K_2Cr_2O_7$-exposed group showed a significant increase in fasting blood glucose level on day- 7, 14 & 21 progressively, increased OGTT levels from 0.0hr to till 2.0hrs than the baseline value, an increase in fasting glucose level with significant decrease of plasma insulin level, significant increase in insulinogenic index and significant decrease in liver glycogen. These findings clearly demonstrate $K_2Cr_2O_7$ impairs glucose homeostasis and Cr(VI) is hyperglycemic. Our results align with prior research.[225, 312] Accumulation of $K_2Cr_2O_7$ in vital organs disrupts glucose metabolism pathways through enzyme and hormone alterations, increasing reactive oxygen species

and impairing antioxidant defenses. This oxidative stress damages islet cells, impacting insulin production and sensitivity, altering the immune system response.[312, 313]

According to the pathophysiology, our research has shown that the decrease in liver glycogen induced by $K_2Cr_2O_7$ could be due to its breakdown through glycogenolysis, leading to an increase in serum glucose levels.[314] This sets in metabolic stress[315] advanced with activation of hepatic liver lactate dehydrogenase (LDH) or pyruvate dehydrogenase (PDH) resulting in depletion of liver glycogen concentration.[312,316,317]

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats showed a significant decrease in fasting blood glucose level from day-14 onwards, improved OGTT levels from 0.0hr to till 2.0hrs remaining within the baseline value, decrease in fasting glucose level with significant increase of plasma insulin level, significant decrease in insulinogenic index and significant increase in liver glycogen. These results indicate that supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats can significantly improve duration dependent hyperglycemia.[318]

$1,25(OH)_2D_3$ deficiency can cause insulin insufficiency due to increased pro-inflammatory cytokines, pancreatic beta cells dysfunction or reduced glucose utilization in peripheral tissues. Studies link $1,25(OH)_2D_3$ to improved insulin synthesis from pancreatic β-cell and insulin sensitivity, with resultant improved glucose homeostasis.[270] Our study found that increase in liver glycogen with supplementation of $1,25(OH)_2D_3$, seem to have improved partially suggesting potential liver protection from $K_2Cr_2O_7$ exposure.[312]

6.5 SERUM LIPID PROFILE

In our study $K_2Cr_2O_7$-exposed rats showed a significant increase in serum TC, TGL, LDL-C and decrease in HDL-C disrupting lipid metabolism. Previous studies

also show that Cr(VI) can raise TC, TGL and LDL-C while lowering HDL-C in rats.[145,319]

Cholesterol homeostasis in the liver is regulated by feedback from the sterols. High levels lead to reduced uptake and synthesis as well as increased bile acid conversion. Low levels activate cholesterogenic enzymes such as 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoAR) and suppression genes of cholesterol metabolism such as cholesterol 7α-hydroxylase (CYP7A1). HMGR gene expression is regulated by sterol regulatory element binding protein (SREBP)-dependent pathways while that of CYP7A1 is by liver X receptor α (LXRα)-dependent pathways.[145,319,320]

$K_2Cr_2O_7$ causes dyslipidaemia by altering gene expression of HMG-CoAR, leading to decreased expression of the LDL receptor gene and increased production of cholesterol absorption. Low lipoprotein lipase activity can also impact triglyceride breakdown, increasing total and LDL cholesterol levels in the bloodstream, with fat either used by tissues or stored in adipose tissue.[145,319] Prior research has demonstrated that Cr(VI)-induced oxidative stress leads to lipid peroxidation via ROS raising serum apolipoproteins while lowering apolipoprotein-A1, by targeting molecules such as GLUT2 (glucose transporter-2; an insulin-independent transmembrane carrier protein expressed in pancreatic β cells and hepatocytes), SREBPs (sterol regulatory element-binding proteins; involved in the metabolism of TGs) and FAS (fat synthase involved in fatty acid synthesis).[321]

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats lowered TC, TGL, LDL-C and raised HDL-C, suggesting improved lipid metabolism, consistent with previous research.[322] Vitamin D regulates lipid metabolism by inhibiting cholesterogenic enzymes like HMG-CoAR, fatty acid synthase and glucose-6-phosphate dehydrogenase. It also impacts calcium, PTH levels

enhancing and protecting against Cr(VI)-induced dyslipidaemia.[270,323]

6.6 SERUM LIVER FUNCTION TESTS

In our study $K_2Cr_2O_7$-exposed rats had a significant increase in serum bilirubin, SGOT/ALT, SGPT/ALP and ALP indicating impaired liver function, matching with previous studies.[324] Various studies have shown that $K_2Cr_2O_7$-exposure leads to hepatocellular injury through chain of events such as oxidative stress, inflammatory responses and mitochondrial dysfunction. This results in leakage of hepatic enzymes AST and ALT causing their increased levels in the blood. Additionally, Cr(VI) disrupts glycolytic pathways, leading to decreased hepatic glycogen levels and further increased liver enzyme levels.[268,311,312,324] $K_2Cr_2O_7$-induced oxidative stress and subsequent cell injury may lead to the recruitment of inflammatory cells and release of cytokines in the liver, contributing to further mitochondrial metabolic stress releasing succinate and isocitrate dehydrogenases.[1]

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats showed a significant decrease in serum bilirubin, SGOT/ALT, SGPT/ALP and ALP. A cross-sectional study found that lower $1,25(OH)_2D_3$ levels were associated with higher levels of liver enzymes such as ALT and AST, suggesting a correlation between vitamin D deficiency and impaired liver function.[325]

In our study $K_2Cr_2O_7$-exposed rats showed a significant decrease in serum proteins and albumin levels, consistent with previous studies,[324] which revealed decrease in synthetic capacity of the liver and increased proteolytic activity due to $K_2Cr_2O_7$-exposure.

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats showed a significant increase in serum proteins and albumin in experimental animals, consistent with a previous study.[326] Supplementation of

$1,25(OH)_2D_3$ is hypothesized to exert its effects on liver health through anti-inflammatory and streamlining metabolic and enzymatic pathways thereby increasing the synthetic activity of the liver in $K_2Cr_2O_7$-exposure.[326]

6.7 SERUM OXIDATIVE STRESS

6.7.1 Serum Malondialdehyde (MDA)

In our study $K_2Cr_2O_7$-exposed rats showed a significant increase in serum Malondialdehyde (MDA), consistent with previous studies which have found similar results.[3, 272, 268] In $K_2Cr_2O_7$-exposure, Cr(VI) is reduced to Cr(III) causing generation of reactive oxygen radicals such as $O_2^{\bullet-}$, H_2O_2, HO• species.[327] ROS trigger oxidative stress which initiates a series of events damaging polyunsaturated fatty acids of the cell membrane, through lipid peroxidation. Simultaneously, unstable lipid hydroperoxides are produced that decompose into reactive aldehydes such as MDA. As the lipid peroxidation progresses, the levels of MDA in the cells increases, that reflects the extent of oxidative damage to cell membranes and lipids.[328]

Cr(VI)-induced higher MDA levels lead to cellular damage, disruption of membrane integrity, enzyme inactivation, altered cellular signaling pathways, dysfunction, cytotoxicity and potential carcinogenesis.[3, 268, 272]

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats decreased serum MDA levels significantly, consistent with previous studies.[329] Vitamin D as an antioxidant, can lower MDA levels and counteract Cr(VI)-induced oxidative stress by scavenging and neutralizing ROS. It boosts antioxidant enzyme activity such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), while blocking pro-oxidant enzymes like NADPH oxidase and lipoxygenase. It also modulates signaling pathways like NF-κB and MAPK, maintains redox balance by adjusting GSH levels and protects cell membranes from peroxidation

by stabilizing membrane lipids. These actions prevent lipid peroxidation and reduce MDA formation.[308]

6.8 TISSUE OXIDATIVE STRESS

6.8.1 Tissue lipid peroxide (LPO)

In our study, $K_2Cr_2O_7$-exposed rats showed a significant increase in tissue lipid peroxide (LPO) of heart aorta and lung tissues.[330] $K_2Cr_2O_7$ can undergo redox cycling and initiate lipid peroxidation by abstracting hydrogen atoms from polyunsaturated fatty acids (PUFAs) in cell membranes enhancing LPO.[331] LPO products act as redox signaling mediators that cause membrane and protein/DNA damage through free radical-independent; enzymatic and non-enzymatic oxidation and iron-mediated lipid peroxidation generating highly reactive lipid alkoxy and peroxy radicals. Many researches demonstrate the connection between rising levels of LPO products and the advancement of diseases related to oxidative stress.[332]

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-rats reduced serum LPO significantly aligning with past research.[333] Vitamin D_3 supplementation can protect from oxidative stress by stabilizing the imbalance between the rate of reactive oxygen species generation and activity of antioxidant enzymes resulting in lowering levels of LPO.[333]

6.9 NITROSATIVE STRESS

6.9.1 Serum Nitric Oxide concentration

In our study, $K_2Cr_2O_7$-exposed rats showed a significant increase in serum NO levels consistent with previous studies.[334] Cr(VI) causes nitrosative stress via intricate interplay of ROS and RNS production. Reduction of Cr(VI) to Cr(III) generates reactive oxygen species (ROS) which cause oxidative stress, alteration of redox state and activation of signaling cascade such as PI3K, NF-κB and MAPKs.[100,199,335] This

enhances nitric oxide synthase (NOS), to produce nitric oxide (NO) from L-arginine. Subsequently, NO reacts with superoxide radicals forming peroxynitrite ($ONOO^-$), a potent RNS that can cause nitration of proteins, lipid peroxidation, mitochondrial dysfunction, DNA damage and apoptosis. Furthermore, Cr(VI) up-regulates inducible NOS (iNOS) amplifying cyclic oxidative / nitrosative cytotoxic stress, disrupting cellular homeostasis linked to chronic diseases including cardiovascular diseases.[271,336]

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats lowered serum NO levels significantly which is similar to a previous study.[337] The relationship between $1,25(OH)_2D_3$ and NO has been thoroughly researched in humans and animals indicating that $1,25(OH)_2D_3$ regulates the NO synthesis and inducible NOS (iNOS) activation predominantly in endothelial cells. Certain researches have reported that $1,25(OH)_2D_3$ directly regulates endothelial NOS and arterial tone.[337] This is achieved through the modulation of pro-inflammatory cytokines via NF-kB pathway resulting in activation of antioxidant enzymes like SOD and GPx and stabilizes cellular homeostasis.[217] The anti-inflammatory effects of $1,25(OH)_2D_3$ block the oxidative stress leading to a reduced synthesis and availability of L arginine necessary for NO production.[338,339]

6.10 SERUM ANTIOXIDANTS

In our study $K_2Cr_2O_7$-exposed rats showed a significant decrease in serum SOD, vitamin- C, D & E levels.

