STUDIES ON EFFECTS OF HIGH CHOLESTEROL AND VITAMIN D_3 ON CARDIOVASCULAR SYSTEM

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University
in partial fulfillment of the requirements for the degree of

MASTER OF VETERINARY SCIENCE
in
VETERINARY PATHOLOGY
(Minor Subject: Veterinary Microbiology)

By

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CERTIFICATE – I

This is to certify that the thesis entitled, **"Studies on Effects of High Cholesterol and Vitamin D_3 on Cardiovascular System"** submitted for the degree of **Master of Veterinary Science (M.V.Sc.),** in the subject of **Veterinary Pathology** (Minor subject: **Veterinary Microbiology**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Kothule Viren Ramlal (L-2008-V-27-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE – II

This is to certify that the thesis entitled, **"Studies on Effects of High Cholesterol and Vitamin D_3 on Cardiovascular System"** submitted by **Kothule Viren Ramlal (L-2008-V-27-M)** to the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in the partial fulfillment of the requirements for the degree of **Master of Veterinary Science** in the subject of **Veterinary Pathology** (Minor subject: **Veterinary Microbiology**) has been approved by Student's Advisory Committee after an oral examination on the same, in collaboration with an external examiner.

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Head of the Department **Major Advisor**

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ABSTRACT

The present experiment was conducted to study the clinical signs, haematological, biochemical, enzymehistochemical, tissue mineral concentration, gross and histopathological changes in rabbits fed high cholesterol alone, high vitamin D_3 alone and high cholesterol with susequent high vitamin D_3. The clinical signs observed in vitamin D_3 fed rabbits and cholesterol + vitamin D_3 fed rabbits included anorexia, diarrhoea and cachexia. The mortality was observed on day 68 and 69 in vitamin D_3 fed rabbits and on day 67, 68 and 70 in cholesterol + vitamin D_3 fed rabbits. The haematological changes in cholesterol + vitamin D_3 fed rabbits included significant decrease in hemoglobin count and TLC while there was significant increase in heterophil count. The biochemical changes in cholesterol + vitamin D_3 treated rabbits were increase in AST, ALT, AKP, Ca, P, total protein, globulin, total cholesterol, triglycerides, HDL, LDL, VLDL, LDL:HDL, total cholesterol: HDL, BUN, creatinine while decrease in albumin and albumin:globulin concentrations. The enzymehistochemical studies revealed increase in AKPase, G6PD, LDH, MDH, NADH-diphorase and NADPH-diphorase activity while decrease in SDH activity in heart, aorta and liver. In cholesterol + vitamin D_3 fed rabbits the tisuue mineral concentration showed increase in tissue Ca, Fe and Na and decrease in Mg, Zn, Cu and K in aorta and heart. Grossly postmortem changes in rabbits fed Cholesterol + vitamin D_3 revealed severe plaques and mineralization in cardiac valves and aorta. The coronary arteries were prominent and the liver was fatty. Intestine and stomach showed white deposits of mineralization, while trachea showed hemorrhages and calcification on the luminal surface. There was marked hypertrophy of adrenals and spleen in rabbits of cholesterol + vitamin D_3 as compared to control rabbits. Microscopically aorta showed atheromatous lesions. Few atheromas showed ruptured endothelial lining of the T. intima. Foam cells were evident and there was smooth muscle cell proliferation. Liver showed moderate to severe fatty changes. Adrenocortical nodular hyperplasia, with nodules of the lipid-laden cells loaded within zona fasciculate and zona reticularis and cholesterol crystals in the nodular region were evident. Spleen showed spleenic foam cells and rarefaction of white pulp along with calcification in spleenic capsule and trabaculae. Kidneys showed partial to complete loss of glomeruli and proteinaecious material accumulation within glomerular spaces. Fibrous tissue proliferation and lymphomononuclear infiltration was also evident. Sudan III staining revealed intense

deposits of fat in aorta, liver and heart. Von kossa staining demonstrated Ca within various tissue sections. It was concluded that high intake of cholesterol along with high intake of vitamin D_3 is more injurious to the cardiovascular and other body systems than cholesterol or vitamin D_3 alone.
Key Words: Cardiovascular system, high cholesterol, high vitamin D_3, hypercalcemia, histopathology, biochemistry, enzymehistochemistry, and tissue mineral assays.

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Signature of Major Advisor Signature of the Student

CONTENTS

ABBREVATIONS

%	:	per cent
@	:	at the rate of
µl	:	microliter
µm	:	micrometer
^{o}C	:	degree centigrade
AAS	:	Atomic Absorption Spectrometry
AchE	:	acetylcholinesterase
AKP	:	alkaline phosphatase
ALT	:	alanine aminotransferase
AMI	:	acute myocardial infarction
ANOVA	:	analysis of variance
AST	:	aspartate aminotransferase
BUN	:	blood urea nitrogen
BW	:	body weight
Ca	:	Calcium
CAD	:	coronary artery disease
CHD	:	coronary heart disease
CK	:	creatinine kinase
CT	:	Collectimg tubule
Cu	:	Copper
CVD	:	Cardiovascular disease
DCT	:	Distal Convulated tubule
DLC	:	differential leukocyte count

E	:	Eosinophils
EDTA	:	Ethylene Diamine Tetra Acetate
eNOS	:	endothelial nitric oxide synthase
Fe	:	Iron
Fig	:	figure
G-6-PD	:	Glucose 6 Phosphate dehydrogenase
GADVASU	:	Guru Angad Dev Veterinary and Animal Sciences University
gm	:	gram
H	:	Heterophils
H&E	:	haematoxylin and eosin stain
Hb	:	hemoglobin
HDFP	:	Hypertension Detection and Follow-up Program
HDL-C	:	high-density lipoprotein-cholesterol
hrs	:	hours
IAEC	:	Institutional Animal Ethics Committee
IDL	:	Intermediate Density Lipoprotein
IU	:	International unit
K	:	potassium
L	:	Leukocytes
LD_{50}	:	lethal dose-50
LDH	:	Lactic dehydrogenase
LPM	:	Livestock Production and Management
M	:	Monocytes
MDH	:	malate dehydrogenase
mg	:	milligram

Mg	:	Magnesium
Na	:	Sodium
nm	:	nanometer
NO	:	nitric oxide
P	:	Phosphorus
PCT	:	proximal convulated tubule
ppm	:	parts per million
RBC	:	red blood cell
ROS	:	reactive oxygen species
SDH	:	Succinic dehydrogenase
SMC	:	Smooth Muscle Cell
TLC	:	total leukocyte count
ULs	:	Upper Intake Levels
UV	:	ultraviolet
WBC	:	white blood cells
WHO	:	world health organization
Zn	:	Zinc

CHAPTER-I

INTRODUCTION

Coronary heart disease resulting from progressive atherosclerosis remains the most common cause of morbidity and mortality in humans all over the world (Yusuf *et al* 2001). India is on the verge of cardiovascular epidemics (Grover *et al* 2003 and Okrainec *et al* 2004). The World Health Organization (WHO) estimates that 60 per cent of the world's cardiac patients will be Indian by 2010 (Gaziano *et al* 2006).

Atherosclerosis/ Arteriosclerosis is a disease of large and medium sized arteries, characterized by endothelial dysfunction, vascular inflammation and accumulation of lipids (mostly cholesterol), blood components, and calcium deposits in the arteries. The earliest detectable changes in vascular diseases are the abnormalities of the endothelium resulting in loss of the endothelium's normal homeostatic functions that normally act to inhibit disease-related processes such as inflammation and thrombosis (Channon and Guzik 2002). Reactive oxygen species (ROS), especially superoxide (O2•-) have been implicated in the pathogenesis of vascular lesion formation in atherosclerosis (Prasad and Kalra 1993, Prasad *et al* 1994 and Steinberg 1992).

Cholesterol (about 80%) is produced in the liver and is important component in the cell membranes, production of hormones, vitamin D_3 and the bile acids. Although multiple risk factors play a significant role in the pathogenesis of atherosclerosis and cardiovascular disease (CVD), dyslipidemia remains a major determining factor for these pathologies (Cavallini *et al* 2009). Thus, factors that lead to increased serum cholesterol would seem to accelerate the development of atherosclerosis (Solberg and Strong 1983). When low-density lipoproteins (LDL) levels are too high, fatty deposits or plaque can start to build up on the walls of the arteries, decreasing the amount of blood that can flow through and thus posing a greater risk of heart attack, stroke, arteriosclerosis, or coronary heart disease.

Vitamin D_3 is a fat-soluble vitamin formed from a reaction between the Sun's ultraviolet-B (UVB) rays and a cholesterol-like steroid in the skin called 7-dehydrocholesterol. It is normally obtained from two sources: sunlight (UVB rays) and in the diet. Vitamin D_3 plays a key role in regulating levels of calcium and phosphorus and control the release of parathyroid hormone in the body (Collins and Norman 2001).

Although not proven causative, studies have linked low vitamin D levels to cardiovascular disease (CVD). Increasing research links vitamin D deficiency to cardiovascular disease, heart failure, elevated VLDL triglycerides, hypertension, and CVD mortality (www.news-medical.net/health/Vitamin-D-Overdose.aspx). The issue of vitamin D in heart health has not yet been settled. Moreover, there may be an upper limit after which cardiac benefits decline. Larger doses administered for a short time or periodically (e.g., 50,000 IU/week for 8 weeks) do not cause toxicity. Rather, the excess is stored and used as needed to maintain normal serum 25(OH)D concentrations when vitamin D intakes or sun exposure are limited (Hathcock *et al* 2007). Several nutrition scientists recently challenged the **Upper Intake Levels (ULs)** (2000 IU/adult), first published in 1997 (Hathcock *et al* 2007). They point to newer clinical trials conducted in healthy adults and conclude that the data support a UL as high as 10,000 IU/day. Although vitamin D supplements above recommended levels are given in clinical trials have not shown harm, most trials were not adequately designed to assess harm (Cranney *et al* 2007).

Vitamin D_3 is an antioxidant vitamin and is helpful in prevention of cardiovascular diseases but excess of it has a hazardous effect on cardiovascular system. Hypervitaminosis D_3 has been incriminated in causation of aortic, coronary and cardiac lesions along with plaque formation in left atrium, coronary artery and aortic and mitral valves. Besides, formation of atherosclerotic intimal thickening in coronary arteries accompanied with calcification of elastic and collagen fibers is also reported (Taura *et al* 1978). In human

beings, formation of calcified plaque in blood vessel (s) of heart and other organs is a common phenomenon and proves fatal (Burke *et al* 2001). Thus there is need to study the putative role of vitamin D_3 in formation of these calcified plaques.

Increase in intracellular calcium in atherosclerosis is due to increased plasma membrane calcium permeability. In atherosclerosis, many of the abnormal cellular processes are regulated by calcium (Phair 1988). Calcium channels are involved either primarily or secondarily, in mediating the effect of cholesterol enrichment on Ca^{++} accumulation, which is linearly related to the extent of cellular free cholesterol (Bialecki *et al* 1991).

Abnormal circulating levels of hepatic enzymes are frequently found in subjects displaying hyperlipidemia or obesity or both. Cardiovascular and metabolic features characterizing the plurimetabolic syndrome, including serum uric acid levels, are associated with significant elevation of hepatic enzyme activities (Bruckert *et al* 2002). Decreased level of kidney function is an independent risk factor for all-cause mortality as well as adverse cardiovascular disease (CVD) outcomes including myocardial infarction, stroke, and progression of heart failure. (McCullough *et al* 2000, Dries *et al* 2000, Best *et al* 2002 and Mann *et al* 2001).

Immunological and inflammatory processes have been recognized as possible pathological mechanisms of the initiation and progression of atherosclerosis (Ross 1999 and Hansson 2001). Atherosclerosis involves hypercholesterolemia, low-density lipoprotein oxidation, increased oxidative stress and leukocyte recruitment and infiltration within the atheroma (Ross 1999 and Libby 2002). Cu deficiency affects endothelial function by depressing nitric oxide (NO) synthesis possibly contributing to the development of atherosclerosis. Zn^{++} plays a major role in atherogenesis and acts as an endogenous protective factor against atherosclerosis perhaps by reducing lesion Fe content, intracellular

and extracellular lipids in the intima, connective tissue formation, and smooth muscle proliferation.

Animal models provide a means for studying the underlying mechanism behind the atherosclerotic disease process, as well as a means for studying the effect of interventions, dietary or otherwise (mechanical or chemical or immunological), on the development or regression of disease, while in controlled conditions. The rabbit exhibits hypercholesterolemia within a few days of an administration of a high cholesterol diet, it is very sensitive to the inducement of atheromatic lesions (Yanni 2004). However, studies on adverse effects of feeding of vitamin D_3 and high cholesterol are lacking.

Therefore, the present study was undertaken with the following objectives:

1) To study the clinic-pathology in high cholesterol fed rabbits and subsequently given vitamin D_3 per oral.
2) To study the enzymehistochemistry of aorta in high cholesterol fed rabbits and subsequently given vitamin D_3 per oral.

CHAPTER-II

REVIEW OF LITERATURE

Keeping in view objectives of the present study the review of literature has been summarized under following headings:

2.1 Synthesis and toxicokinetics of vitamin D_3 (cholecalciferol)
2.2 Mechanism of vitamin D_3 toxicity

2.3 LD_{50} in rabbits and other species
2.4 Rabbits in cardiovascular disease studies
2.5 Hematological studies
2.6 Biochemical studies
2.7 Atomic absorption mineral estimation studies
2.8 Histochemical studies
2.9 Histopathological studies
2.10 Effects of vitamin D_3 in atherosclerosis

2.1 Synthesis and toxicokinetics of vitamin D_3 (cholecalciferol)

Smith (1982) reported that the vitamin D was not a single compound but different compounds were reported to have a vitamin D activity. Among them vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol) were important. Ergocalciferol (vitamin D_2) is formed by UV irradiation of ergosterol in plants. In animals cholecalciferol (vitamin D_3) is synthesized in the epidermis under the influence of sunlight from its precursor 7-dehydrocholesterol. The technical procedures to prepare vitamin D compounds are based on irradiation of ergosterol to prepare ergocalciferol and of 7-dehydrocholesterol to form cholecalciferol.

Vitamin D_3 (cholecalciferol) was initially registered in U.S.A. [E.P.A Reg. No. 12455-39] and was first used as rodenticide in 1980's (Marshall 1984) in U.S.A. Cholecalciferol is a solid resin with molecular weight of 384.62 and melting point 84-85^{0}C. It is soluble in most of the organic solvents, slightly to moderately soluble in vegetable oil and virtually insoluble in water (Deluca 1979).

Beasley (1999) reported that the absorption of vitamin D was rapid and complete in the small intestine, especially if the animal's diet was rich in lipids. Bile was essential for adequate absorption of vitamin D. Vitamin D_2 and D_3 are processed to D_2-calcitriol and D_3-calcitriol respectively by the same enzymatic pathways in the body. Vitamin D binding protein transports cholecalciferol from the skin into the blood. Vitamin D_3 from dietary sources is absorbed by facilitated diffusion and becomes bound to alpha-2-globulin in blood.

Cholecalciferol is then enzymatically (calciferol-25-hydroxylase) converted to 25, hydroxy-cholecalciferol (calcifediol, 25-OH-D_3) in the liver. Then 25-OH-D_3 is transported to the kidney where further metabolism (by 25-OH-D_3-1 hydroxylase) to 1,25di-hydroxy-cholecalciferol (calcitriol, 1,25-(OH)2-D_3) occurs. Conversion of 25-OH-D_3 to 1,25-(OH)2-D_3 (calcitriol) is rate-limiting and partially explains the delay in biological activity of dietary vitamin D_3. In the liver 1,25-(OH)2-D_3(calcitriol) can also be converted by 24-hydroxylase to 24,25 di-hydroxy-cholecalciferol [24,25-(OH)2-D_3]. The 24,25-(OH)2-D_3 is less active and is preferentially formed when high serum calcium levels exist. In general cholecalciferol and ergocalciferol parent compounds are lipid soluble and are extensively stored in the adipose, liver and muscle tissue. Vitamin D_3 metabolites are excreted primarily via bile and in faeces with about one-third in urine.

2.2 Mechanism of vitamin D_3 toxicity

Kwatra *et al* (1974) reported an unusual disease in about 90% of sheep at the breeding farm at Mattewara, Ludhiana, India. Fifty-three out of 57 adult Corriedale sheep examined after death showed plaque formation in the aorta and other arteries. Histopathological examination revealed intense calcification of the medial layer and proliferation of intimal connective tissue. In some cases the lung and kidney also showed calcareous deposits. Increase in blood calcium and phosphorus levels were noted. The disease was suspected to be due to ingestion of some poisonous plant, with effects similar to those of *Solanum malacoxylon* which is known to cause *enteque seco* disease in sheep in Argentina.

Stevenson *et al* (1976) reported vitamin D_3 toxicosis in rabbits due to consumption of commercial feed supplements containing excessive vitamin D_3 concentration.

Morrissey *et al* (1977) studied relative toxicity and metabolic effect of cholecalciferol and 25-hydroxycholecalciferol in chicks. 100-fold increase in toxicity was seen when the hydroxylated form of cholecalciferol is fed.

Harrington and Page (1983) studied acute vitamin D_3 toxicosis in horses. They reported extensive mineralization of cardiovascular and other soft tissues with severe clinical signs and clinicopathologic changes of toxicosis like limb stiffness and tachycardia, anorexia, weakness, and recumbency, loss of 29% of body weight, hyperphosphatemia, hypercalcemia, polydipsia and polyuria. They showed fractures, which were demonstrated radiographically and histologically, in the costochondral junctions of horses.

Long (1984) reported a case of acute toxicosis in swine associated with excessive dietary intake of vitamin D (2,500 IU/kg). The affected pigs showed clinical signs like lethargy, emesis and some of them died. The post mortem lesions reported were hemorrhagic gastro-enteritis, diffuse interstitial pneumonia, myocardial degeneration and nephrosis. The histopathological findings reported were necrosis and variable degrees of mineralisation in number of organs. They also found increased levels of calcium, phosphorus and magnesium.

Adam and Daniel (1987) reported sporadic atherosclerosis of aorta in seven cattle from slaughter houses and farms. Aorta walls were thickened with many white and yellow mineralized plaques on the intimal surface. Microscopically aortas had mineral deposits in tunica intima and media, surrounded by fibrous tissue. The disease was associated with hypervitaminosis D_3 in three cows and one heifer.

Jiang *et al* (1991) studied radiologic, microangiographic and pathologic correlation of bone remodelling in hypervitaminosis D_3 in rabbits. Radiograph showed subperiosteal bone resorption and porotic cortical bone which on metastatic calcification fills these intracortical caverns or the intertrabecular spaces in the metaphysis and physis, thus showing a diffuse increased density on radiographs. Microangiograms showed proliferating vessels in the

periosteum and cortical bone with associated dilatation of the Haversian and Volkmann's canals.

Peterson *et al* (1991) reported accidental cholecalciferol rodenticide toxicity in a cat. The cat was hypercalcemic, hyperkalemic, and acidotic. Despite management of hypercalcemia and preservation of renal function with physiologic saline solution, furosemide, dopamine, and calcitonin, the cat died, apparently as a result of extensive pulmonary mineralization.

Morita *et al* (1995) studied the natural outbreak of vitamin D toxicosis (systemic calcinosis) in cats resulted from long term feeding of pet food containing excessive amount of vitamin D (6,370/100 g diet). Clinically, chronic weight loss, anorexia, episodic vomiting and signs of respiratory disturbance such as cough and difficulty in breathing were noted. Hematology and serum analyses showed the elevated values of phosphorus, blood urea nitrogen and serum creatinine. X-ray examination disclosed the increased density of systemic bones. Histologically, marked calcification was present at the vascular walls of almost all the organs including the lungs, trachea, kidneys, heart, aorta, alimentary tracts, choroid plexus and bones.

Beasley (1999) reported that the vitamin D metabolites increased serum calcium concentration (hypercalcemia) in vitamin D_3 toxicity by following ways:

i) Vitamin D and its active metabolites function to increase the absorption of calcium and phosphorus from the intestines. The major target tissue for calcitriol is the small intestine where it increased the active trans-cellular transport of calcium and phosphorus. The absorptive capacity of the intestine for calcium is a direct function of the amount of calcium binding protein (CaBP) present. Synthesis of CaBP was stimulated by vitamin D metabolites.

ii) The active metabolites of cholecalciferol also act on bone. Vitamin D (in the presence of PTH) was required for osteoclastic resorption and calcium mobilization from bone.

Cholecalciferol, 25-OH-D_3 and calcitriol stimulate osteoclastic proliferation and resorption of bone. On a per weight basis calcitriol is 100 times more potent in stimulating bone resorption than is 25- OH-D_3.

iii) Active metabolites of vitamin D also stimulate the retention of calcium by increasing its renal distal tubular reabsorption.

iv) Death of toxicated animal is thought to be result from hypercalcemia, calcification of tissues and renal and heart failure.

Tischler *et al* (1999) reported vitamin D_3 induced autonomous proliferative lesions in rat adrenal medulla. Lesions were multicentric, bilateral and peripheral in location along with hyperplastic nodules and pheochromocytomas.

Braun *et al* (2000) reported enzootic calcinosis in goats caused due to feeding of hay containing golden oat grass (*Trisetum flavescens*) which contains high concentration of vitamin D_3 analogue (1,25-dihydroxycholecalciferol). The affected goats showed weight loss, frequent recumbency, difficulty in rising and movement, kneeling after rising, stilted gait, arched back, shifting weight from one leg to another, intermittent carrying of an extremity, occasional swinging of a limb back and forth and reluctance to remain standing after roused. The heart rate found markedly increased with evidence of cardiac arrhythmia. The milk production of affected animals found markedly reduced. They also observed elevated calcium, phosphorus and alkaline phosphatase levels. The radiographic examination of affected goats showed marked calcification of the wall of the aorta and increased density of pulmonary parenchyma.

Hilbe *et al* (2000) reported a case of metastatic calcification in dog due to ingestion of tacalcitol ointment (topical antipsoriatic preparation containing synthetic vitamin D analogue tacalcitol). They observed the death of dog after history of lethargy, recumbency, paresis of the hind limbs, increased rectal temperature, dyspnea and hematemesis. Histopathological

lesions reported were metastatic calcification in the kidneys, lungs, myocardium, brain, stomach and tear glands.

Morrow (2001) reported that, with unregulated increase in plasma calcium and phosphorus in vitamin D_3 toxicity, their product (calcium x phosphorus) can rise above 60 which cause mineralization of tissues/organs like kidneys, GIT, cardiac muscles, skeletal muscles, blood vessels and ligaments and cause structural damage that lead to decreased functional capacity of these tissues and organs. The loss of function contributes to the development of ongoing end stage clinical signs as well as long term signs in animals that survive. The cause of death reported in vitamin D_3 toxicity includes cardiac and renal failure.