6.10.1 Serum superoxide dismutase

SOD is a crucial enzyme in the antioxidant defense system that catalyzes the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen.[340] Studies indicate that chromium exposure can result in a decrease in SOD activity through several mechanisms, consistent with previous studies.[341] Cr(VI)-exposure increases superoxide ($O_2^{\bullet-}$) disrupting cell signaling pathways. $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ harm the biological macromolecules, leading to reduction in superoxide dismutase (SOD) and catalase (CAT), resulting in DNA damage, lipid peroxidation.[342]

SOD uses trace elements and prosthetic groups to carry out enzymatic detoxification of ROS, making them susceptible Cr(VI)-toxicity, which deactivates metal cofactors (Cu, Zn, or Mn) and oxidizing sulfur-containing amino acids (cysteine, methionine) in SOD, altering structure and function. Additionally, Chromium may impede Nrf2 activation, reducing SOD gene expression and synthesis.[343] Superoxide dismutase is found in almost all the cells in the body which receive oxygen. SOD counteracts the harmful effects of superoxide and safeguards the cells.[100]

6.10.2 Serum vitamin C (Ascorbic Acid)

Vitamin C plays a vital role as the primary antioxidant in reduction of Cr(VI) to Cr(III) and gets oxidized to dehydroascorbic acid during this process. This rapid consumption leads to vitamin C depletion.[344] Studies indicate that chromium exposure can result in a decrease in vitamin C concentration. Cr(VI) also impairs regeneration of ascorbic acid from its oxidized form, further contributing to its depletion. Besides, Cr(VI) may interfere with the sodium-dependent vitamin C transporters (SVCTs), reducing cellular uptake of ascorbic acid.[345]

6.10.3 Serum vitamin D

$1,25(OH)_2D_3$ is essential for cellular health and antioxidant support, while Cr-

exposure can lower $1,25(OH)_2D_3$ levels. Cr interferes with the enzymes of vitamin D synthesis and activation, like enhancing the activity of 24-hydroxylase, an enzyme that catabolizes active vitamin D and suppressing 1α-hydroxylase required for conversion of active form of vitamin D in the kidneys. Cr disrupts the vitamin D receptor (VDR), impairing cellular responses to vitamin D. Reduced $1,25(OH)_2D_3$ levels are linked to higher NF-κB and IL-6 expression in vascular endothelial cells, inhibiting their function.[346]

6.10.4 Serum vitamin E (α-Tocopherol)

Fat soluble vitamin E is an antioxidant crucial for protecting cell membranes and its estimation is the indicator of circulatory antioxidant status. It reacts with lipid radicals of lipid peroxidation chain reaction and gets oxidized to α-tocopheroxyl radicals[347] The oxidized α-tocopheroxyl radicals can be converted back to their active reduced state by other antioxidants like ascorbate, retinol, or ubiquinol.[348,349]

Studies indicate that Cr-induced ROS can decrease vitamin E by accelerating its consumption and interfering with its regeneration by depleting other antioxidants like vitamin C that play a role in this mechanism. Cr(VI)-induced lipid peroxidation in cell membranes and lipoproteins can lead to the destruction of vitamin E molecules embedded in these structures. Cr(VI)-induced oxidative stress can interfere with the hepatic metabolism of vitamin E, affecting its distribution and storage in the body.[350]

To sum up, the series of processes ultimately leads to a substantial reduction in antioxidant levels (Serum SOD, Vit- C, D & E), resulting imbalance between pro-oxidants and antioxidants that may result in extensive cellular harm and multiple pathological conditions linked to chromium toxicity.

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats increased serum SOD, vitamin- C, D & E levels significantly.

$1,25(OH)_2D_3$ helps counteract chromium toxicity by activating the Nrf2 (Nuclear factor erythroid 2-related factor 2) pathway, which regulates antioxidant response. This leads to increased expression of genes involved in antioxidants production. Various studies suggest, $1,25(OH)_2D_3$ can up-regulate the expression of antioxidant enzymes like superoxide dismutase (SOD) and catalase.[216]

$1,25(OH)_2D_3$ boosts glutathione production, a key cellular antioxidant. This can counteract reactive oxygen species produced during chromium reduction, saving other antioxidants like Vitamin C and E. $1,25(OH)_2D_3$ affects enzyme expression in Vitamin C recycling during oxidative stress. Vitamin D and Vitamin E may team up to protect cell membranes from lipid peroxidation, maintaining Vitamin E levels during chromium-induced oxidative stress. Vitamin D metabolites could also chelate chromium.[100,351,352]

Vitamin D contributes to the overall antioxidant defense system and may help preserve the function and levels of other antioxidants indirectly. Nonetheless, the exhaustion of cellular antioxidant reserves is marked by higher ROS and RNS production; lower free-radical scavengers (Vitamins E and C) and cellular antioxidants (GSH) and inhibition of the activity of enzymes such as GPx, GSH-R, GSH-T, CAT and SOD that contribute to removal of harmful oxygen molecules (ROS) through detoxification.

6.11 SERUM MOLECULAR MARKERS

6.11.1 Serum VEGF gene expression

In our study $K_2Cr_2O_7$-exposed rats showed a significant increase in serum VEGF levels. VEGF is a signaling protein that controls the proliferation, migration and survival of endothelial cells, which is vital in both physiological processes and pathological conditions. Studies have indicated that Cr-exposure, can lead to an up-

regulation of VEGF expression. Cr-induced oxidative state activates hypoxia-inducible factor-1α (HIF-1α) which translocates to the nucleus where it binds to hypoxia-response elements (HRE) facilitating transcriptional up-regulation of VEGF. Furthermore, Cr activates VEGF signaling pathways like the extracellular signal-regulated kinases (ERK) and phosphoinositide 3-kinase (PI3K)/Akt pathways. Activation of these pathways promotes cell survival, proliferation and enhances VEGF expression through the phosphorylation of intracellular VEGF promoting proteins contributing to the robust upregulation of VEGF in Cr-exposed cells. [352, 353]

Elevated levels of VEGF resulting from chromium exposure may enhance vascular permeability and stimulate the growth of tumor-associated blood vessels, thus facilitating metastasis.

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed decreased serum VEGF levels significantly.

$1,25(OH)_2D_3$ binds to the vitamin D receptor (VDR) present in numerous tissues, including endothelial cells.[216] The VD-VDR complex modulates gene expression by interacting with transcriptional co-factors and binding to specific DNA sequences. Vitamin D may down-regulate the expression of VEGF by inhibiting the NF-κB pathways.[199] Vitamin D reduces the transcription of VEGF and other pro-inflammatory cytokines, thus mitigating the angiogenic response stimulated by chromium. Furthermore, vitamin D may enhance the expression of suppressor genes that regulate angiogenesis, contributing to a protective effect against chromium-induced toxicities.[354]

6.12 SERUM CHROMIUM CONCENTRATION

In our study $K_2Cr_2O_7$-exposed rats had a significantly increased serum chromium levels. Serum chromium concentration can be an important biomarker for

assessing exposure to hexavalent chromium (chromium VI). In unexposed individuals, serum chromium levels are <0.5 μg/L in humans. Occupational exposure can result in serum chromium concentrations ranging from 1-10 μg/L or higher, depending on the extent and duration of exposure.[298]

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats showed a significant decrease in serum chromium levels. Some studies have suggested that vitamin D supplementation levels may be associated with lower serum chromium concentrations.[225]

6.13 HISTOPATHOLOGICAL EVALUATIONS OF TISSUES

6.13.1 Chromium-induced histopathologic changes in the heart:

In our study $K_2Cr_2O_7$-exposed rats showed significant histopathologic changes in the cardiac tissue.

Cr(VI)-exposure leads to oxidative stress in the heart, generating ROS that induce apoptosis in cardiomyocytes. Chromium's inflammatory properties cause increase in cardiac enzymes, MDA, IL-1β, and TNF-α levels, and decrease in GSH, CAT, SOD, ATP and levels, promoting inflammation, tissue injury and fibrosis. It also disrupts nitric oxide production, worsening endothelial dysfunction and ischemic conditions. Histopathologic features in myocardial disease include myocyte degeneration, hypertrophy, interstitial fibrosis and inflammatory cell infiltrates causing structural disruptions and impaired cardiac function.[271-273]

In our study, supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-induced rats showed partial improvements in cardiac histopathology which is attributed by various mechanisms. $1,25(OH)_2D_3$ down-regulated pro-inflammatory signaling pathways like TLR4, NF-κB and TNF-α,[303,304] suppressed adhesion molecules like P-selectin, β1- and β2-integrin[305] and inhibited inflammatory cytokines like IL-6,[306] Alongside it

also enhanced antioxidative enzymes and anti-inflammatory cytokine like IL-10[194] resulting in reduced cardiac oxidative stress. $1,25(OH)_2D_3$ modulates sympatho-vagal balance,[272,277] attenuates cardiac autonomic dysfunction[290,291] and heart rate variability,[291,292] and inhibits RAAS activity[281] with net effects of lowering hypertension, amelioration of cardiac hypertrophy and its remodelling.[272,275]

Considerable studies suggest a correlation between vitamin D_3 deficiency and the cardiovascular diseases [201,202] and improved cardiovascular health on supplementation of vitamin D.[205]

6.13.2 Chromium-induced histopathologic changes in coronary arteries

In our study $K_2Cr_2O_7$-exposed rats showed significant histopathologic changes in the coronary arteries. Exposure to chromium can lead to histopathologic changes in coronary arteries through oxidative stress and inflammation, resulting in endothelial dysfunction, vascular remodeling, and atherosclerotic plaque formation, increasing coronary artery disease risk.[272,273,355]

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats showed partial improvements in the histopathology of coronary arteries. $1,25(OH)_2D_3$ inhibits cholesterogenic enzymes like HMGCoAR, FAS, G6PD thereby reducing coronary dyslipidemia, vascular smooth muscle hyperplasia and its remodeling. [270] $1,25(OH)_2D_3$ can also inhibit NADPH enzyme expression in endothelial cells of coronary artery reducing vascular stress.[206] The inflammatory inflammatory molecules in endothelial cells activates $1,25(OH)_2D_3$[285-287] which inturn reduces calcium influx resulting in relaxation of smooth muscle stiffness and lowered diastolic function.[282,283]

6.13.3 Chromium-induced histopathologic changes in aorta

In our study $K_2Cr_2O_7$-exposed rats showed significant histopathologic changes in the aorta. Exposure to chromium leads to aortic wall changes through oxidative stress, inflammation and apoptosis. This includes endothelial and smooth muscle cell degeneration, disrupting the extracellular matrix. These alterations contribute to intimal hyperplasia and vascular remodeling, potentially causing atherosclerosis and cardiovascular diseases. Chromium also increases inflammatory cell infiltration, worsening tissue damage.