Price *et al* (2001) reported the mechanisms of hypercalcemia in vitamin D_3 toxicity. In vitamin D_3 toxicity the active metabolites of cholecalciferol have been reported to increase the blood calcium level (hypercalcemia) by increased resorption/mobilization of calcium from bone, increased absorption of calcium from intestine and decreased calcium excretion by kidney. The net result is high concentration of blood calcium level (hypercalcemia) and death reported due to renal and cardiac failure. The effect of hypercalcemia on cells includes altered cell membrane permeability, altered calcium pump activity and decreased cellular energy production and cellular necrosis.

Melamed *et al* (2008a) determined the association between 25-hydroxyvitamin D (25(OH)D) levels and the prevalence of peripheral arterial disease (PAD) in the general United States population. For each 10 ng/mL lower 25(OH)D level, the multivariable-adjusted prevalence ratio of PAD was 1.35. Low serum 25(OH)D levels were thus associated with a higher prevalence of PAD.

Gimeno *et al* (2000) reported effect of plant (Solanum glaucophyllum-high levels of 1,25-dihydroxyvitamin D_3 as glycoside derivatives) induced hypervitaminosis D on cutaneous structure, cell differentiation and cell proliferation in cattle. Sg-intoxicated cattle

showed atrophy of epidermis and severe involution of hair follicles and of sebaceous and sweat glands with reduction in cellular proliferation.

Rumbeiha *et al* (2000) developed a novel approach to postmortem diagnosis of cholecalciferol (CCF) toxicosis in dogs using kidney, bile, and urine samples and to differentiate CCF from ethylene glycol toxicosis. Results of this study show that biliary and renal 25(OH) D_3 concentrations and renal calcium to phosphorus ratio were of diagnostic value in dogs exposed to toxic concentrations of CCF.

Guo *et al* (2008) observed the effect of hyperlipidemia and atherosclerosis on rat myocardial expression of calcium-sensing receptor and apoptosis. They found that hyperlipidemia and atherosclerosis can up-regulate myocardial calcium-sensing receptor expression, promote myocardial apoptosis, aggravate oxidative stress and myocardial ischemia.

Melamed *et al* (2008b) studied the association between low serum 25(OH)D levels and mortality in the general population. In cross-sectional multivariate analyses, increasing age, female sex, nonwhite race/ethnicity, diabetes, current smoking, and higher body mass index were all independently associated with higher odds of 25(OH)D deficiency while greater physical activity, vitamin D supplementation, and non-winter season were inversely associated. The lowest quartile of 25(OH)D level (17.8 ng/mL) was independently associated with all-cause mortality in the general population.

2.3 LD_{50} in rabbits and other species

Eason (1993) evaluated the toxicity of cholecalciferol in rabbit and found to be very susceptible to cholecalciferol toxicity. The LD_{50} for cholecalciferol toxicity was evaluated as 9mg kg-1and LD_{95} as 18mg kg-1 body weight.

Henderson and Eason (2000) studied the acute toxicity of cholecalciferol to the European rabbit, *Oryctolagus cuniculus.* Although rabbits showed a considerable variation in

their individual response to cholecalciferol, they were very susceptible to the toxicant (LD_{50} = 4.4 mg kg^{-1}) and it was lethal to almost all rabbits ingesting the doses greater than 15 mg kg^{-1} body weight. However, concentrations of 0.04% and 0.18% cholecalciferol in bait were not readily eaten by rabbits.

There were lot of species variation for LD_{50} values of cholecalciferol. The single-dose of LD_{50} for cholecalciferol in Norway rats was 42.5 mg/kg and that in house mice, it was 43.6 mg/kg. Possums and rabbits were particularly sensitive to cholecalciferol (Eason 1991, 1993 and Jolly *et al* 1995). Cats were usually studied for secondary toxicity studies of cholecalciferol. It was found that they were less susceptible than possums, and toxicity was less consistent, with some cats surviving doses up to 200 mg/kg, while others died after doses of 50 mg/kg (Eason 1991). Fish-eating marine mammals, such as seals were quite resistant to high dietary levels of cholecalciferol (Keiver *et al* 1988). Dermal toxicity studies in rabbits revealed LD_{50} values to be 2000 mg/kg. Avian species are very resistant to cholecalciferol toxicity. LD_{50} values in mallard duck by oral route was estimated to be more than 2000 mg/kg and dietary LC_{50} came out as 4000 ppm in mallard duck and 2000 ppm in bobwhite quail The LD_{50} of vitamin D in dogs (the dose that will kill half the animals) is 88 mg/kilogram.

2.4 Rabbits in cardiovascular disease studies

Drew (2001) explained the importance of animal models in the study of atherosclerotic diseases. Animal models of atherosclerosis develop lesions either spontaneously or by interventions such as dietary, mechanical, chemical or immunological induction. They provide a means for studying the underlying mechanisms behind the atherosclerotic disease process, as well as a means for studying the effect of interventions, dietary or otherwise, on the development or regression of disease, while under controlled conditions. They also provided valuable information regarding, factors contributing to

disease progression and regression, diagnostic and therapeutic strategies, with extensive investigation of events occurring in the artery wall throughout these procedures.

Yanni (2004) described the laboratory rabbit as the widely used animal model for atherosclerosis research. Because the rabbit exhibits hypercholesterolaemia within a few days of an administration of a high cholesterol diet, it is very sensitive to the inducement of atheromatic lesions. The administration of different types of diets caused different types of lesions. The generation over recent years of transgenic rabbits with alterations in species genes was expected to help with the elucidation of the mechanisms underlying the initial and developmental stages of the disease. The laboratory rabbit significantly broadened our understanding on the pathogenesis of atherosclerosis.

Dhanya and Hema (2008) reviewed the animal models used for atherosclerosis research now a day. Several animals have been used for the study of atherosclerosis, such as rabbit, non human primates, swine, mice, guinea pigs and hamsters. But it was found that several characteristic of the rabbit made it an excellent model for the study of atherosclerosis. Due to the fact that rabbit is very sensitive to the inducement of atheromatic lesions through a high cholesterol diet, the “cholesterol fed rabbit” was deemed to be one of the most important animal models for the study of atherosclerosis. With cholesterol feeding they developed hypercholesterolemia (>2000mg/dl) and accumulated cholesterol in intima of the large conduit arteries especially aortic arch and thoracic aorta.

Shimizu *et al* (2009) investigated the appropriate conditions for induction of lesions in the rabbit atherosclerosis model. Rabbits were given high cholesterol feed at two different levels, at two different ages, and did or didn’t undergo ballon injury. Histological investigation for plaque formation was done. The study indicated that the simplest conditions for inducing the rabbit atherosclerosis model were 1% high cholesterol diet, non-ballon

injury and early start of high cholesterol diet. This type of model is considered to be suitable for experiments with new therapeutic devices.

2.5 Hematological studies

Ensrud and Grimm (1992) reviewed that the WBC count demonstrated in several epidemiologic studies is a strong independent predictor of future coronary heart disease. White blood cell, in particular the neutrophil, is instrumental in the pathogenesis of myocardial ischemia. Patients who develop acute myocardial ischemia had abnormal leukocyte function before the onset of the acute event, which provided a pathophysiologic milieu for the progression of the atherosclerotic process.

Facchini *et al* (1992) evaluated the relationship between peripheral white blood cell (WBC) count, insulin-mediated glucose uptake, and several risk factors for coronary heart disease (CHD). WBC count correlated with plasma glucose response to oral glucose, fasting plasma triglyceride and HDL-cholesterol concentrations. The data indicated that WBC count was significantly correlated with changes in carbohydrate and lipoprotein metabolism and blood pressure that increased the risk of CHD.

Lee *et al* (2001) examined the association between white blood cell (WBC) count and incidence of coronary heart disease and ischemic stroke and mortality from cardiovascular disease. The patients in the highest quartile of WBC count (≥7,000 cells/mm3) had 1.9 times the risk of incident coronary heart disease, 1.9 times the risk of incident ischemic stroke, and 2.3 times the risk of cardiovascular disease mortality as their counterparts in the lowest quartile of WBC count (<4,800 cells/mm3). An elevated WBC count was directly associated with increased incidence of coronary heart disease and ischemic stroke and mortality from cardiovascular disease.

Madjid *et al* (2004) reviewed the epidemiological and clinical evidence for a relationship between the leukocyte count and coronary heart disease (CHD). Leukocytosis

was an independent predictor of future cardiovascular events, both in healthy individuals free of CHD at baseline and in patients with stable angina, unstable angina, or a history of myocardial infarction. Elevated differential cell counts, including eosinophil, neutrophil and monocyte counts, also predicted the future incidence of CHD. Leukocytosis affected CHD through multiple pathologic mechanisms that mediate inflammation, cause proteolytic and oxidative damage to the endothelial cells, plug the microvasculature, induce hypercoagulability and promote infarct expansion.

Jia *et al* (2005) explored the relationship between differential leukocyte count and coronary atherosclerosis in human. The independent association of neutrophil count with the angiographical characteristics of coronary atherosclerosis strongly suggested that granulocytosis played a major role in development of coronary atherosclerosis.

Dijk *et al* (2006) studied whether hemoglobin levels are related to different vascular indicators of atherosclerosis. Each mmol/l increase in hemoglobin was associated with a lower carotid IMT (CIMT) and a lower prevalence of >/=50% internal carotid artery stenosis (ICAS) after adjustment for age, gender and potential confounders. Hemoglobin was not related to prevalence of peripheral arterial disease. Thus, the study showed that increasing hemoglobin levels was associated with reduced severity of atherosclerosis in patients with manifestation of arterial disease.

2.6 Biochemical studies

Newman and Zilversmit (1962) studied the quantitative aspects of cholesterol flux in rabbit atheromatous lesions. The study revealed that plasma cholesterol was the primary source of the cholesterol in the atheromatous aorta and that the accumulation of cholesterol in the atherosclerotic lesion was not a static process but was subjected to continuous turnover. Larger quantities of cholesterol in the lesion were associated with higher influxes. Animals

maintained on the cholesterol diet for longer times showed higher influx rates at the same serum cholesterol concentration than those who had received cholesterol for shorter intervals.

Vijayakumar and Kurup (1974) reported that hypervitaminosis D causes increased total cholesterol, phospholipids and triglyceride levels of the serum, liver and aorta in both normal and high fat cholesterol diet-fed rats, the extent of increase was more in the latter group.

Solberg and Strong (1983) reviewed the relationship of risk factors to atherosclerotic lesions. The findings among the studies showed that, elevated serum cholesterol and blood pressure were positively and significantly related to atherosclerotic lesions. High-density lipoprotein cholesterol was inversely related to coronary and probably also to cerebral atherosclerosis. In most studies no consistent association was found between obesity or physical activity and the degree of atherosclerosis.

Geurian *et al* (1992) examined the mechanism of triglyceride-induced atherogenesis in coronary heart disease (CHD) and investigated an appropriate therapeutic approach to hypertriglyceridemia. Hypertriglyceridemia is a theoretical risk factor for CHD because of the increased production of atherogenic chylomicron and VLDL remnants. Inverse relationship was presented between serum triglyceride and HDL. The resultant increase in LDL was attributed to remnant-reduced hepatic LDL-receptors as well as the formation of more dense and, therefore, more atherogenic LDL, and to the interaction between serum triglyceride and the fibrinolytic/coagulation system. Role of triglyceride-lowering to reduce CHD risk remained controversial.