Elevated oxidative stress, primarily caused by increased NADPH oxidase activity, leads to enhanced production of ·O2− that promotes H_2O_2 build-up and peroxynitrites while diminishing NO availability. These mechanisms are linked with hypertension, as they cause luminal narrowing of the arteries resulting in higher peripheral resistance and blood pressure. **[272,273,355]**

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats showed partial improvements in the histopathology of aorta.**[272, 273, 356]** Various studies have reported $1,25(OH)_2D_3$ deficiency can cause endothelial dysfunction.**[203,204]** $1,25(OH)_2D_3$ supplementation reduces oxidative stress and suppresses endothelial cell apoptosis by cytokines and tumour necrosis factor-α (TNF-α) and angiotensin II.**[205,272]**

In oxidative stress response, $1,25(OH)_2D_3$ activates MEKs/ERKs/SIRT-1 axis in endothelial cells thereby reducing caspases, superoxides and apoptosis.**[213]** Recent studies reveals that $1,25(OH)_2D_3$ can inhibit advanced glycation end-products (AGEs) buildup in the aortic tissue.**[207]** $1,25(OH)_2D_3$ also impacts calcium and PTH levels enhancing protection against Cr(VI)-induced dyslipidaemia.**[270,323]**

6.13.4 Chromium-induced histopathologic changes in lungs

In our study $K_2Cr_2O_7$-exposed rats showed significant histopathologic changes in the lungs. Cr exposure is associated with a range of histopathologic changes in lung tissue. The initial response includes the activation of alveolar macrophages, which release pro-inflammatory cytokines. This inflammatory response is characterized by the accumulation of immune cells, resulting in bronchial hyperactivity and potentially leading to chronic conditions such as asthma or chronic obstructive pulmonary disease (COPD).[272,273]

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats showed partial improvements in the histopathology of the lungs.[197] $1,25(OH)_2D_3$ decreases oxidative stress in human bronchial epithelial cells (HBEC) [100] by inhibiting PARP-1, MAPK, Wnt/ß-catenin, ROS, cytokines, PG's, matrix metalloproteinase (MMP)[192,193] and the NOS-signaling systems[276] improving bronchial inflammation and pulmonary remodelling.[192,193] $1,25(OH)_2D_3$ protects surfactant by enhancing interleukin (IL)-10 levels [194] and inhibiting phospholipase A2 and B cell proliferation.[195] Studies in humans have revealed $1,25(OH)_2D_3$ can reduce pulmonary exacerbations through VDR present in the HBEC.[100,197]

6.14 NORMALIZED WALL INDEX

In our study $K_2Cr_2O_7$-exposed rats showed significantly increased NWI, consistent with other studies.[272,273] Raised normalised wall index (NWI) of the coronary arteries, in the present study is caused by hyperplasia of vascular smooth muscle cells (VSMCs) induced by $K_2Cr_2O_7$-exposure and hence considered as a marker of arterial remodelling. $K_2Cr_2O_7$-exposure induces production of ROS in cells in the vessel wall (endothelial, smooth muscle and adventitial cells) which activate extracellular signal regulated kinases (ERKs), mitogen-activated protein kinases

(MAPKs), receptor and non-receptor tyrosin kinases, and protein tyrosin phosphatases and transcription factors, such as NFkB and AP-1. Additionally, high NADPH oxidase, superoxide and peroxynitrites and lower NO availability contributing hyperplasia of vascular smooth muscle cells (VSMCs) and resulting in increased vascular wall thickness and NWI.[355] Blood pressure independent increased sympathetic overactivity.[272,273,357] inactivation and non-bioavailability of NO are some of the contributory factors in VSMC proliferation and vascular remodeling.[358]

In our study, simultaneous supplementation of $1,25(OH)_2D_3$ in $K_2Cr_2O_7$-exposed rats showed a significantly reduced NWI. Vitamin D regulates nitric oxide (NO) through a VDR-dependent pathway. eNOS is a member of NOS family of enzymes expressed in endothelium. The ability of NOS to release NO relies on phosphorylation (p-NOS). NO is a potent vasodilator, plays a critical role in the endothelium that causes relaxation of the underlying VSMC. $VitD_3$ up-regulates the expression of VDR dependent AMPK, OPN and eNOS phosphorylation pathways in aorta leading to protection against arterial remodeling.[358,359]

Chapter-7

Summary and Conclusion

7.1 SUMMARY

Heavy metals are natural heterogenous group of elements which have specific weight of more than 5 g/cm^3 and are 5 times denser than water. Studies reveal a link between heavy metal exposure and chronic diseases, including cardiovascular, respiratory, renal diseases, metabolic disorders and cancers. Chromium is one of the essential trace elements essential for cellular functions, but when exposed in excessive amounts can cause toxic effects on the health and well-being of living beings including humans.

Amongst environmental heavy metal pollutants, hexavalent chromium (Cr(VI)) is most widely spread environmental contaminant due to its ever increasing demand and use in various industrial applications especially in stainless steel, metal alloy electroplating of metals, metallurgy and metal alloys preparations. This ever increasing use not only leads to occupational exposure but also resulting in non-occupational exposure through water food and air. Exposure to heavy metal Cr(VI) causes generation of free radicals and induction of oxidative and nitrosative stress with depletion of antioxidants leading to sequels of cell injury. Cr(VI) is a known hematotoxic, immunotoxic, cardiotoxic, pulmotoxic, hepatotoxic, nephrotoxic and carcinogenic agent. $1,25(OH)_2D_3$ is a fat-soluble vitamin and its extensive antioxidant actions have been widely recognised. The role of vitamin D as an anti-oxidant in reducing harmful effects of reactive oxygen and nitrogen species could open up new possibilities in chromium-induced cardiovascular pathophysiology.

In the present research, we aimed to evaluate the possible protective role of antioxidative $1,25(OH)_2D_3$ against heavy metal Cr(VI)-induced pathophysiology in the heart, aorta and lungs of male albino rats with a hypothesis that Cr(VI)-exposure causes cardiovascular and respiratory changes and $1,25(OH)_2D_3$ supplementation may improve cardiovascular and respiratory changes in male albino rats exposed to Chromium(VI). The present experimental study was designed by sourcing twenty-four adult male

Albino Wistar rats (Rattus Norvegicus) which were randomly allocated into four groups; each with six rats (n=6) namely as group-1 (control); group-2 ($K_2Cr_2O_7$, 5.0 mg/kg body weight intraperitoneally on every alternate day), group-3 ($1,25(OH)_2D_3$, 12.5µg/kg/d, orally daily) and group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$). The intervention was carried out for 21 days. The study material consisted of pre and post -interventions. Before initiating the interventions, gravimetry for initial body weight, initial cardiac electrophysiology (cardiovascular autonomic balance), and initial blood sample for glucose homeostasis were collected as pre-intervention parameters, on day-1. During the interventions blood samples were collected for blood glucose estimation on day-7, 14 and 21. Post-intervention final gravimetry, final cardiac electrophysiology (BP, ECG, HR & HRV analysis) and sample collection for blood investigations was carried out on day-22. Subsequently the animals were sacrificed and tissue specimens of heart, aorta, lungs and liver were collected for tissue homogenate preparation and histopathology studies. Blood samples were processed and hematology (CBC), glucose homeostasis (FBG, OGTT, plasma insulin, insulinogenic index liver glycogen), lipid profile (serum- TC, TGL, HDL & LDL), liver function tests (Serum- Bilirubin, SGOT, SGPT, ALP, protein & albumin) serum oxidative stress markers (serum MDA), tissue-oxidative marker in heart, aorta and lungs (tissue LPO), nitrosative stress marker (serum NO), antioxidant markers (serum SOD, vit- C, D & E), vascular marker (serum VEGF), chromium toxicity marker (serum chromium) were estimated. This was followed by sacrifice of the rats for histopathology study of heart, aorta and lungs and NWI of coronary arteries and aorta. The data of results was analyzed group-wise and expressed in terms of M $\pm$ SD, ANOVA test, followed by post-hoc t-test.

After 21 days of respective interventions, it revealed statistically significant impairment in the observations. The Group-2 ($K_2Cr_2O_7$-exposed) had significantly affected impairment of; gravimetry (loss of body weight and increased organosomatic index), cardiac autonomic functions (increased HR, MAP, altered HRV causing sympathetic and decreased parasympathetic causing vasovagal imbalance),

hematologic (reduced Hb, red cell indices, Plt, and increased TLC), glucose homeostasis (Increased FBG, OGTT, decreased plasma insulin, increased insulinogenic index and decreased liver glycogen), lipid profile (decreased TC, TGL, LDL and decreased HDL), LFT (increased serum- bilirubin, SGOT, SGPT, ALP, decreased protein and albumin), serum oxidative stress (increased serum MDA), tissue oxidative stress (increased tissue LPO in heart, aorta and lungs), nitrosative stress (increased serum NO), antioxidative markers (decreased SOD, vitamin- C, D & E), molecular marker (increased serum VEGF), chromium (increased serum chromium), histopathogic changes (morphologic tissue remodeling of heart, aorta and lung tissues) and NWI (increased in coronary arteries and aorta). The results clearly substantiate the chromium-induced generation of ROS and NOS, causing oxidative and nitrosative stress affecting pathophysiologic changes in cardiovascular and respiratory systems.

The group-4 (K_2Cr_2O7+$1,25(OH)_2D_3$) rats showed significant improvements in; gravimetry, cardiac autonomic functions, hematologic, glucose homeostasis, lipid profile, LFT, serum oxidative stress, tissue oxidative stress, nitrosative stress, antioxidative markers, molecular marker, chromium, NWI and improved histopathologic changes. Whereas there was no significant change in group-1 (control) and group-3 ($1,25(OH)_2D_3$) rats.

The results corroborate the effects of antioxidant properties of vitamin D, which was able to ameliorate the chromium-induced toxicity and can possibly be used as an add-on antioxidant supplementation against chromium toxicity related pathophysiology.

7.2 CONCLUSION

Present study shows that chromium-exposure causes enhanced generation of ROS and RNS inducing oxidative, nitrosative and intermediary metabolic stress and depletion of enzymatic as well as nonenzymatic anti-oxidants resulting in sequel of imbalanced vasovagal activity, hypertension, cardiovascular and pulmonary remodelling. Results also reveal ameliorating effects of vitamin D may possibly reduce Cr-induced cardiovascular and pulmonary pathophysiology.

7.3 CLINICAL IMPLICATIONS

The outcome of this study may have clinical value in humans and deserves further exploration.

7.4 LIMITATIONS

1. Chromium exposure was of shorter duration (sub-chronic). The long term adverse effects have not been studied.
2. Effects in females have not been evaluated.

7.5 FUTURE PERSPECTIVES

1. Assessment of interactions between Cr(VI), vitamin D and VDR can be done by molecular studies such as DNA sequencing using real-time PCR for evaluation of genetic interaction and patterns of DNA sequencing.
2. More studies should be conducted to explore the role of vitamin D in non-heavy metal metabolic induced cardiovascular and respiratory pathophysiology.

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Annexures

Annexure-I

Plagiarism verification certificate

1. Name of the student:		Dr. Saeed M. Yendigeri
2. Reg. No	:	14PHD009
3. Title of the thesis	:	Effect of 1,25-$(OH)_2D_3$ on pathophysiology of heart, aorta and lungs in male albino rats exposed to chromium(VI).
4. Department	:	Pathology.
5. Name of the Guide	;	Dr. Surekha U. Arakeri
6. Designation	:	Professor Department of Pathology
7. Name of the Co-Guide:		Dr. Kusal K. Das
8. Designation	:	Professor Laboratory of Vascular Physiology and Medicine, Department of Physiology.

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

Software used	:	i-authenticate.
Date	:	24-10-2024
Similarity index (%)	:	5%
Total words count	:	35711

The report is attached for the review by the student and Guide. The plagiarism report of the above thesis has been reviewed by the undersigned. The similarity index is below the accepted norms.The thesis may be considered for submission to the university. The software report is attached.

Co-Guide	Guide	Student
Dr. Kusal K. Das.	**Dr. Surekha U. Arakeri.**	**Dr. Saeed M. Yendigeri**
Distiguished Professor	Professor	Reg. No:14PHD009
Laboratory of Vascular Physiology and Medicine	Department of Pathology	Department of Pathology
Department of Physiology		

Verified by:

Shri B. M. Patil Medical College, Hospital and Research Centre.
BLDE (Deemed to be University). Vijayapura.

Annexure-II

Al Ameen Medical College & Hospital
Owned & Managed by : Al-Ameen Charitable Fund Trust, Bangalore
Athani Road, Bijapur - 586108, Karnataka. Phone : 08352-270067 / 08352-270045, Fax : College : 270184 Hospital : 270251
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Dr. B.S. Patil M.D.
Dean

O/w.No : AMC/IAEC/2014-15/02/dated29/06/15

Institutional Animal Ethical Committee (IAEC)

Date: 29-06-2015

In the continuation of the clearance of Institutional Animal Ethical Committee, the following research project entitled ***"Effect of 1, 25-$(OH)_2$ D_3 on pathophysiology of heart and lungs in male albino rats exposed to chromium VI"***, to be undertaken by ***Dr. Saeed M. Yendigeri,*** in the department of Pathology, Al Ameen Medical College, Vijayapur and provisionally registered for PhD in Pathology in BLDE University; has further been cleared from Institutional Animal Ethical Committee of this institution to carry out PhD research work henceforth.

Chairman
Institutional Animal Ethical Committee (IAEC)
Al Ameen Medical College, Vijayapur

Presentations

Annexure-III

PRESENTATIONS

[1] "Chronic sustained hypoxia and its impact on cerebral ischemia in rodent model".

Participated as Guest Speaker in 3rd Annual Conference of Association of Physiologists of India (ASSOPICON-2016), September 2016 at B.L.D.E. (Deemed to be University), S.B.M.P. Medical College, Vijayapur.

[2] "Effects of supplementation of Vitamin-D on alteration of cardiovascular pathophysiology in male albino rats treated with hexavalent chromium".

Poster presentation in South zonal conference of "Association of Clinical Biochemist of India", at Kasturba Medical College, Manipal, Karnataka in December 2018.

[3] "Effect of 1, 25-Dihydroxy vitamin D3 supplementation on hexavalent chromium (Cr (VI)) induced hepatoxicities in male albino rats".

Oral presentation in XXXId Annual National Conference of the Physiological Society of India (PSI), November 2023 at At B.L.D.E. (Deemed to be University), S.B.M.P. Medical College, Vijayapur.

AWARDS

[1] "Cerebral Ischemic Rodent Model-Hypoxic Exposure".

Paper Presented in Research Day, 06-06-2018, at B.L.D.E. (Deemed to be University), S.B.M.P. Medical College, Vijayapur.

Awarded with first prize in PhD Scholars category.

Publications

Annexure-IV

PUBLICATIONS

[1] Yendigeri SM, Arakeri SU, Das KK. Effect of 1,25-dihydroxy vitamin D ($1,25(OH)_2D_3$) on hexavalent chromium (Cr (VI)) induced alteration of glucose homeostasis in Wistar rats. *JKIMSU*. 2023;12 (3):1-8.

[2] Yendigeri SM, Arakeri SU, Das KK. Effect of 1,25-Dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) supplementation on hexavalent chromium [Cr(VI)] induced hepatoxicities in male albino rats. *Med. J. Dr. D.Y. Patil Vidyapeeth*. 2024;(17):S186-92.

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ORIGINAL ARTICLE

Effect of 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) on hexavalent chromium (Cr (VI)) induced alteration of glucose homeostasis in Wistar rats

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Abstract

Background: 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) is a fat-soluble known antioxidant vitamin to protect cardiovascular health. Hexavalent chromium (Cr (VI)) as a heavy metal has adverse effects on vascular system. *Aim and Objectives*: To evaluate the possible protective effects of $1,25(OH)_2D_3$ supplementation on Cr (VI) induced altered glucose regulation. *Materials and Methods:* Twenty-four adult Wistar male rats were divided into four groups (n=6 in each group) as following: Group-1 (control); Group-2 received $K_2Cr_2O_7$ 5.0 mg/kg body weight intraperitoneally for 10 dosages on every alternate day for 20 days; Group-3 received $1,25(OH)_2D_3$ 12.5µg/kg/d, orally daily till 20 days; and Group-4 received both $K_2Cr_2O_7$ and $1,25(OH)_2D_3$ with dosages as above. At the end of 10th dosage after overnight fasting i.e. (on day 21) blood samples were collected from tail vein of all the rats. Oral Glucose Tolerance Test (OGTT), serum glucose and insulin concentrations were estimated. Insulinogenic index was also calculated. Liver glycogen concentrations were assessed after sacrificing the animals. *Results:* OGTT showed an increase of fasting blood glucose levels in Cr (VI) treated Group-2 rats till at the end of 2.0 hrs. The Cr (VI) treated and simultaneously supplemented with $1,25(OH)_2D_3$ Group-4 rats showed lesser elevation of blood glucose level till 2.0 hrs. A decrease in plasma insulin level and increase in insulinogenic index were also found in Cr (VI) treated Group-2 rats but in case of vitamin D_3 supplemented Group-4 rats, both plasma insulin levels and insulinogenic index were found to be improved remarkably. Liver glycogen concentrations in $K_2Cr_2O_7$ treated rats were also found to be reduced significantly but $1,25(OH)_2D_3$ supplemented Group-4 rats showed improvement in liver glycogen concentrations. *Conclusion:* $1,25(OH)_2D_3$ is found to be beneficial against hexavalent chromium induced alteration of glucose homeostasis.

Keywords: Chromium (VI), Glucose Homeostasis, 1,25-dihydroxy vitamin D_3

Introduction

Chromium is a heavy metal routinely used in alloy industries. Although chromium (III) is an important trace element required for insulin synthesis, Cr (VI) or hexavalent chromium possesses several toxicological symptoms. Industrial uses of chromium are mainly as a hexavalent form. Several studies on humans and animals found that hexavalent chromium has both acute and chronic toxicities, which include even cancer-like diseases [1]. Cr (VI) toxicity depends on the dosages and duration of exposure. Long time exposure with higher dosages of Cr (VI) upregulates apoptosis genes and down regulates antioxidant genes like Superoxide Dismutase (SOD), Glutathione (GSH) etc. [2]. Cr (VI), which is a powerful oxidant that gets converted into Cr (III) generates Reactive Oxygen Species

(ROS) and is capable of damaging intracellular organelles and developing toxicities. Cr (VI) targets multiple physiological systems in the body i.e. hematological and digestive, respiratory system, besides metabolically active organs like liver, kidney and brain, which results in serious toxic manifestations [3]. There are very few reports on Cr (VI) induced hyperglycemia in experimental setup with suggested possibilities of remediation by some phytochemical supplementations due to their antioxidant properties [4]. As Cr (VI) is not stable in the body, it is reduced to relatively nontoxic Cr (III) in the presence of antioxidants [5]. Normally reduction of Cr (VI) is the process of detoxification which may occur inside or outside the cell. The detoxification process of Cr (VI) within the cell is hazardous as it damages the cell organelles and DNA, whereas detoxification outside of the cell by the reduction of Cr (VI) is relatively less toxic [6].

1,25-Dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) is synthesized indigenously in the body and plays an antioxidant defense against oxidative stress in the body. Vitamin D_3 ($1,25,(OH)_2CC$), which is the biologically active form of vitamin D_3 is synthesized in the kidney and is found to be a protective factor as a possible antioxidant against metabolic diseases, including cardiovascular ailments [7]. Some studies showed the protective action of antioxidants against Cr (VI) induced oxidative stress. There are some reports on Cr (VI) and alteration of glucose metabolism due to oxidative stress but the role of antioxidants like $1,25(OH)_2D_3$ against Cr (VI) toxicities has not been fully understood. Hence the present study assessed the protective role of $1,25(OH)_2D_3$ supplementation against Cr (VI) treated alteration of glucose homeostasis.

Material and Methods

Experimental Animals: Twenty-four adult Wistar strain of male rats (*Rattus novergicus*) of 8-10 weeks old, weighing 180-220 g body weight, were procured from the Central Animal House of Shri B. M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapura. Before experimental protocols, rats were acclimatized for 7 days in laboratory conditions (temperature of $22^0C \pm 2^0C$ and 12 hrs of light-dark cycles). Institutional Animal Ethics Committee clearance was taken.

All the experimental animals were pair-fed with normal laboratory stock diet and water *ad libitum*. 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.

Experimental groups: Animals were divided into four groups with six in each group and given intervention as described in Table 1.

Table 1: Experimental groups and interventions for 21 days

Groups	Number of rats	Intervention
Group-1 (Control)	n=6	Placebo, oral gavage daily
Group-2 ($K_2Cr_2O_7$)	n=6	$K_2Cr_2O_7$ 5.0 mg/kg b.wt., i.p. for 10 dosages on every alternate day [8].
Group-3 ($1,25(OH)_2D_3$)	n=6	$1,25(OH)_2D_3$ 12.5 µg/kg/d, orally daily till 20 days [9].
Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$)	n=6	$K_2Cr_2O_7$ 5.0mg/kg b.wt., i.p. for 10 dosages on every alternate day + $1,25(OH)_2D_3$ 12.5 µg/kg/d, orally daily till 20 days.

b.wt: body weight; i.p: intraperitoneally

Glucose homeostasis analysis

Blood glucose estimation: Blood was collected from the tail vein of all the rats and fasting blood glucose levels were measured by using the oxidase-peroxidase method on day 1, day 7, day 14 and day 21 [10].