Matts *et al* (1993) reported serum creatinine as an independent predictor of coronary heart disease mortality in normotensive survivors of myocardial infarction. The baseline serum creatinine values in the control group patients ranged from 0.7 to 1.9 mg/dL (60 to 170 [mu]mol/L), and were found to be independent predictors of both overall mortality and

atherosclerotic coronary heart disease mortality. Each 0.1 mg/dL (9 [mu]mol/L) increment in the baseline serum creatinine increased the relative risk for subsequent overall mortality by 36% and the relative risk for subsequent atherosclerotic coronary heart disease mortality by 47%. Thus serum creatinine value, obtained in normotensive, nonobese, normoglycemic survivors of a myocardial infarction without preexistent renal disease or heart failure, provides independent prognostic information regarding subsequent overall and atherosclerotic coronary heart disease mortality.

Narang *et al* (1997) determined the relationship of serum calcium, phosphorus and albumin levels with the angiographic severity of coronary artery disease (CAD). On univariate analysis, total serum calcium and serum albumin levels had a negative association with the number of diseased vessels. Serum albumin had an independent negative and serum phosphorus had an independent positive association with the angiographic severity of CAD. Serum phosphorus level also showed highly significant positive associations with the presence of total or subtotal occlusion and with most severe stenosis observed on angiography.

Tozer and Carew (1997) studied the residence time of low-density lipoprotein in the normal and atherosclerotic rabbit aorta. The concentration of radiolabeled arterial LDL was measured in New Zealand White rabbits killed at several different times (0.5 to 72 hours) after injection of labeled LDL. The arterial LDL residence times in normolipidemic and hyperlipidemic rabbits before lesion formation were similar in both the lesion-prone and resistant sites. However, on development of early fatty streak lesions, the arterial LDL residence time increased dramatically. After only 16 days of cholesterol feeding, the residence time was 10 times longer in the lesioned aortic arch. After 21 days of cholesterol feeding, the residence time increased to >25-fold that of normolipidemic tissue. This early

retention of LDL suggested that significant changes took place within the arterial wall during the critical stage of early lesion development.

Bruckert *et al* (2002) studied the relationships between serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma glutamyl transferase (GGT) and cardiovascular and metabolic risk factors. Both men and women who exhibited ALT levels superior to the upper limit of the normal range had elevated systolic (SBP) and diastolic blood pressure (DBP), body mass index (BMI), alcohol intake, and serum levels of blood glucose, uric acid, total cholesterol, and triglycerides. BMI, uric acid, and blood glucose remained significantly associated with ALT levels in men and women. Thus, cardiovascular and metabolic features characterizing the plurimetabolic syndrome, including serum uric acid levels, were associated with significant elevation of hepatic enzyme activities.

Koba *et al* (2002) reported an association between increased number of very-low-density lipoprotein particles and coronary heart disease in Japanese men, independently of intermediate-density lipoprotein or low-density lipoprotein. Although total- and LDL-cholesterol levels were similar in CHD and control participants, triglyceride levels were significantly higher and high-density lipoprotein (HDL)-cholesterol levels were lower in CHD patients. IDL-triglyceride levels were significantly elevated in CHD, but IDL-cholesterol level was not. This suggested that an increased number of VLDL particles were strongly associated with CHD, independently of traditional risk factors or newly recognized atherogenic lipoproteins, such as IDL or small, dense LDL, in Japanese men.

Hulten *et al* (2005) reported that macrophages may play a dual role in atherogenesis, i.e. both by promoting and limiting LDLoxidation. Macrophages respond to oxidative stress by endogenous antioxidant activity, which is sufficient to decrease reactive oxygen species

both in LDL and oxLDL. This suggested that the antioxidant activity of macrophages was insufficient during atherosclerosis development.

Tonelli *et al* (2005) studied the relation between serum phosphate level and cardiovascular event rate in people with coronary disease. A graded independent relation between higher levels of serum phosphate and the risk of death and cardiovascular events was found in people with prior myocardial infarction, most of whom had serum phosphate levels within the normal range.

Hsu and Culley (2006) studied the effects of dietary calcium on atherosclerosis, aortic calcification and icterus in rabbits fed a supplemental cholesterol diet. It was seen that Ca supplementation significantly decreased the lesions by 41% ($p < 0.05$) and markedly inhibited calcification by 62% ($p < 0.05$); Whereas Ca deficiency significantly increased the lesions by 2.7-fold ($p < 0.05$) and caused a small but significant calcification. Ca supplementation caused a significant 30% decrease in serum cholesterol ($p < 0.05$). Calcium deficiency increased serum cholesterol by 57% ($p < 0.001$). Serum cholesterol and LDL-cholesterol levels in Ca deficient rabbits were 2-fold higher than those with high Ca diets. Ca supplementation decreased soluble Ca and P content in aortas, suggesting that the effect may underlie the effects of Ca supplementation on calcification. Calcium deficiency increased icterus by 33% ($p < 0.05$), which may affect hepatic clearance of cholesterol, while calcium supplementation decreased it by 43% ($p < 0.001$). Thus, Ca supplementation to an atherogenic diet inhibits atherosclerosis, aortic calcification, and icterus, whereas a Ca deficient-diet promotes them.

Hsu and Tawfik (2006) studied the role of calcifying vesicles in mechanisms of focal calcification in rabbit aortas. Focal calcification in rabbit aortas and diffuse mineralization in human fibrointima were found to be not associated with osteogenesis, Blockade of the blood

supply to SMCs triggered the cells to produce more calcifying vesicles, thereby leading to site-specific calcification in rabbit aortas.

Jiaa *et al* (2006) studied the relationship between total cholesterol/high-density lipoprotein cholesterol ratio, triglyceride/high-density lipoprotein cholesterol ratio, and high-density lipoprotein subclasses. The results indicated that TC/HDL-C and TG/HDL-C ratios together may be a good indicator of HDL subclass distribution. When these 2 ratios increased simultaneously, the trend toward smaller HDL size was obvious, which, in turn, indicated that the maturation of HDL might be impeded and the reverse cholesterol transport might be weakened. In addition, the TG/HDL-C ratio might be a more powerful factor to influence the distribution of HDL subclasses.

Madhumathi *et al* (2006) evaluated the changes induced in rabbits feed high cholesterol diet for 12 weeks. High cholesterol fed rabbits showed significantly elevated levels of serum Total Cholesterol (TC), Low Density Lipoprotein (LDL), Very Low Density Lipoprotein (VLDL) and also an increase in Low Density Lipoprotein (LDL)/High Density Lipoprotein (HDL) ratio. Histopathology of aorta revealed well formed, thick atheromatous plaques on the intimal surface of aorta with presence of foam cells and increased amount of cholesterol and lipid deposition in intimal and subintimal areas of the aorta.

Dhingra *et al* (2007) studied the relations of serum phosphorus and calcium levels to the incidence of cardiovascular disease in the community. In multivariable analyses and adjusting for established risk factors and additionally for glomerular filtration rate and for hemoglobin, serum albumin, proteinuria, and C-reactive protein levels, a higher level of serum phosphorus was associated with an increased cardiovascular disease (CVD) risk in a continuous fashion. Higher serum phosphorus levels are associated with an increased CVD risk in individuals free of chronic kidney disease (CKD) and CVD in the community, however, serum calcium was not related to CVD risk.

Kadono *et al* (2007) reported serum albumin level as an indicator of atherosclerosis with differnet pathogenesis. Serum albumin level was a marker of nutritional status, and was reported to possess antioxidative properties. The results demonstrated that extremes of nutritional state were linked to atherosclerosis. Low serum albumin condition increased the prevalence of atherosclerosis through reduced defense oxidative stress, and high serum albumin may link to metabolic syndrome, leading to atherosclerosis.

Onufrak *et al* (2007) examined the association of serum phosphorus level with carotid atherosclerosis in the general population. Phosphorus levels were significantly associated with age, female gender, diabetes mellitus, hypertension, hypercholesterolemia, and fibrinogen levels, but not with estimated glomerular filtration rate (eGFR). In a population-based cohort of subjects free of overt cardiovascular and renal disease serum phosphorus was positively associated with carotid intima medial thickness (cIMT) independent of traditional risk factors for atherosclerosis and glomerular filtration rate.

Rubin *et al* (2007) investigated the association between serum calcium level and carotid plaque thickness, a powerful early predictor of clinical coronary and cerebrovascular events. Subjects with carotid plaque had higher corrected serum calcium levels within the normal range than those without carotid plaque. Subjects in the top quintile of maximal carotid plaque thickness were more likely to be in the highest quintile of serum calcium level.

Abdelhalim and Alhadlaq (2008) studied the effect of feeding high cholesterol diet on hematological and biochemical profile in rabbits. Rabbits fed high cholesterol showed significantly increased level of serum Total Cholesterol (TC), Low Density Lipoprotein (LDL), Very Low Density Lipoprotein (VLDL), High Density Lipoprotein (HDL), Total Glycerides (TG), White Blood Corpuscles (WBC) and lymphocyte percentage. However, there was significant decrease in Hemoglobin and neutrophils percentage with no significant change in eosinophil and monocyte percentage. The study indicated that blood parameters

helped in diagnosis and monitoring the progression of atherosclerosis in rabbits fed high cholesterol.

Abdelhalim *et al* (2008) studied the effects of feeding high cholesterol and saturated fat diet on blood biochemistry in rabbits. The results showed that TC, LDL and TG were significantly increased while HDL levels were decreased in cholesterol-fed rabbits as compared with control rabbits. Thus the study concluded that TC, LDL, TG, in serum helped in the diagnosis and monitoring the progression of arteriosclerosis.

Foley *et al* (2008) reported that calcium-phosphate levels, linked to vascular dysfunction in chronic kidney disease, may represent novel risk factors for coronary heart disease, stroke, and death in community-dwelling adults. Although calcium, phosphate, and calcium-phosphate product levels exhibited complex associations with traditional cardiovascular risk factors and outcomes, they may be potentially modifiable risk factors for stroke and death in community-dwelling adults.

Adeney *et al* (2009) examined associations of serum phosphate concentrations with vascular and valvular calcification in individuals having moderate chronic kidney disease (CKD) and no clinical cardiovascular disease. Each 1-mg/dl increment in serum phosphate concentration was associated with a 21%, 33%, 25% and 62% greater prevalence of coronary artery, thoracic, aortic valve, and mitral valve calcification, respectively. Adjustment for traditional risk factors for atherosclerosis, parathyroid hormone, or 1,25-dihydroxyvitamin D levels did not alter these associations. Thus, higher serum phosphate concentrations, although still within the normal range, was associated with a greater prevalence of vascular and valvular calcification in people with moderate CKD.

Al-Hakeim (2009) investigated the possible correlation between lipid profile components and serum cations Ca and Mg. The results showed a significant decrease in high-density lipoprotein-cholesterol (HDL), total and ionized Mg in hypothyroid patients as

compared to control group. There was a significant increase in serum total cholesterol (TC), low-density lipoprotein-cholesterol (LDL) and (LDL)/(HDL) ratio in hypothyroid patients as compared with control group. However, no correlation was found between the cation levels and lipid profile of the studied groups. Thus, patients with hypothyroidism exhibited elevated atherogenic parameters (TC and LDL) and high risk of cardiovascular diseases.

Charnow (2009) studied the role of higher phosphorus levels in atherosclerosis. Higher phosphorus levels were associated with peripheral atherosclerosis and arterial stiffness, independent of traditional risk factors for cardiovascular disease. These associations accounted for a recognized association between phosphorus and CVD events. Compared with the reference group, the low and high ankle-brachial index groups had significantly higher serum phosphorus levels.