Oral Glucose Tolerance Test (OGTT): All the rats of four groups were orally fed with glucose (3.5 g/kg b.wt.) on day 21. Just immediately before glucose administration (0.0 hrs), blood samples were collected from the tail vein of all the rats on every 0.5 hrs intervals till 2.0 hrs. Blood glucose level were immediately measured by using a glucometer (Accu-chek active, Roche diagnostics, Germany)

Plasma insulin and insulinogenic index: Collected blood samples from all the four groups were kept in heparinized microtubes and immediately placed in ice bath for 20 minutes and centrifuged for 7-10 minutes at 4000 rpm for plasma separation. Plasma insulin concentrations were measured at 0 and 30 min (0.5 hr) after glucose administration by ELISA kit (ERINS, Thermo Fischer Scientific, Life technologies). The insulinogenic index was also calculated by using the following formula:

Insulinogenic index = (30 min plasma insulin – fasting plasma insulin) / (30 min plasma glucose – fasting plasma glucose) [11].

Liver glycogen estimation: Animals were sacrificed after blood collection, and liver tissue was isolated from each rat. Liver glycogen was estimated by using a glycogen assay kit (Abnova, Taiwan; catalogue number KA0861 [12].

Statistical analysis

All analysis were done by using SPSS 2 version of software. Mean ± SD of each group was done. One-way ANOVA followed by Post Tukey's multiple comparison tests were also done to find out significant differences between groups ($p < 0.05$).

Results

Hexavalent chromium ($K_2Cr_2O_7$) treatment (5.0 mg/kg b.wt., i.p.) in Group-2 progressively increased fasting blood glucose level on D7, D14 and D21 (Figure 1). Hexavalent chromium treated and simultaneously $1,25(OH)_2D_3$ supplemented (12.5 µg/kg/d, orally) rats in Group-4 showed a decrease in fasting blood glucose level after D14 onwards till D21 (Figure 1).

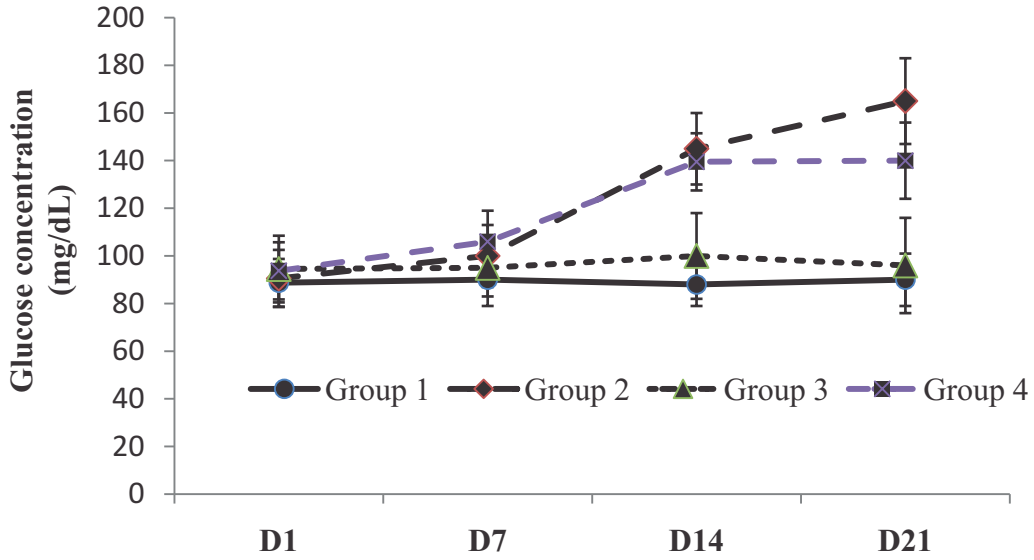

Figure 1: Effect of vitamin D_3 (12.5 µg/kg/d, orally) supplementation on $K_2Cr_2O_7$ (5.0 mg/kg b.wt., i.p. 10 dosages) treated rats on fasting blood glucose concentration (mg/dL) among four groups of rats on day 1 (D1), day 7 (D7), day 14 (D14) and day 21 (D21). Group-1, control; Group-2, $K_2Cr_2O_7$; Group-3, vitamin D3; Group-4, $K_2Cr_2O_7$ + vitamin D_3; n=6 rats in each group.

Table 2 shows a normal OGTT in Group-1 (control) rats i.e. gradual increase of glucose level till 1.0 hr and followed by decrease of blood glucose levels. At 2.0 hr the value became near normal level. OGTT also shows that Group-2 blood glucose levels from 0.0 hr to till 2.0hrs remained higher than baseline value. All the values of Group-2 were also found to be significantly higher than Group-1 from baseline FBS to till 2.0 hr at any given duration during OGTT ($p<0.05$). In Group-4 $1,25(OH)_2D_3$ supplemented $K_2Cr_2O_7$ treated rats showed significant decrease in blood glucose level at every interval till 2.0 hr although it never reached to similar baseline value even at 2.0 hr but as compared to Group-2 ($K_2Cr_2O_7$) all the values of Group-4 at any interval were significantly lower ($p<0.05$).

Table 3 shows fasting plasma glucose, insulin, insulinogenic index and liver glycogen concentrations. Results show an increase of fasting glucose level with significant decrease of plasma insulin level in hexavalent chromium treated rats (Group-2) as compared to controls (Group-1) (($p < 0.05$)). In case of Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$), plasma level of glucose was found to be decreased and plasma level of insulin was found to be increased as compared to Group-2 i.e. hexavalent chromium treated rats ($p<0.05$). Insulinogenic index indicates an increased value in Group-2 ($K_2Cr_2O_7$) rats whereas in case of Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$) it was found to be decreased. Results from Table-3 are also showing a decreased value of liver glycogen in group-2 hexavalent chromium treated rats as compared to Group-1 (control). The liver glycogen concentration in Group-4 ($K_2Cr_2O_7$ + $1,25(OH)_2D_3$) rats shows a significant increase as compared to Group-2 rats ($p<0.05$) although it remained lower as compared to Group-1 (control).

Table 2: $1,25(OH)_2D_3$ (12.5 µg/kg/d, orally) supplementation on OGTT in sub chronic $K_2Cr_2O_7$ (5.0 mg/kg b.wt., i.p.) treated rats on D21 (at the end of 10 dosage)

Treatment groups	0.0 hr	0.5 hr	1.0 hr	1.5 hr	2.0 hr	*p*
Group 1	$89.00 \pm 10.56^{a, x}$	$110.54 \pm 10.56^{b, x}$	$113.76 \pm 10.65^{b, x}$	$100.54 \pm 10.76^{c, x}$	$84.45 \pm 10.43^{a, x}$	< 0.05
Group 2	$30.54 \pm 14.75^{a, y}$	$156.76 \pm 15.43^{b, y}$	$158.76 \pm 12.45^{b, y}$	$170.56 \pm 12.65^{c, y}$	$170.75 \pm 11.24^{c, y}$	< 0.05
Group 3	$78.96 \pm 6.06^{a, x}$	$113.76 \pm 10.65^{b, x}$	$113.75 \pm 15.75^{b, x}$	$107.76 \pm 16.50^{b, x}$	$95.56 \pm 16.34^{c, x}$	< 0.05
Group 4	$112.75 \pm 10./65^{a, z}$	$125.75 \pm 12.50^{b, z}$	$134.56 \pm 10.87^{c, z}$	$120.76 \pm 10.97^{b, z}$	$119..65 \pm 9.56^{b, z}$	< 0.05
p	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	

Group-1, control; Group-2, $K_2Cr_2O_7$; Group-3, $1,25(OH)_2D_3$; Group-4, $K_2Cr_2O_7$ + $1,25(OH)_2D_3$. n=6 rats in each group. D21, day 21. In each row, values with different superscripts (a, b, c) are significantly different from each other ($p < 0.05$). Vertical columns indicate variation of blood glucose level among four different groups at different time interval till 2 hrs. In each column, values with different superscripts (x, y, z) are significantly different from each other ($p<0.05$).

Table 3: $1,25(OH)_2D_3$ supplementation (12.5 µg/kg/d, orally) on fasting plasma glucose, insulin and insulinogenic index and liver glycogen in $K_2Cr_2O_7$ treated rats (5.0 mg/kg b.wt., i.p.) D21 (at the end of 10 dosages)

Treatment	Fasting plasma glucose (mg/dL)	Fasting Plasma Insulin (µg/L)	Insulinogenic index	Liver glycogen (mg/g)
Group-1	87.27 ± 3.85^{a}	1.07 ± 0.17^{a}	0.0034 ± 0.00052^{a}	20.56 ± 1.49^{a}
Group-2	137.65 ± 12.29^{b}	0.54 ± 0.09^{b}	0.0058 ± 0.00064^{b}	14.14 ± 1.18^{b}
Group-3	86.91 ± 3.65^{a}	1.11 ± 0.14^{a}	0.0031 ± 0.00029^{a}	20.93 ± 1.55^{a}
Group-4	113.08 ± 3.42^{c}	0.75 ± 0.22^{c}	0.0052 ± 0.00035^{c}	17.53 ± 2.11^{c}

Group-1, control; Group-2, $K_2Cr_2O_7$; Group-3, $1,25(OH)_2D_3$; Group-4, $K_2Cr_2O_7$ + $1,25(OH)_2D_3$. n=6 rats in each group. D21, day 21. Values with different superscripts are significantly different from each other ($p<0.05$)

Discussion

Results from figure 1 indicate a steady elevation of fasting blood glucose level in hexavalent chromium ($K_2Cr_2O_7$) treated rats from day 7 onwards, and it remained in elevated status even at the end of the experiment (D21). It indicates that hexavalent chromium is a hyperglycemic metal, but simultaneous supplementation of $1,25(OH)_2D_3$ can control hyperglycemia from D14 onwards. The results also reflect heavy metals like $K_2Cr_2O_7$-induced duration-dependent steady rise of blood sugar may be positively ameliorated by $1,25(OH)_2D_3$ supplementation [13].

OGTT on day 21 showed a diabetic glucose tolerance in hexavalent chromium ($K_2Cr_2O_7$) treated rats (Table 2). The results also revealed a much-improved glucose tolerance in case of $1,25(OH)_2D_3$ supplemented rats. Increased serum glucose level with a concomitant decrease of serum insulin level and resultant increased insulinogenic index in Cr (VI) treated rats indicated an impairment of glucose homeostasis (Table 3). $1,25(OH)_2D_3$ supplementation clearly showed a possible amelioratic action of $1,25(OH)_2D_3$ against hexavalent chromium induced hyperglycemia. $1,25(OH)_2D_3$ was found to be linked with increased insulin sensitivity and insulin generation, which help in better glucose homeostasis [14]. $1,25(OH)_2D_3$ deficiency-related insulin deficiencies may be due to increased levels of rise of pro-inflammatory cytokines, impairment of pancreatic beta cell functions, or even reduced glucose absorption in peripheral tissues [15].

Possible damage of pancreatic beta cells by Cr (VI) might have been partially reversed due to $1,25(OH)_2D_3$ supplementation in present study [16-17]. Study also revealed that many vitamins as antioxidants are highly protective against heavy metal induced oxidative stress in metabolically active organs [18]. These findings were further supported by our study on liver glycogen concentration in $1,25(OH)_2D_3$ supplemented Cr (VI) treated rats (group-4) against only hexavalent chromium treated rats. Depletion of liver glycogen by hexavalent chromium may be due to Cr (VI) induced increase in liver glycogenolysis and resultant increase in serum glucose levels [19]. This result is further suggestive of the breakdown of liver metabolism due to Cr (VI) induced metabolic stress [20].

Study also supported the observations on antioxidant supplementation as protective against ROS induced oxidative stress in vascular health [21]. Further, Cr (VI) may alter liver lactate dehydrogenase or pyruvate dehydrogenase activities which result in depletion of liver glycogen concentration [22].

Conclusion

$1,25(OH)_2D_3$ supplementation possibly counteracts this liver breakdown and reduces glycogen depletion in Cr (VI) treated rats. These findings clearly indicate $1,25(OH)_2D_3$ as a possible therapeutic nutrient against Cr (VI) induced alteration of glucose homeostasis.

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Original Article

Effect of 1,25-Dihydroxy Vitamin D_3 ($1,25(OH)_2D_3$) Supplementation on Hexavalent Chromium [Cr(VI)]-Induced Hepatoxicities in Male Albino Rats

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Abstract

Background: Hexavalent chromium [Cr(VI)] is a heavy metal, extensively used in the stainless steel industry. It causes various metabolic oxidative stress and is hepatotoxic. 1,25-Dihydroxy vitamin-D_3 ($1,25(OH)_2D_3$) synthesized in the body is an antioxidant against metabolic oxidative stress. **Objectives:** The present study aimed to assess the possible protective effects of $1,25(OH)_2D_3$ supplementation on Cr(VI) induced hepatotoxicity in albino rats. **Methods:** Twenty-four male adult Wister rats were divided into four groups (n = 6 in each group) as follows: Group-1 (control); group-2 ($K_2Cr_2O_7$, 5.0 mg/kg body weight intraperitoneally for 10 dosages on every alternate day), group-3 ($1,25(OH)_2D_3$, 12.5 µg/kg/d, orally daily till 20 days) and group-4 ($K_2Cr_2O_7$+$1,25(OH)_2D_3$). At the end of the 10th dosage after overnight fasting, i.e., (on day 21) gravimetry was obtained, blood samples were collected for analysis of hematology, liver function tests, serum oxidative stress markers (MDA and SOD), nitrosative stress markers like nitric oxide (NO), antioxidants (ascorbic acid, α-tocopherol, and vitamin D_3) were estimated. Histopathology of the liver was done after sacrificing the animals. **Results:** Cr(VI)-treated rats showed a significant alteration of hematological parameters, liver enzymes, and oxidative and nitrosative stress parameters as compared to their respective controls. Simultaneous supplementation of $1,25(OH)_2D_3$ with Cr(VI) showed significant beneficial effects on rodent hematology, oxidative stress, and antioxidant defence system against hexavalent chromium-induced hepatotoxicities. Histopathology of the liver showed several hepatic damages in Cr(VI)-induced group. The recorded data showed that liver damages in the $1,25(OH)_2D_3$ supplemented group were partially protected hepatic tissues. **Conclusion:** This study showed $1,25(OH)_2D_3$ as a hepatoprotective antioxidant against hexavalent chromium-induced hepatotoxicity.

Keywords: *$1,25(OH)_2D_3$, chromium (VI) [Cr(VI)], hepatotoxicity and oxidative stress*

Introduction

Hexavalent chromium [Cr(VI)] is a heavy metal used in stainless steel industries. It ranks 17th in ATSDR listing as an environmental pollutant. Chromium gets distributed to all organs of the body with the highest concentrations in the liver.[1] Reduction of chromium (VI) to chromium (III) increases the generation of free radicals, which cause toxic manifestations in the liver.[2] Vitamin D plays a vital role in several biological functions in the body. 1,25-Dihydroxy vitaminD_3 ($1,25(OH)_2D_3$) is an active form of vitamin-D_3 and an antioxidant.[3]

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Hence, the present study is designed to assess the protective role of $1,25(OH)_2D_3$ supplementation against Cr(VI)-induced hepatotoxicities in albino rats.

Materials and Methods

Study design: Twenty-four laboratory-bred experimental Wister rats *(Rattus novergicus)* of 8-10 weeks old weighing 180–220 gms body weight were included in the study.

Ethical clearance: Institutional Animal Ethical Committee clearance with approval No: O/w No.AMC/IAEC/2014-15/02 dated 29/06/15 was obtained.

Experimental Animals: The animals were housed in wire-bottomed cages and were maintained for a week in a laboratory at 22 º C ± 2 ºC temperature and 12 hrs (light–dark) cycle for acclimatization. The animals were pair-fed with a normal laboratory diet and water *ad libitum* for the entire study period, according to Committee for Control and Supervision of Experiments on Animals (CPCSEA) guidelines, and Government of India protocols.

Experimental groups: The acclimatized experimental animals were randomly allotted into four groups with six rats in each group and were treated to 10 dosages [Table 1]. The entire experimental protocol was approved by the Institutional Ethical Committee, and utmost care was taken during the experimental procedure, as well as at the time of sacrifice, according to ICMR guidelines.[4]

Gravimetry: The body weight of each rat of all the groups was measured on the day of intervention and the day of sacrifice by using Sartorius electronic balance (Model: Practum 1102-10IN), and the percentage body weight gain was calculated.

Sample collection: At the end of 21 days of intervention, the animals were kept on overnight fasting, and anesthetized, blood samples from the tail were collected and stored in EDTA and plain tube with a clot activator. The samples were kept at room temperature for 45 min and centrifuged at 600×g for 15 min. The serum was separated, protected from light, and stored at −20°C for further biochemical analysis.

Table 1: Dosages of potassium dichromate ($K_2Cr_2O_7$) and vitamin D_3 ($1,25(OH_2)$ D_3) on rats (n=6 in each group) for 20 days

Groups	Treatment	Intervention
Group-1	Control	Placebo, oral gavage daily.
Group-2	$K_2Cr_2O_7$	5.0 mg/kg b.wt., i.p. for 10 dosages on every alternate day.[5]
Group-3	$1,25(OH_2D_3$	12.5µg/kg/d, orally daily till 20 days[6]
Group-4	$K_2Cr_2O_7$+1, $25(OH)_2D_3$	5.0mg/kg b.wt., i.p. for 10 dosages on every alternate days +12.5µg/kg/d, orally daily till 20 days

b.wt. (bodyweight); i.p.(intraperitoneal); K_2Cr_2O (potassium dichromate); $1,25(OH_2D_3$ (vitamin D_3)

Animal sacrifice: After collection of blood samples, rats were painlessly sacrificed as per CPCSEA guidelines between 09.00 AM and 11.00 AM.

Determination of hematological parameters: All the hematological parameters, i.e. hemoglobin (Hb) concentration, total red blood corpuscles (RBC) count, packed cell volume (PCV), red cell indices, total white blood corpuscles (WBC) count, and platelets count were measured by using fully automated hematology analyzer (Sysmax K-4500).[7]

Analysis of liver function tests: Liver function parameters, i.e., serum bilirubin, ALT (SGOT), AST (SGPT), Alk. phosphatase (ALP), total protein, and albumin were measured by using a fully automated biochemical analyzer (Erba EM 200).

Biochemical determination of oxidative stress markers and antioxidant vitamins: Blood samples were centrifuged at 3000 rpm for 15 min to remove the plasma and buffy coat. The product of lipid peroxidation, malondialdehyde (MDA) in serum samples was estimated by the thiobarbituric acid (TBA) method, in which MDA in combination with TBA in hot acidic media forms in a pink-to-red colored complex, the color intensity of which is measured at 530 nm of absorbance and was directly proportional to the concentration of MDA.[8] The concentration of MDA (nmol/g Hb) was calculated using a standard curve obtained from the reaction between varying MDA concentrations. The Total (Cu–Zn and Mn) superoxide dismutase activity was determined according to the method of Misra and Fridovich.[9] The ability of superoxide dismutase to inhibit the auto-oxidation of epinephrine at pH 10.2 has been used as the basis of a convenient and sensitive assay for this enzyme. One unit of SOD activity was defined as the amount of enzyme that inhibited the oxidation of epinephrine by 50%. The activity was expressed as units/mg of protein.

Biochemical determination of nitrosative stress marker: Nitrosative stress marker nitric oxide (NO) in its stable 164 form nitrate in serum reduced to nitrite by cadmium reduction and forms a colored complex with *N*-naphthalene diamine. The color absorbance was measured by a UV-Visible spectrophotometer (Shimadzu, 168 Model: UV1800).[10,11]

Anti-oxidants: Ascorbic acid was estimated in serum by the method of Roe and Koether.[12] Serum

alpha-tocopherol concentrations were estimated by the method of Jargar *et al.*[13] In the protein-free serum samples, alpha-tocopherol was extracted into the xylene layer, which reduces ferric to ferrous ions and forms red colored complex with 2,2′-bipyridyl reagent. The intensity of red colour developed was red at 492 nm against blank by using a microplate reader (Meril EIAQuant, Meril Diagnostics Pvt. Ltd., India).[13]

Histopathology of liver: After the blood collection, animals were sacrificed and liver tissue was dissected and washed in cold saline to remove the excess blood; 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 hematoxylin and eosin (H and E).[14] The stained tissue sections were observed under a photomicroscope and photographed (Olympus CH-20*i* with Olympus digital color camera, Model No. SDC-242).

Statistical analysis: Mean ± SD values were calculated for each group. To determine the significance of intergroup differences, we analyzed each parameter separately. A one-way analysis of variance (ANOVA) followed by a post-hoc'*t*' test was done to determine which of the groups differed among themselves using statistical software (StatPac for Windows, Version 11.0).