Shuto *et al* (2009) investigated the effect of acute phosphorus loading on endothelial function *in vitro* and *in vivo*. Exposing bovine aortic endothelial cells to a phosphorus load increased production of reactive oxygen species, which depended on phosphorus influx via sodium-dependent phosphate transporters, and decreased nitric oxide production via inhibitory phosphorylation of endothelial nitric oxide synthase. Phosphorus loading inhibited endothelium-dependent vasodilation of rat aortic rings. The high dietary phosphorus load increased serum phosphorus at 2 h and significantly decreased flow-mediated dilation in healthy men. These findings suggested that endothelial dysfunction mediated by acute postprandial hyperphosphatemia may contribute to the relationship between serum phosphorus level and the risk for cardiovascular morbidity and mortality.

Tonelli *et al* (2009) studied relation between alkaline phosphatase, serum phosphate, and all-cause or cardiovascular mortality. An independent relation between higher levels of AKP and adverse outcomes among survivors of myocardial infarction was found. The excess

risk of death was present in people without evidence of kidney disease and was particularly high among people with higher levels of both AKP and serum phosphate.

Wang *et al* (2009) reported that elevation of serum aminotransferase activity increased risk of carotid atherosclerosis in patients with non-alcoholic fatty liver disease. It was observed that the risk of carotid atherosclerosis increased with increment of every 10 IU/L in serum ALT levels. The study suggested that the serum ALT levels could serve as a surrogate marker of cardiovascular risk.

2.7 Atomic absorption mineral estimation studies

Dubick *et al* (1987) analyzed the concentrations of Fe^{++}, Cu^{++}, Zn^{++}, and Mn^{++} in tissue samples from patients with abdominal aortic aneurysms (AAA) and patients with atherosclerotic occlusive disease (AOD). It was observed that the Fe and Mn concentrations in AAA and AOD tissue were higher than the levels in nondiseased control aorta, whereas Cu and Zn levels in AAA and AOD tissue were similar to the levels in controls. The Zn:Cu ratio was significantly lower in the AAA tissue in comparison to both AOD and control tissue. Although these observations did not directly support the hypothesis that AAA is associated with aortic Cu deficiency they do suggest a role for oxygen radicals or increased lipid peroxidation in occlusive and aneurysmal disease of the aorta.

Phair (1988) reviewed the role of cellular calcium in atherosclerosis. Arterial calcium was increased in atherosclerosis. This increase in tissue calcium content is largely intracellular. Increased intracellular calcium content is caused by increased plasma membrane calcium permeability which is causally related to atherogenesis. Many of the cell physiological, cell biological, biochemical, and molecular biological processes, known to function abnormally in atherosclerosis, are also known to be calcium regulated. These processes are activated or inactivated in atherosclerosis in a manner consistent with increased cell calcium.

Strickberger *et al* (1988) reported that increased intracellular calcium is a possible mediator of cholesterol-induced atherogenesis. Calcium fluxes and contents were determined in aortic segments from cholesterol-fed rabbits and age-matched controls to identify the possible causes of this accumulation. Total intracellular calcium increased from 269 ±11.6 to 1,300 ±352 Dmol/g in cholesterol-fed animals compared with controls. The fraction of tissue calcium that was intracellular increased significantly from 0.065 ±0.006 to 0.223 ±0.048 (/X0.01) in experimental atherosclerosis. These changes were brought about by a 4.8-fold increase in the plasma membrane calcium permeability of aortic smooth muscle cells.

Cichocki *et al* (1989) reported that artery wall calcification was responsible for the mineralization in atherosclerosis. The amount of P^{+} and Ca^{++} increased with age approaching at places 9% and 20% and mineral deposits were detected in tunica media. At the same time an increase in the Ca/P ratio and in the crystallinity of deposits was observed. The concentrations and localization of Zn showed artery wall layer-dependent changes. In some places of the artery wall, minerals were also found in young persons.

Bialecki *et al* (1991) studied the effect of cholesterol enrichment on vascular smooth muscle cell (VSMC) calcium homeostasis by evaluating calcium uptake, efflux, and intracellular content in cultured VSMC derived from the rat pulmonary artery. Incubation of VSMC with liposomes consisting of free cholesterol (FC) and phospholipid (2:1 molar ratio, 1 mg FC/ml medium) for 24 h resulted in a 69±19% increase ($P < 0.01$; $n = 10$) in FC which was associated with a 73±11% increase ($P < 0.005$; $n = 10$) in intracellular calcium content. Cholesterol enrichment caused a marked increase in the unidirectional calcium uptake rate from 0.026±0.03 to 0.158±0.022 nmol calcium/s per mg protein, but had no effect on calcium efflux. Exposure of cholesterol-enriched VSMC to cholesterol-poor liposomes for 24 h returned both FC and calcium contents to control levels. Thus, VSMC FC content played a

role in regulating cellular calcium homeostasis, both under basal conditions and in response to selected agonists.

Oster *et al* (1993) measured the element concentrations (selenium, copper, zinc, iron, magnesium, potassium, phosphorous) in heart tissue of patients with coronary heart disease and correlated with physiological parameters of the heart. There was no relationship between the element concentrations and the number of vessels stenosed or the occurrence of myocardial infarction. When the element concentrations of the heart were related with parameters characterizing cardiac output, such as ejection fraction and cardiac index, positive statistically significant correlations were found for iron, copper, zinc, phosphorus and selenium.

Vlad *et al* (1994) studied the concentration of copper, zinc and iron in the abdominal aorta of patients diseased with coronary heart disease. The Cu^{++} and Zn^{++} concentrations were significantly lower in the atherosclerotic plaques of abdominal aorta of the diseased patients with ischemic heart disease (IHD) and acute myocardial infarction (AMI) than in the control group. Iron had the tendency to rise but not significantly. The low values of copper in the atherosclerotic aortic tissue in IHD and AMI was attributed to a shift of copper from aortic tissue into the blood.

Haigney *et al* (1995) studied the correlation of tissue magnesium concentration with cardiac levels. Sublingual epithelial cell $[Mg]_i$ correlated well with atrial $[Mg]_i$ but not with serum magnesium. $[Mg]_i$ levels were low in patients undergoing cardiac surgery and those with acute myocardial infarction (AMI). Intravenous magnesium sulfate corrected low $[Mg]_i$ levels in AMI patients.

Vijaya *et al* (1995) analysed trace metal concentration in the aorta with and without atherosclerotic lesions. The tissue concentrations of zinc, copper and manganese was estimated in atomic absorption spectrophotometer. High concentration of zinc was observed

in atherosclerotic lesions tissue. Low copper and manganese concentration was also observed in atherosclerotic lesions tissue. Thus, low copper and manganese in the tissue of lesions seem to be a shift of these minerals into the serum. The high concentration of zinc in the tissue of lesions shows the prevalence of deficiency of zinc in atherosclerosis.

Thong *et al* (1996) studied elemental changes in atherosclerotic lesions using nuclear microscopy. Unstained freeze-dried tissue sections from the aorta of New Zealand white rabbits fed with a 1% cholesterol diet for 12 weeks were scanned with a 2 MeV proton beam nuclear microscope. There was an average of seven-fold increase in iron and an average of nearly two-fold increase in phosphorus in the atherosclerotic lesion compared with healthy tissue. The increase in iron suggested that iron-catalyzed free radical reactions might be associated with the development of atherosclerosis. Occurrence of granules rich in sodium, chlorine and potassium at the interface between lesioned and non-lesioned tissue were also observed.

Weginwar *et al* (2002) determined the uptake of trace elements in the liver, kidney and blood of hypercholesterolemic model mice. The uptake of Fe^{++} in liver increased with an increasing feeding period of a cholesterol-rich diet, whereas the uptake of Zn^{++} decreased. Feeding of the diet resulted in a marked increase in serum total cholesterol, triglycerides and low-density lipoprotein cholesterol. A significant positive correlation was found between the concentration of serum triglycerides and liver uptake of Fe and a negative correlation for the uptake of Zn^{++}. The results suggested that cholesterolemia have some specific effects on the metabolism of some elements.

Koksal *et al* (2007) compared the tissue Fe^{++}, Cu^{++} and Zn^{++} levels (as a marker of lipid peroxidation) in the abdominal aorta in relation to the development of aneurysmal and occlusive disease in the infrarenal aorta. The comparison of tissue Zn^{++} levels showed no significant difference. Tissue levels of Fe^{++} and Cu^{++} were found to be higher in the

abdominal aortic aneurysm (AAA) group, compared with the aortic occlusive disease (AOD) group. These results suggest that higher oxidative stress as a result of higher Fe and Cu levels in the AAA, compared with AOD, may be one of the contributing factors in aneurysmal formation as a result of promoted wall erosion.

Stadler *et al* (2008) quantified metal ion and protein oxidation levels in human carotid and abdominal artery specimens containing early-to-advanced lesions, to determine whether zinc concentrations correlate inversely with iron levels and protein oxidation. Elevated levels of zinc (6-fold) were detected in advanced lesions compared to healthy tissue or early lesions. Zinc did not correlate negatively with iron or copper levels suggesting that zinc did not displace these metal ions. Highly significant positive correlations were detected between zinc and calcium levels. Thus, the data indicated that zinc did not prevent protein oxidation in advanced lesions. The reported protective effect of zinc accumulation was proposed to be associated with lesion calcification.

Jia *et al* (2009) studied an association of serum sodium concentration with coronary atherosclerosis in China. The serum sodium concentration showed a statistically significant negative association with coronary events and all-cause mortality in subjects with coronary atherosclerosis.

Abdelhalim *et al* (2010) elucidated the effects of a high fat diet on trace elements in rabbit tissues using atomic absorption spectroscopy. Comparing high fat diet (HFD) rabbits to control rabbits, percentage change of increase of Fe^{++} was 7% in kidney tissue. The highest percentage change of decrease of Cu^{++} was 16% in aortic tissue, while the lowest percentage change of decrease of Cu^{++} was 6% in kidney tissue. The percentage decrease of Zn^{++} was 71% in kidney tissue. These results suggested that Fe played a major role in atherogenesis; it may accelerate the process of atherosclerosis probably through the production of free radicals, deposition and absorption of intracellular and extracellular lipids

in the intima, connective tissue formation, smooth muscle proliferation, lower matrix degradation capacity and increased plaque stability. Cu^{++} played a minor role in atherogenesis and Cu^{++} supplements might inhibit the progression of atherogenesis, by reducing the migration of smooth muscle cells from the media to the intima. Zn^{++} played a major role in atherogenesis by acting as an endogenous protective factor against atherosclerosis perhaps by reducing lesion Fe^{++} content, intracellular and extracellular lipids in the intima, connective tissue formation, and smooth muscle proliferation. The results suggested that it may be possible to use the measurement of changes in trace elements in different tissues of rabbits as an important risk factor during the progression of atherosclerosis.

2.8 Histochemical studies

Mrhovx *et al* (1963) determined the activity of aorta dehydrogenase systems (lactic acid dehydrogenase, succinic acid dehydrogenase and malic acid dehydrogenase) biochemically in early stages of experimental cholesterol atherosclerosis in rabbits. There was a significant decrease of SDH activity as early as 4 weeks and 10 weeks after the start of cholesterol feeding. The activity of the dehydrogenase systems revealed a decrease of borderline significance on 10^{th} weeks (LDH, MDH) following daily administration of the cholesterol-fat diet.

Maier and Haimovici (1965) studied the oxidative capacity of the succinic oxidase and cytoehrome oxidase systems in aortas from rabbits and dogs subjected to an atherogenic regimen. In both animals, at an early stage in the atherosclerotic process, the oxidative capacity of both systems was increased in the atherosclerotic intima-media layer, separated at the cleavage plane, while at a later stage it was decreased. The intima-media of the uninvolved portions of the aorta showed a decrease in the oxidative capacity of the cytochrome oxidase system, whereas that of the succinic oxidase system remained

unchanged. Findings indicated that metabolic alterations of the arterial wall are associated with the development of atherosclerosis.