Results

Gravimetry: Table 2 shows that the mean ± SD of the initial body weight of all the groups of rats was statistically insignificant. In group-2 (Cr(VI))-exposed rats the final body weight gain is −11.76% as compared to group-I (control), which is gained by 19.11%. While supplementation of $1,25(OH)_2D_3$ alone in group-3 and supplementation of $1,25(OH)_2D_3$ in Cr(VI)-exposed rats (group-4) showed improvement in body weight gain of 14.40 and 8.35%, respectively.

Hematological parameters: Table 3 shows that intra-peritoneal Cr(VI) administration resulted in a significant decrease in Hb concentration, RBC count, and hematocrit value (PCV%) in group-2 rats when compared to untreated control (group-1). Group-4 (Cr(VI) + $1,25(OH)_2D_3$) also showed a significant decrease in Hb concentration, RBC count, and hematocrit value (PCV%) in comparison with group-1, but when compared with group-2 rats a significant increase of all above-mentioned parameters were noticed. No significant alterations of any of those parameters were found in the case of group-3 (only $1,25(OH)_2D_3$ treated) rats when compared with untreated control (group-1) rats. Table 3 also depicts a decrease of platelet count and an increase in WBC count in group-2 rats in comparison to untreated control (group-1). In the case of group-4 rats, a significant decrease in platelet count and increase in WBC count were noticed when compared with group-1 rats, but when it was compared with group-2 rats a significant improvement by increase in platelet and decrease in WBC count were found. No significant changes were observed in any of these parameters in group-3 rats when it was compared with untreated control (group-1).

Liver function tests: Table 4 shows the activity of serum bilirubin total, bilirubin direct, Alanine transaminase (ALT/SGOT), and aspartate aminotransferase (AST/SGPT) and alkaline phosphatase (ALP) in rats after Cr(VI) treatment (group-2) had increased significantly from that of control group-1. In group-4 rats, although the enzyme activity was significantly high in comparison with the untreated control, when compared to Cr(VI) treated group both the activity of AST and ALT were significantly decreased after simultaneous treatment with $1,25(OH)_2D_3$. Table 4 also showed serum total protein and albumin in rats after Cr(VI) treatment (group-2) had decreased significantly from that of control group-1. In group-4 rats, although the levels were significantly low in comparison with the untreated control, when compared to Cr(VI)-treated group, both the levels were significantly increased after simultaneous treatment with $1,25(OH)_2D_3$.

Oxidative stress: Table 5 shows increased serum MDA levels after Cr(VI) treatment in group-2 rats in comparison to group-1 control. Simultaneous supplementation of $1,25(OH)_2D_3$ in group-3 and in

Table 2: Gravimetry (initial body weight, final body weight, percentage weight gain/loss) of the experimental groups

Parameters	Group-1 (*n*=6)	Group-2 (*n*=6)	Group-3 (*n*=6)	Group-4 (*n*=6)
Initial body weight (gms) (Day 1)	$189.11\pm6.43^{a,x}$	$188.23\pm4.20^{a,x}$	$188.30\pm5.59^{a,x}$	$186.46\pm4.39^{a,x}$
Final body weight (gms) (Day 22)	$225.25\pm12.52^{a,y}$	$166.08\pm4.43^{b,y}$	$215.41\pm7.15^{a,y}$	$202.46\pm5.87^{c,y}$
Percentage body weight gain/loss	19.05 ± 3.84^{a}	$(-)\ 11.76\pm2.69^{b}$	14.49 ± 5.49^{a}	8.59 ± 3.08^{c}

Group-1: normal control, Group-2:Cr (VI), Group-3:$1,25(OH)_2D_3$ & Group-4: Cr (VI) +1, $25(OH)_2D_3$; Each value is mean±SD of six observations in each group. In each row, values with different superscripts (a, b, c) indicate statistically significant differences from each other (P<0.05). In each column values with different superscripts (x, y) indicate statistically significant from each other (P>0.05). *Post-hoc t*-test analysis was used to test for differences among the means when analysis of variance (ANOVA) indicated a significant P value (P<0.05). * Final body weight significantly differs from respective initial body weight group (P<0.05) (Paired *t*-test done)

group-1 decreases serum MDA in group-IV rats as compared to group-2 rats. Table 5 shows a significant increase in serum SOD concentration in Cr(VI)-treated rats (group-2) as compared to group-1 control. Simultaneous supplementation of $1,25(OH)_2D_3$ decreases serum SOD in group-4 rats as compared to group-2 rats. Table 5 shows a significant increase in serum nitric oxide concentration (nitric oxide) in Cr(VI)-treated rats (group-2) as compared to their respective controls (group-1). Simultaneous supplementation with $1,25(OH)_2D_3$ (group-4) shows a significant increase as compared to control (group-1), but when compared with Cr(VI) treated group-2, it showed a significant reduction of serum nitric oxide concentration.

Anti-oxidants: Table 6 shows a decrease in serum Vit-C, Vit-D, andVit-E levels after Cr(VI) treatment in group-2 rats in comparison to group-1 control. Simultaneous supplementation of $1,25(OH)_2D_3$ in group-3 and group-1 increases serum Vit-C, Vit-D and Vit-E levels in group-IV rats as compared to group-2 rats.

Histopathology: Table 7 shows the various alterations of liver histopathology in experimental groups as compared to control in detail.

Table 3: Hematological parameters (Hb, RBC, PCV%, MCV, MCH, MCHC, WBC and Platelet counts) of the experimental groups

Parameters	Group-1 (*n*=6)	Group-2 (*n*=6)	Group-3 (*n*=6)	Group-4 (*n*=6)
Hemoglobin (gm/dL)	14.56±1.13[a]	10.63±0.85[b]	14.78±0.97[a]	12.53±0.44[c]
RBC Count (10^6 cells/µL)	5.60±0.41[a]	4.08±0.31[b]	5.61±0.33[a]	4.83±0.17[c]
Hct (%)	43.70±3.41[a]	31.9±2.55[b]	44.35±2.92[a]	37.6±1.32[c]
MCV (fL)	83.4±3.51[a]	74.61±2.75[b]	88.28±3.73[a]	80.4±0.84[a]
MCH (pg)	29.0±2.15[a]	23.41±1.39[b]	30.18±1.83[a]	27.83±1.13[c]
MCHC (gm/dL)	33.21±1.38[a]	28.41±2.55[b]	34.0±1.02[a]	30.78±0.82[c]
WBC (10^3 cells/µL)	6.81±0.64[a]	13.31±2.85[b]	7.15±1.02[c]	9.36±0.90[c]
Platelet (10^3 cells/µL)	3.11±0.46[a]	1.49±0.31[b]	3.12±0.52[a]	2.00±0.22[c]

Group-1: normal control, Group-2: Cr (VI), Group-3: 1, $25(OH)_2D_3$ & Group-4: Cr (VI) + 1, $25(OH)_2D_3$; Each value is mean±SD of six observations in each group. In each row, values with different superscripts (a, b, c) indicate statistically significant differences from each other (P<0.05). *Post-hoc t*-test analysis was used to test for differences among the means when analysis of variance (ANOVA) indicated a significant P value (P<0.05)

Discussion

Though chromium is an essential trace element for normal physiological functioning of our body, causes final body weight loss in rats exposed to Cr(VI). These observations are in line with the findings of other workers that reveal the adverse effect of heavy metals, which may be due to gastritis and malabsorption leading to less calorie consumption; and low protein levels leading to loss of body weight.[15] Cr(VI) induces anemia (decreased RBC count, Hb concentration, and PCV) in rats.

Table 4: Liver function parameters (Bilirubin Total, Bilirubin Direct, ALT (SGOT), AST (SGPT), ALP, Total protein, Albumin) of the experimental groups

Parameters	Group-1 (*n*=6)	Group-2 (*n*=6)	Group-3 (*n*=6)	Group-4 (*n*=6)
Bilirubin Total (mg/dL)	0.87±0.05[a]	1.13±0.05[b]	0.83±0.04[a]	1.02±0.04[c]
Bilirubin Direct (mg/dL)	0.25±0.06[a]	0.79±0.10[b]	0.24±0.09[c]	0.62±0.10[b]
ALT (SGOT) (IU/dL)	24.98±5.61[a]	99.00±11.38[b]	22.61±5.54[a]	58.15±8.35[c]
AST (SGPT) (IU/dL)	85.0±4.52[a]	142.03±5.76[b]	82.06±2.23[a]	119.20±7.73[c]
ALP (IU/dL)	61.21±6.28[a]	157.35±10.47[b]	60.25±6.09[a]	84.36±6.76[c]
Total protein (gm/dL)	10.41±1.24[a]	5.26±0.45[b]	10.51±1.23[a]	7.21±0.61[c]
Albumin (gm/dL)	7.61±0.51[a]	3.50±1.72[b]	7.81±0.70[a]	5.11±0.47[c]

Group-1: normal control, Group-2: Cr (VI), Group-3: 1, $25(OH)_2D_3$ and Group-4: Cr (VI) + 1, $25(OH)_2D_3$; Each value is mean±SD of six observations in each group. In each row, values with different superscripts (a, b, c) indicate statistically significant differences from each other (P<0.05). *Post-hoc t*-test analysis was used to test for differences among the means when analysis of variance (ANOVA) indicated a significant P value (P<0.05)

Table 5: Oxidative and nitrosative stress parameters evaluations parameters (Serum MDA, Serum SOD, Serum Total nitric oxide) of the experimental groups

Parameters	Group-1 (*n*=6)	Group-2 (*n*=6)	Group-3 (*n*=6)	Group-4 (*n*=6)
Serum MDA (µmol/L)	1.52+0.10[a]	3.35+0.49[b]	1.34+0.07[a]	2.11+0.32[c]
Serum SOD (U/mL)	37.93+4.42[a]	19.01+2.08[b]	36.56+3.37[a]	25.60+1.65[c]
Serum Total nitric oxide (µmol/L)	4.58+0.43[a]	14.33+1.87[b]	4.19+0.33[a]	8.56+0.89[c]

Groups: Group-1: normal control, Group-2: Cr (VI), Group-3: 1, $25(OH)_2D_3$& Group-4: Cr (VI) + 1, $25(OH)_2D_3$; Each value is mean±SD of six observations in each group. In each row, values with different superscripts (a, b, c) indicate statistically significant differences from each other (P<0.05). *Post-hoc t*-test analysis was used to test for differences among the means when analysis of variance (ANOVA) indicated a significant P value (P<0.05)

Previous studies have shown that heavy metals may adversely affect hematopoiesis.[16] In our study, a decrease in RBC count, PCV%, and Hb concentration may be due to non-regenerative anemia arising due to chromium-induced direct injury of hematopoietic stem cells.[17] Cr(VI) exposure to rats resulted in the reduction of RBC count and hematocrit value along with a decrease in hemoglobin concentration,[18] which is due to inhibition of its biosynthesis by decreasing the succinyl and glycine pool.[19] Simultaneous treatment with $1,25(OH)_2D_3$ decreased the toxic effect of Cr(VI) by showing increased erythrocyte parameters, which may be due to the stimulation of erythropoiesis in the bone marrow. In this study, a decrease in platelet count occurred may be due to decreased production or increased consumption of them. Simultaneously, in treatment with $1,25(OH)_2D_3$, the total leucocyte count was also found to be lowered, suggesting anti-inflammatory activity of the $1,25(OH)_2D_3$. Previous studies have shown that $1,25(OH)_2D_3$ down-regulates the inflammatory pathway mediated by Toll-like receptor 4, reducing the expression of TLR4, NF-kB, and TNF-a in the liver tissue.[20]

Table 6: Antioxidative parameters evaluations parameters (Serum Vit-C, Serum Vit-D, Serum Vit-E) of the experimental groups

Parameters	Group-1 (*n*=6)	Group-2 (*n*=6)	Group-3 (*n*=6)	Group-4 (*n*=6)
Serum Vit-C (mg/dl)	1.11+0.16[a]	0.61+0.05[b]	1.01+0.06[a]	0.84+0.09[c]
Serum Vit-E (pg/mL)	1.91+0.08[a]	1.48+0.12[b]	1.98+0.05[a]	1.73+0.05[c]
Serum Vit-D (ng/mL)	37.16+3.80[a]	19.60+2.75[b]	70.72+5.30[a]	30.79+2.99[c]

Groups: Group-1: normal control, Group-2: Cr (VI), Group-3: 1, $25(OH)_2D_3$& Group-4: Cr (VI) + 1, $25(OH)_2D_3$; Each value is mean±SD of six observations in each group. In each row, values with different superscripts (a, b, c) indicate statistically significant difference from each other (P<0.05). *Post-hoc t*-test analysis was used to test for differences among the means when analysis of variance (ANOVA) indicated a significant P value (P<0.05)

In this study, increased activity of both SGOT and SGPT after Cr(VI) treatment was observed, which may be due to leakage of enzymes from liver cytosol into the blood stream, indicating the hepatotoxic effect of Cr(VI).[21] Further, the improvement of SGOT and SGPT activity toward control value in the rats treated simultaneously with $1,25(OH)_2D_3$ proved its antioxidant properties against Cr(VI) toxicity. Previous studies have shown that high doses consumption of $1,25(OH)_2D_3$ can improve aspartate aminotransferase (ALT) and aspartate aminotransferase (AST).[22] Increased SGOT and SGPT indicate increased protein catabolism in the liver due to Cr(VI) toxicity. The improvement of hepatocellular enzymatic activity after $1,25(OH)_2D_3$ supplementation indicates its beneficiary role against Cr(VI) induced alteration of protein metabolism.

In the present study, we observed increased MDA concentrations of Cr(VI) treated rats, which is an early indicator of increased oxidative stress due to metal intoxication and has been testified in various reports. This increase in MDA concentration was accompanied by increased Reactive oxygen species (ROS) like superoxide, hydroxyl radical, and hydrogen peroxide formation, which terminates in oxidative stress.[23] Increased activity of SOD successfully appeases the initial converting it into hydroxy radical and hydrogen peroxide to be further reduced to water by catalase. The hydroxy radicals generated lead to lipid peroxidation.[2,24,25] Simultaneous supplemented with $1,25(OH)_2D_3$ was found to be beneficial against Cr(VI)-induced hepatotoxicity.

Table 7: Shows histopathological findings of liver parenchyma

Histopathological findings	Group-1 (Control)	Group-2 (Cr (VI))	Group-3 (Vit-D_3)	Group-4 (Cr (VI) +Vit-D_3)
Hepatic lobular architecture of liver	Maintained	Distorted diffusely in Zone 1(periportal area), Zone 2 (midzonal) area &Zone 3 (centrilobular area)	Maintained	Affected zonally in Zone 3 (centrilobular area)
Central vein dilatation	Not seen	Abnormally dilated	Normal	Not seen
Central vein congestion	Not seen	Abnormally congested	Not seen	Not seen
Hepatocytes	Normal	Diffuse ballooning degeneration involving all the 3 zones	Normal	Focal ballooning degeneration in zone 3.
Fatty change	Not seen	Seen in zone 1 & 2	Not seen	Seen in zone 3
Liver cell necrosis	Not seen	Diffuse (massive) in all the 3 zones	Not seen	Zonal in zone 1
Inflammatory cell infiltration	Absent	Present in zone 1, 2 & 3	Absent	Present in zone 1
Portal triads (Portal vein and duct) congestion	Absent	Present (severe)	Absent	Present (mild)
Portal triad and duct fibrosis	Absent	Present (severe)	Absent	Present (mild)
Hepatocytic regeneration	Absent	Absent	Absent	Present

Zone-1=periportal, zone-2=midzonal and zone-3=centrilobular area

Our present study also reveals an increase in serum NO concentrations in Cr(VI)-treated rats, which may be due to heavy metal-induced hepatotoxicities, which leads to excessive generation of inducible nitric oxide synthase (i-NOS) activity.[26] High levels of nitric oxide react with the superoxide anion (O_2) and form peroxynitrite anion ($ONOO^-$), both of which are potent oxidants and may further trigger lipid peroxidation, inhibit mitochondrial electron transport, and oxidize thiol compounds. Supplementation of $1,25(OH)_2D_3$ may decrease the NO production by possible inhibition of the NF-κB pathway in heavy metal-treated rats.[27]

In the present study, the histopathology [Figure 1a-h] in Cr(VI)-treated rats, the liver parenchyma showed diffuse distortion of hepatic lobular architecture with abnormally dilated and congested central vein, ballooning degeneration, fatty change and liver cell necrosis, inflammatory cell infiltration, and periportal fibrosis. Observed dilation is an adaptation to increasing the flow of blood to tissue for swift filtration and detoxification by the organism. Lymphocyte accumulation has also been reported, with other heavy metals that might mark the beginning of inflammatory liver disease. Chromium intoxication leads to the accumulation of fats along with hepatocyte vacuolation. Focal necrosis has also been reported earlier with Cr(VI) exposure. It indicates the toxic injury is possibly accompanied by ROS and the production of free radical-inducing enzymes and protein synthesis inhibition. The results obtained from liver sections in group-4 (Cr(VI) +$1,25(OH)_2D_3$) showed normal restoration and regeneration of hepatic lobules, which is suggestive of $25(OH)_2D_3$ as an antioxidant and anti-fibrotic agent against Cr(VI) liver damage. Similar reports suggest the anti-inflammatory and anti-fibrotic properties of the vitamin D.[22]

Conclusion

The present study on experimental animals indicates a severe hepatotoxicity by hexavalent chromium [Cr (VI)], which is widely used in various industries. However routine supplementation of Vit-D3 may protect the hepatocellular functions against Cr(VI)-induced toxicities and suggest vitamin D_3 as a therapeutic agent against heavy metal toxicities.

Author contributions statement (CRediT Statement)

Term	Yendigeri SM*[1a, 2]	Arakeri SU[1a]	Das KK[1b]
Conceptualization	√	√	√
Methodology/Study design	√	√	√
Software	√	√	√
Validation	√	√	√
Formal analysis	√	√	√
Investigation	√	√	√
Resources	√	√	√
Data curation	√	√	√
Writing—original draft	√	√	√
Writing—review and editing	√	√	√
Visualization	√	√	√
Supervision		√	√
Project administration		√	√
Funding acquisition	x	x	x

Data availability statement from the authors

The data that support the findings of this study are available with the authors and can be shared by the corresponding author, upon reasonable request.

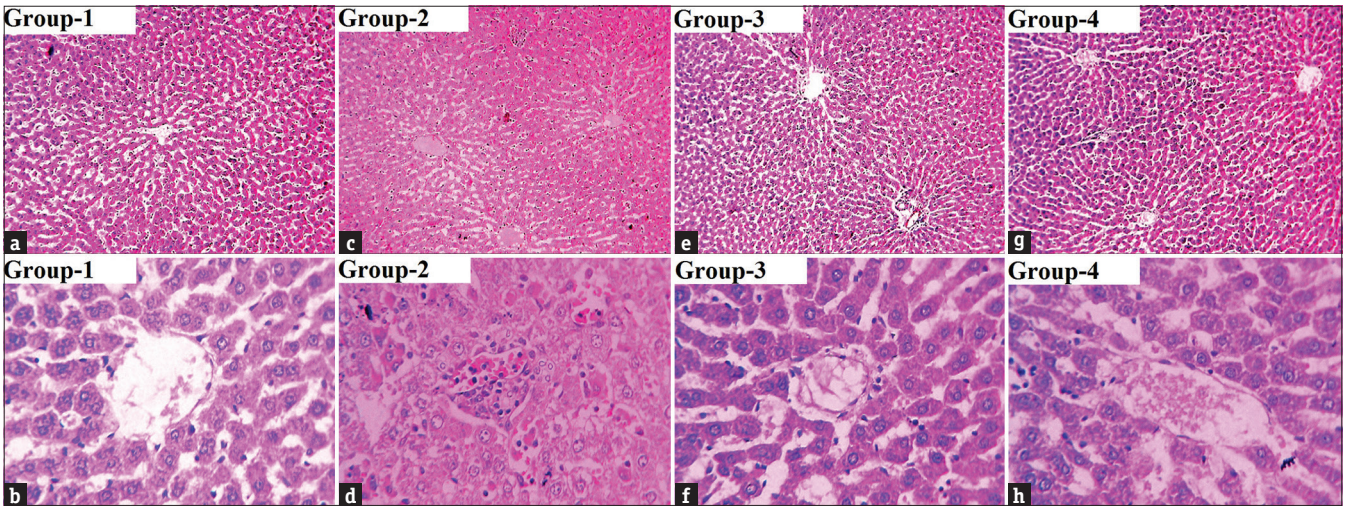

Figure 1: (a-h) Shows the histopathology of the rat liver after Cr(VI) treatment and supplementation of 1,25,(OH)2D3. Group 1 (control) (a: 100 x & b: 400x); Group 2 (Cr VI) (c: 100x & d: 400x), Group 3 (Vit D3) (e: 100x & f: 400x) and Group 4 (Cr VI + Vit D3) (g: 100x & h: 400x)

Abbreviations

1,25(OH)2 D3	:	1,25 Dihydroxy vitamin D3.
ALP	:	Alkaline phosphatase.
ALT/SGOT	:	Alanine transaminase.
AST/SGPT	:	Aspartate aminotransferase.
b.wt.	:	Body weight.
Cr(VI)	:	Chromium VI.
i.p.	:	Intraperitoneally.
MDA	:	Malondialdehyde.
NO	:	Nitric oxide.
ROS	:	Reactive oxygen species.
SOD	:	Super oxide dismutase.

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Conflicts of interest

There are no conflicts of interest.

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