Chazov *et al* (1969) studied the serum lactic dehydrogenase isoenzyme patterns in coronary atherosclerosis. The data obtained suggested that the determination of the LDH isoenzymes in serum serve as a quantitative test for evaluating the degree of myocardial injury in patients with coronary atherosclerosis.

Rosnowski and Kujawa (1977) studied the succinic dehydrogenase activity of the myocardium in experimental atherosclerosis. The activity of succinic dehydrogenase (SDH) in the myocardium of rabbits with experimental atherosclerosis was investigated in the electron microscope from the second to the 16th week of the experiment. An increase in the number of mitochondria in the heart muscle was noted in the second month. From the fourth week SDH activity did not change in the mitochondria, whereas a decrease of SDH was observed beginning in the eighth week of the experiment.

Garbarsch *et al* (1978) investigated the succinate dehydrogenase activity in the wall of rabbit aorta. The level of succinate dehydrogenase per se in the smooth muscle cells was found to be fairly high, while the mitochondrial level of carrier CoQ was low. The CoQ explained the cause of low level or lack of activity of succinate dehydrogenase in these cells.

Shioi *et al* (2002) studied the roles of tumor necrosis factor-α and oncostatin M derived from macrophages in induction of bone-type alkaline phosphatase in human vascular smooth muscle cells. Thermostability and immunoassay showed that AKP induced in human vascular smooth muscle cells (HVSMCs) was bone-specific enzyme. 1,25-dihydroxyvitamin D_3 had a stimulatory effect on in-vitro calcification through increasing the expression of alkaline phosphatase (AKPase), an ectoenzyme indispensable for bone mineralization, in vascular smooth muscle cells. Tumor necrosis factor-α (TNF-α) and oncostatin M (OSM) was identified as major factors inducing ALP in HVSMCs. TNF-α and OSM, only when

applied together, increased AKP activities and in vitro calcification in HVSMCs in the presence of IFN-γ and 1,25(OH)2D_3. Thus, macrophages contribute to the development of vascular calcification through producing various inflammatory mediators, especially TNF-α and OSM.

Leopold *et al* (2003) reported that glucose-6-phosphate dehydrogenase overexpression decreased endothelial cell oxidant stress and increased bioavailable nitric oxide (NO). Overexpression of G6PD in vascular endothelial cells decreases reactive oxygen species accumulation in response to exogenous and endogenous oxidant stress and improves levels of bioavailable NO.

Fuhrman *et al* (2004) examined AChE capacity to protect LDL against oxidation. Acetylcholine esterase (AChE) is serum ester hydrolases, which is associated with the prevalence of myocardial infarction. Preincubation of LDL with AChE retarded the onset of copper ion-induced LDL oxidation in a concentration-dependent manner. AChE significantly reduced the formation of lipid peroxides and TBARS during the course of LDL oxidation, by up to 45%. This effect was associated with AChE-mediated hydrolysis of lipid peroxides, which accounts for the inhibition in the onset of LDL oxidation, the oxidative propagation phase, and aldehyde formation. Thus, AChE can hydrolyze lipid peroxides and prevent the accumulation of oxidized LDL and attenuate atherosclerosis development.

Matsui *et al* (2006) examined whether Glucose-6-phosphate dehydrogenase (G6PD) deficiency affects vascular oxidants and atherosclerosis in high-fat fed apolipoprotein (apo) $E^{-/-}$ mice. Lower NADPH production in G6PD deficiency resulted in lower NADPH oxidase-derived superoxide anion, and thus lower aortic lesion growth. The association of higher blood pressure with lower serum cholesterol levels in this mouse model was indicative of the complex effects that G6PD deficiency may have on vascular disease.

Silva *et al* (2008) investigated the in vitro acute effects of compounds used for the prevention or treatment of cardiovascular diseases on total AKP activity from male Wistar rat heart homogenate. Rat heart expressed intestinal AKP type II and had high total AKP activity. Compounds used in the prevention of cardiovascular pathology inhibited AKP activity. Thus, AKP manipulation in vivo constituted an additional target for intervention in cardiovascular diseases.

2.9 Histopathological studies

Albrecht *et al* (1965) studied the influence of hypercholesterolaemia on adrenal function. Adrenocortical function was assessed by reference to the corticosterone output. A decrease in adrenal function was found after cholesterolfeeding for 50 days. There was no direct connection between adrenocortical hypertrophy and corticosterone secretion; suggesting that the decrease in corticosterone production was not responsible for the adrenocortical hypertrophy. Despite the diminution in corticosterone secretion, the concentration of corticosterone in the peripheral blood, and its protein binding remained unchanged. In the adrenal homogenate, the alkaline phosphatase activity showed no change, whereas the acid phosphatase activity displayed an increase. Findings appeared to be related to the deposition of lipids in the adrenals.

Bondjers and Bjorkerud (1973) determined the concentration and *in vivo* uptake of free and esterified cholesterol in regions with intact and defective endothelium in the aorta of normal rabbits. The cholesterol content and the uptake of free and esterified cholesterol were higher in regions with *defective* endothelium suggesting filtration of lipoproteins in regions with *defective* endothelium. In regions with *intact* endothelium, an inverse relationship was found between uptake and content of cholesterol, suggesting an adjustment of the cholesterol uptake to different requirements in different regions of the aorta. The results indicated that

the intact endothelial lining represented a structural barrier against the excessive influx of cholesterol, and that the integrity of this barrier is decreased during atherogenesis.

Panganamala *et al* (1974) studied the gross and histological appearance and the lipid composition of normal intima and lesions from human coronary arteries and aorta. Normal intima and individual lesions from coronary arteries and aorta did not differ in absolute or relative triglyceride content. Intracellular lipid lesions and foam cell lesions had the same total and relative lipid composition. The absolute amount and the relative amount of cholesteryl ester increased when these lesions were compared with normal intima. The absolute amount and the relative amount of cholesteryl ester were increased when fibrous plaques were compared with normal intima but were not increased when fibrous plaques were compared with intracellular lipid lesions and foam cell lesions. The relative amount of free cholesterol was increased when fibrous plaques were compared with both normal intima and foam cell lesions but not intracellular lipid lesions. Thus, fibrous plaques did not correlate with either a progressive increment in total lipid or a progressive change in relative lipid composition.

Parke *et al* (1978) reported short-term induction of atherosclerosis in rat using large doses of vitamin D_2 and cholesterol. The higher dose of vitamin D_2 (480,000 iu/kg) and cholesterol (60 mg/kg) produced almost a 3-fold increase in aorta weight and a 57 percent increase in plasma cholesterol levels but resulted in a mortality rate of 83 percent. The lower dose regimen over 5 and 7 days produced similar increases but the aorta weights were not significantly different from the controls. A reduced vitamin D_2 treatment resulted in decreased mortality however aorta weights and plasma cholesterol levels were increased significantly. Reducing the vitamin D_2 treatment to one day prevented mortalities, did not alter aorta weights but did increase plasma cholesterol levels.

Taura *et al* (1978) reported ultrastructural changes of cardiovascular lesions induced by hypervitaminosis D. Calcification of medial and internal elastic lamina of thoracic aorta, pulmonary trunk and coronary artery was noted. Aortic valves showed cartilage formation along with minimal calcification.

Kunitomo *et al* (1981) studied biochemical aspect of histopathological observation in vitamin D and cholesterol supplemented rats. He suggested significance of vitamin D supplementation in deposition of cholesterol and calcium in aorta of rats. Macroscopic lesion in heart, aorta (roughness, thickening and stiffening) and coronary artery was noted. Microscopy revealed degeneration and calcification of intima and media of aorta and artery. Biochemical observation showed significant decrease in serum triglycerides and elevated levels of calcium (three times).

Buja *et al* (1983) studied cellular pathology of progressive atherosclerosis in the WHHL rabbit. The rabbits showed evidence of progressive disease of the aorta with accumulation of strongly birefringent lipid in intimal and medial lesions, including fatty streaks, raised foam cell lesions, and plaques (atheromas). Ultrastructurally the cellular population of the intimal lesions consisted predominantly of smooth muscle cells with lipid deposits and lipidladen foam cells. Lipid deposits occurred as cytoplasmic neutral lipid droplets and as multilamellar bodies. The New Zealand white rabbits fed a high cholesterol and fat diet for 2 weeks showed early intimal lipid accumulation in the aorta and prominent lipid accumulation in hepatocytes and macrophages of the liver and spleen.

Kunitomo *et al* (1983) studied synergism between high cholesterol and excess vitamin D_2 for the induction of atherosclerotic lesion in guinea pigs. Histopathological observation revealed intimal proliferation and calcification of the intima and media, but no atheroma was present at the sites of arterial injury. However, the biochemical findings revealed accumulation of cholesterol (mainly esterified) and calcium in the aorta.

Civen *et al* (1984) studied the effects of excess dietary cholesterol on adrenal cholesterol accumulation and steroidogenesis. Rats maintained on diets containing 3% cholesterol for 10 weeks show marked increase in adrenal cholesterol content. The greatest part of the increase was in the cholesterol ester fraction (329%), although free cholesterol was also elevated (140%). Morphologically, a marked increase in the lipid droplet content of the cells was observed. Neutral cytosolic cholesterol esterase was unaffected by the diet. No significant increase in cholesterol occurred in the mitochondrial fraction. Cholesterol supplementation elevated adrenal corticosteroid levels (43%). Mild stress resulted in greater increase in adrenal corticosteroids after dietary supplementation.

Thomas *et al* (1985) studied effects of supplemental dietary vitamin D and calcium on lipid distribution and aortic mineralization in young goats. Goats in the basal milk plus Ca^{++} diet group had plasma cholesterol concentrations that were 16.6% of those of the basal group. Generally, total lipid and cholesterol concentrations were unaltered in liver, other viscera, and carcass tissues. Dietary cholecalciferol increased concentrations of cholesterol and total lipid in aortas, whereas dietary calcium decreased total lipids in aortas. Concentrations of calcium, magnesium and total ash were increased in aortas by dietary treatment, with a marked increase observed in the basal and Ca plus vitamin D_3 diet group. Sudan IV and gross calcium staining in aortas revealed both lipid and mineral deposition.

Matsukuma *et al* (1998) studied histopathology of pancreatic ischemic lesions induced by cholesterol emboli. Pancreases had well-demarcated patchy lesions composed of degenerating acinar cells showing deeply eosinophilic cytoplasm and pyknotic nuclei, indicative of fresh ischemia. Few pancreases had patchy fibrotic foci containing small ductules with slightly retraction features. These patchy fibrotic foci were the healed ischemic lesions. The existence of remnant intralobular ductules and the patchy retraction features may be useful histological markers for the determination of healed ischemic lesions.

Ikezaki *et al* (1999) studied influences of long-term administration of 24R, 25-dihydroxyvitamin D_3, a vitamin D_3 derivative in rats. It was found that urinary calcium levels were significantly elevated; weights of the adrenals and femur were increased. Thickening of cortical bone in the femurs, and medullary hyperplasia and pheochromocytoma of the adrenals was reaveled histopathologically.

ClubbJr *et al* (2001) evaluated temporal distribution of leukocytes, macrophages, foam cells, vascular smooth muscle cells, and subendothelial lipid in Watanabe heritable hyperlipedimic (WHHL) rabbit aortas. Results indicated that lipid accumulation (extra- and intracellular) is important in the early development of atherosclerotic lesions; a corresponding, slower accumulation of adherent cells on the lesion surface promotes lipid conversion from fatty streak to plaque.

Rajashree *et al* (2002) reported upregulation of vitamin D_3 receptor in aortic smooth muscle cells in hypervitaminosis D, which lead to aortic calcification and aneurysm along with fragmentation of elastic fibers and extensive loss of elastic layers.

Togashi *et al* (2003) studied comparison between sclerotic changes of cardiac valve and blood vessel. Grossly, there was a significant correlation in sclerotic change between aorta (Ao) and aortic valve (AV), Ao and mitral valve (MV), AV and MV, coronary artery (CA) and AV, and CA and MV respectively. On gross observation, all valvular sclerosis showed yellowish thickening and/or calcification. Microscopically, hyalinous change of the fibrosa was observed in the yellowish lesion of the valves. Accumulations of foamy macrophages were found focally at the surface area of the fibrosa, but no atheromatous change was observed in the valves. Calcified deposits, if present, were found in the fibrous valvular ring or fibrosa with hyalinous degeneration. In MV, calcification was usually localized in the fibrous ring. However, in AV, valvular calcification extended diffusely in the fibrosa and caused stenosis in some cases. These lesions were similar to calcified area in the

intima with fibrous thickening of Ao and/or CA, but were different from atheromatous lesion of these tissues. Main causes of these differences are thought to be not only the shear stress, but also intramural pressure and mechanical stress with opening and closing, which may interfere with the sclerotic change of cardiac valves. Mechanism of valvular sclerosis may be different from arteriosclerosis because medial smooth muscle cells are absent in the valves.

Napolia and Palinski (2005) reviewed the pathogenic mechanisms from atherosclerosis in neurodegenerative diseases. Several pathogenic mechanisms promoting atherosclerosis are also involved in neurodegenerative diseases, and insight into the factors determining the susceptibility to, and long-term progression of, atherosclerosis may be of interest for the evolution of diseases such as Alzheimer's. Atherosclerosis of intracranial arteries or thromboembolic consequences of atherosclerotic extracranial arteries are responsible for most ischemic events in the brain.

Vance *et al* (2005) reviewed the mechanism of cholesterol homeostasis in neurons and glial cells. Cholesterol is highly enriched in the brain compared to other tissues. Whereas the average concentration of cholesterol in fresh tissues of whole animals is ~2.2 mg/g (Dietschy and Turley 2004) in the brain the concentration of cholesterol is much higher in the range of 15–20 mg/g (Dietschy and Turley 2004). The major source of cholesterol in most mammalian cells is de novo synthesis (Spady and Dietschy 1983, Dietschy *et al* 1993 and Dietschy and Turley 2002) although cholesterol can also be acquired from exogenously supplied plasma lipoproteins. Low density lipoproteins (LDLs) that contain cholesteryl esters are internalized by receptor-mediated endocytosis via the LDL receptor and related receptors. Elimination of the LDL receptor in mice and rabbits does not alter the rate of cholesterol synthesis, or the concentration of cholesterol, in the brain [Osono *et al* 1995 and Dietschy *et al* 1983]. Thus, cholesterol derived from HDLs and LDLs is not transferred from the plasma, across the blood–brain barrier, and into the CNS during either fetal or postnatal development

[Turley *et al* 1996]. When the rate of cholesterol synthesis in the CNS exceeds that required for cholesterol homeostasis there is a net export of cholesterol from the CNS to the plasma.Hess *et al* (2006) investigated the association between atherosclerosis and glomerulopathy in dogs. Dogs with atherosclerosis had significantly more glomerulopathy than the dogs with no histopathological evidence of atherosclerosis.

Madhumati *et al* (2006) evaluated serum lipid profile, gross and histopathological changes in experimentally induced atherosclerosis in rabbits. Serum levels of total cholesterol, triglycerides, low-density lipoprotein and very low density lipoprotein were found to be elevated. The histopathological changes were accumulation of the foam cells, atheromatous plaque formation and replacement fibrosis.

Tang *et al* (2006) investigated the role of hypercholesterolemia in vascular calcification and attributed oxidative stress to be its potential mechanism. Rats given vitamin D with high cholesterol diet significantly enhanced vessel calcium deposition and the activity and mRNA expression of vessel alkaline phosphate (AKPase) compared with vitamin D alone. Serum levels of total cholesterol, oxidized LDL, malondialdehyde and superoxide anion were elevated.

Targher *et al* (2006) studied the relations between carotid artery wall thickness and liver histology in subjects with nonalcoholic fatty liver disease. Nonalcoholic fatty liver disease (NAFLD) patients had a markedly greater carotid intima-media thickness (IMT) than control subjects. Carotid IMT was strongly associated with degree of hepatic steatosis, necroinflammation, and fibrosis among NAFLD patients. The results suggested that the severity of liver histopathology among NAFLD patients is strongly associated with early carotid atherosclerosis, independent of classical risk factors, insulin resistance, and the presence of metabolic syndrome.

Trivino *et al* (2006) reported that cholesterol-enriched diet induces ultrastructural changes in retinal and macroglial rabbit cells. Rabbits fed 0.5% cholesterol-enriched diet for 8 months exhibited alterations in all the retinal layers that were more intense in areas overlying altered retinal pigment epithelium (RPE). RPE changes showed no preferential location. Bruch's membrane was thicker as a result particle build-up in the collagen layers. Excess cholesterol induced ultrastructural changes in the rabbit retina similar to those in human age-related macular degeneration.

Ishizaka *et al* (2007) studied the association between serum albumin, carotid atherosclerosis, and metabolic syndrome in Japanese individuals. Serum albumin is a maker of nutritional status and possesses antioxidative properties. After adjusting for age, total cholesterol, and smoking status, the highest quartile of serum albumin was associated with increased prevalence of metabolic syndrome with an odds ratio of 1.80 in women, and 1.60 in men, when compared to the lowest serum albumin quartile. By contrast, when compared with the lowest quartile, the highest quartile of serum albumin was associated with reduced prevalence of carotid plaque with an odds ratio of 0.62 in women, and 0.76 in men, and for carotid intima-media thickening with an odds ratio of 0.57 in women, and 0.71 in men. Thus, higher serum albumin was inversely associated with the prevalence of early carotid atherosclerosis, although it was positively associated with the prevalence of metabolic syndrome.

Mackey *et al* (2007) reviewed the association between intimal or medial vascular calcification and arterial stiffness. Vascular calcification can occur in either the intimal or medial layers of the arterial wall. Intimal calcification is associated with atherosclerosis, which is characterized by lipid accumulation, inflammation, fibrosis and development of focal plaques. Medial calcification is associated with arterio sclerosis, i.e. age- and metabolic disease-related structural changes in the arterial wall which are related to increased arterial

stiffness. Medial calcification of elastic fibers contributes to increased arterial stiffness; however association between intimal calcification and arterial stiffness is less definitive.

Wouters *et al* (2008) reported that dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. Wild-type (WT) mice displayed only steatosis after a short-term high-fat diet with cholesterol (HFC), however female low-density lipoprotein receptor-deficient [ldlr(-/-)] and APOE2ki mice showed steatosis with severe inflammation characterized by infiltration of macrophages and increased nuclear factor kappaB (NF-kappaB) signaling. An HFC diet induced bloated, "foamy" Kupffer cells in male and female ldlr(-/-) and APOE2ki mice. Hepatic inflammation was found to be linked to increased plasma very low-density lipoprotein (VLDL) cholesterol levels. Omitting cholesterol from the HFC diet lowered plasma VLDL cholesterol and prevented the development of inflammation and hepatic foam cells.

Marechaux *et al* (2009) reported that hypercholesterolemia and Vitamin D induced rabbits developed abnormal leaflet thickening, with a significant alteration of aortic valve performance in Vitamin D animals. Leaflet thickening was related to the development of fatty plaque neolesions on the aortic side of the leaflets, displaying extracellular matrix disorganization, lipid and cellular infiltration and calcification in Vitamin D animals. Tissue Factor was found on the leaflet aortic side in control animals and was identified in aortic valve sclerosis lesions in both hypercholesterolemia and Vitamin D animals.

Kadono *et al* (2010) reported that serum albumin levels predict vascular dysfunction with paradoxical pathogenesis in healthy individuals. The highest tertile of albumin level (4.6–5.4 g/dl) was associated with increased odds ratios for hyperglycemia of 1.35 (1.07–1.70) compared to the middle tertile (4.4–4.5 g/dl), whereas the lowest tertile (3.3–4.3 g/dl) was associated with reduced odds ratios for hyperglycemia of 0.80 (0.65–0.99). The highest

tertile was also associated with increased odds ratios for metabolic syndrome of 1.30 (0.96–1.76) compared to the middle tertile, whereas the lowest tertile was associated with reduced odds ratios of 0.70 (0.51–0.95). Thus, the extremes of serum albumin levels were linked to vascular dysfunction among healthy individuals and serum albumin is paradoxically linked to vascular disease under conditions both of overnutrition and of malnutrition and inflammation complex.

Liu *et al* (2010) reviewed the role of cholesterol in the pathogenesis of neurodegenerative diseases. Cholesterol is an essential component of cell membranes which plays an important role in the maintenance of cellular homeostasis and transmembrane communication within and between cellular compartments. In the brain that contains the highest levels of cholesterol in the body, cholesterol traffic occurs between nerve cells and between intracellular organelles in neurons to subserve normal brain function. Whereas glial cells produce the largest quantities of cholesterol, neurons also acquire cholesterol synthesized by astrocytes. Cholesterol is implicated in regulating diverse cellular metabolisms, compartmental homeostasis, and molecular interactions in extracellular and intracellular communication. Deregulated cholesterol trafficking appears to be involved in the pathogenesis of Alzheimer's diseas, Parkinson's disease and Niemann–Pick disease type C diseases. Under the pathological conditions of these neurodegenerative diseases, aberrant molecular interactions or particular depositions of cholesterol have been observed as critical causes to precipitate neuronal cell death. Polar lipid cholesterol is also toxic to its host cell, and when accumulated, it causes cell death.

2.10 Effects of vitamin D_3 in atherosclerosis

Jurgens *et al* (1971) studied the influence of dietary supplements of vitamin D_3 or cholesterol, or both, upon blood serum cholesterol and the cholesterol and fatty acid content of certain tissue in rats. Rats fed cholesterol had elevated levels of serum and liver

cholesterol and increased per centage of body fat. The inclusion of excessive vitamin D_2 when fed in the presence of dietary cholesterol resulted in lowered serum cholesterol levels ($P < 0.01$) and dramatically increased liver cholesterol content suggesting that the serum cholesterol lowering effect of vitamin D_2 may be related in part to increased retention of cholesterol by the liver.

Major *et al* (2007) studied role of calcium and vitamin D supplementation on beneficial effect of weight loss on plasma lipid and lipoprotein concentrations. Significantly greater decreases in total: LDL and LDL:HDL and of LDL cholesterol were observed in the calcium and vitamin D group than in the placebo group. A tendency for more beneficial changes in HDL cholesterol, triacylglycerol, and total cholesterol was also observed in the calcium and vitamin D group.

Blum *et al* (2008) measured and reported the vitamin D_3 concentration in serum and subcutaneous fat samples from obese individuals and examined the association of vitamin D_3 in fat with vitamin D_3 in serum. Vitamin D_3 concentrations of fat tissue and serum were positively correlated. Fat tissue vitamin D_3 can be measured by LC/MS and is detectable in obese subjects with suboptimal vitamin D status. Compatible with the long-standing concept that fat tissue is a storage site for vitamin D, fat tissue and serum vitamin D_3 concentrations were positively correlated.

Zittermann A *et al* (2009) investigated the effect of vitamin D on weight loss and traditional and nontraditional cardiovascular disease risk markers in overweight subjects. A more pronounced decrease occurred in the vitamin D group than in the placebo group in blood concentrations of parathyroid hormone, triglycerides, and the inflammation marker-tumor necrosis factor. The results indicated that a vitamin D supplement of 83μg/d does not adversely affect weight loss and is able to significantly improve several cardiovascular disease risk markers in overweight subjects.

CHAPTER-III

MATERIAL AND METHODS

The proposed research work entitled "Studies on Effect of High Cholesterol and Vitamin D_3 on Cardiovascular System" was carried out at Department of Veterinary Pathology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, India. The experiment was conducted after approval by Institutional Animal Ethics Committee (IAEC), GADVASU, Ludhiana.

3.1 Materials

3.1.1 Procurement of experimental animals

Twenty-four adult Soviet Chinchilla rabbits (2.5-3.0 Kg in weight) used in this study were obtained from the Rabbit farm, Department of Livestock Production and Management (LPM), College of Veterinary Science, GADVASU, Ludhiana.

3.1.2 Maintenance of experimental animals

The rabbits were housed in individual cages with room temperature of around 18-22°C with a 12:12 h light-dark cycle. The rabbits were provided *ad libitum* feed (standard rabbit feed supplied by Godrej Agrovet Limited, Khanna) and drinking water. The feed composition was as follows:

Total protein	19%
Crude fibre	6%
Ether extract	3.4%
Calcium	1%
Phosphorus	0.4%
Acid insoluble ash	1.3%

3.1.3 Chemicals used for experiment

The cholesterol extra pure was purchased from Loba Chemie pvt. Ltd. Mumbai, India. Vitamin D_3 *(cholecalciferol)* was purchased from Sigma Life Science, St.Louis, USA. Vitamin D solution was prepared by dissolving known amount of cholecalciferol (27 mg) in groundnut oil (10 ml). The solution was kept in refrigerator and was brought to room temperature before use.

3.2 Methods

Protocol of Experiment

3.2.1 Name of Experiment: Studies on effect of hypervitaminosis D_3 in high cholesterol fed rabbits.

The rabbits were kept for 7 days to acclimatize in laboratory condition prior to start of sampling protocols. On day 7 (considered as day zero of the experiment), the rabbits were randomly divided in 4 groups (n=6 rabbits) as follows:

Group	No. of rabbits	Treatment
A	6	Control group with normal diet and was administered groundnut oil daily for 70 days.
B	6	Control group given vitamin D_3 @ 0.9 mg/Kg BW dissolved in groundnut oil from day 56 of experiment until death or maximum for 2 weeks.
C	6	Control group given cholesterol @ 400 mg/Kg BW dissolved in 4% groundnut oil in feed daily for 70 days.
D	6	Group given cholesterol @ 400 mg/Kg BW dissolved in 4% groundnut oil daily in feed daily + vitamin D_3 @ 0.9 mg/Kg BW dissolved in groundnut oil from day 56 of experiment until death or maximum for 14 days.

The rabbits were anaesthetized by using xylazine-ketamine combination anesthesia and blood samples were collected from the ear vein on day 0, 28, 56 and 70 or just before death in 1% heparin for biochemical and in EDTA for hematological analysis. The necropsy of dead rabbits was performed and relevant tissues were collected in 10% buffered formalin saline for histopathological studies and for atomic absorption assays of minerals in tissues. All the surviving rabbits were sacrificed on day 70 and samples from two rabbits of each group were collected in liquid nitrogen for enzymehistochemical studies.

3.2.2 Estimation of haematological parameters

The rabbits were sedated by using xylazine-ketamine combination anesthesia and blood samples were collected from the ear vein of rabbit on day 0, 28, 56 and 70 or just before death in EDTA vials for estimation of hematological parameters. The blood samples were immediately processed for estimation of hematological parameters viz. hemoglobin (Hb), total leukocyte count (TLC) and differential leukocyte count (DLC) as described below:

Sr. No.	**Parameters**	**Method/ Staining**	**Reference**	**Unit**
1	Hemoglobin (Hb)	Sahli's Hemoglobinometer	Benjamin (1985)	g/dL
2	Total Leukocyte Count (TLC)	Neubaur Chamber method (manually)	Benjamin (1985)	/µl
3	Differential Leukocyte Count (DLC)	Battlement method (100 cells counted) Giemsa-Wright staining	Benjamin (1985)	/µl

3.2.3 Estimation of biochemical parameters

The rabbits were sedated by using xylazine-ketamine combination anesthesia and blood samples were collected from the ear vein of rabbit on day 0, 28, 56 and 70 or just before death in 1% heparin solution for estimation of biochemical parameters. The collected samples were centrifuged immediately at 1500 rpm for 15 minutes and plasma was separated

and stored at 4^0C. These plasma samples were used for estimation of biochemical parameters. The biochemical parameters were estimated using auto analyzer (BIOTRAN BTR-830) and diagnostic reagent kits (Autopak) supplied by Bayer Diagnostics India Limited, Gujarat, India and (Labkit) CHEMELEX, S.A. Spain. The biochemical methods used for analysis of biochemical various parameters are given below:

Biochemical	**Method**	**Wavelength**	**Reference**	**Unit**
SGPT (ALT)	UV Kinetic (IFCC) method	340 nm	Expert panel of the IFCC on enzymes	U/L
SGOT (AST)	UV Kinetic (IFCC) method	340 nm	Expert panel of the IFCC on enzymes	U/L
AKP	PNPP method	405 nm	Klin 1972	U/L
Total Cholesterol	CHOD-POD enzymatic-colorimetric	505 nm	Meiattini *et al* 1978	mg/dL
HDL Cholesterol	Phosphotungstate method	500 nm	Miller *et al* 1977	mg/dL
LDL Cholesterol	Colorimetric enzymatic-liquid	600 nm	Okada *et al* 1998	mg/dL
Triglycerides	Enzymatic Colorimetric method	546 nm	Annoni *et al* 1982	mg/dL
Glucose	GOD POD method	546 nm	Trinder, 1964	mg/dL
Calcium	Cresolphthalein Complexone Method	575 nm	Henry and Dryer 1963	mg/dL
Phosphorus	UV endpoint method	340 nm	Amador and Urban 1977	mg/dL
Albumin	Bromocresol Green (BCG) method	628 nm	Webster 1977	g/dL
Total Protein	Biuret method	546 nm	Henry *et al* 1974	g/dL
BUN	UV method	340 nm	Talke and Schubert 1965	mg/dL
Creatinine	Picrate method	500 nm	Henry *et al* 1974	mg/dL

3.2.4 Tissue mineral estimation

Concentrations of different macro and micro-minerals like calcium, magnesium, copper, zinc, iron, sodium and potassium in aorta, heart, liver and kidney of rabbits were estimated using atomic absorption spectrometer (Aanalyst-700, Atomic Spectrometry, Perkin Elmer precisely). Formalin fixed tissue samples each weighing 200 mg were subjected to digestion by adding concentrated nitric acid and hydrochloric acid as 4.5 ml and 0.5 ml respectively and digested in Microwave Reaction System (Multiwave-300, Antron Parr) at 150°C with 1400 power for 40 minutes (10 min-Ramp, 10 min-Hold fan 1 and 20 min- Hold fan 2). Samples were diluted to a final volume of 10 ml with double distilled water and stored in Tarsons polyethylene bottles for later analysis by instrumental techniques.

The instrument measures the change in intensity of the UV light of a specific wavelength. Computerized calibration curve was constructed by running standards of various concentrations (10, 15 and 20 PPM) on the AAS and observing the corresponding absorbance. The concentration of trace elements in each tissue sample was calculated by comparing the absorbance produced by the sample with that produced by a series of standards.

3.2.5 Enzymehistochemistry

The necropsy of sacrificed rabbits was performed and fresh aorta, heart and liver tissue samples were quickly collected and stored in liquid nitrogen for histoenzymological studies. These tissue samples were subjected to cryostat sectioning at -20^{o}C on the cryostat microtome (Damon/ I.B.C. Division, Mass: U.S.A.). The 10-12 µm thick sections were obtained on clean glass slides and these were stored temporarily in deep freezer before incubation for the histoenzymic demonstration of enzymes as detailed below:

Enzyme	**Substrate**	**Method**	**Reference**

Alkaline Phosphatase (AKPase)	Napthol AS-MX phosphate disodium salt in combination with, Fast Blue R.R	Simultaneous coupling azo dye method using substituted napthols	Barka and Anderson (1963)
Succinic dehydrogenase (SDH)	Di-Na-succinate	Standard method of bond enzyme by Nitro BT method	Pearse (1972)
Lactic dehydrogenase (LDH)	Na-DL-lactate	-do-	-do-
Malic dehydrogenase (MDH)	L- Malic acid	-do-	-do-
Glucose 6 Phosphate dehydrogenase (G-6-PD)	Di-Na- Glucose 6 Phosphate	-do-	-do-
NADPH-diaphorase	Co-enzyme (NADPH)	-do-	-do-
NADH-diaphorase	Co-enzyme (NADH)	-do-	-do-
Acetyl cholinesterase (ACHEase)	Acetyl Thiocholine Iodide	Thiocholine method	El-Badawie and Schenk (1967)

3.2.6 Lipid demonstration in tissue sections

The fresh aorta, heart and liver tissue samples were subjected to cryostat sectioning at 10-12 µm thickness and were stained with Sudan-III stain (Luna 1968).

3.2.7 Collection, processing and staining of tissue samples for histopathology

The necropsies of dead rabbits due to toxicity were performed immediately and the tissue samples of various organs were collected in 10% buffered formalin saline for histopathological examination. The rabbits in the control group were anaesthetized by using xylazine-ketamine combination anesthesia and killed by exsanguinations. The formalin fixed tissues were washed overnight in running tap water, dehydrated in ascending grades of alcohol, and cleared in benzene. The 4-5 micron thick tissue sections were cut from the paraffin embedded tissues and were stained with haematoxylin and eosin stain (H&E) for routine histopathology. Von Kossa stain (Luna 1968) was used to demonstrate calcium in tissue sections.

3.2.8 Statistical analysis:

The results were expressed as mean ± standard error (SE). To assess the significance of the differences between the four groups of rabbits, statistical analysis was performed using one-way analysis of variance (ANOVA) for repeated measurements, with significance assessed at 5% confidence level. Microscopic lesion scoring was statistically determined by using Mann-Whitney *U* test.

CHAPTER-IV

RESULTS AND DISCUSSION

4.1 Clinical signs

On day zero of the experiment, rabbits from groups A, B, C and D were normal and alert. The feed and water intake was normal. Rabbits from group A (control feed) remained normal without any noticeable clinical signs until the end of the experimental period. Rabbits from group B (control feed + vitamin D_3) also did not show any sign until day 56. On day 70, these animals became dull and showed reduced appetite. Group C (control feed + cholesterol) rabbits did not show any abnormal signs of illness until day 70. Group D rabbits (cholesterol + vitamin D_3) showed decrease in feed and water intake and lethargy on day 60 onwards. Group B and D rabbits showed ruffled body coat, cachexia and diarrhea on day 65 onwards. Changes in body weight pattern are given in Table 1.

Group B and group C rabbits showed non-significant decrease in body weight however group D rabbits showed significant decrease on day 70. Group A rabbits did not show any significant change in body weight throughout the experiment. Harrington and Page (1983) reported 29% body weight loss in horse due to vitamin D_3 toxicity. The clinical signs in group B and D rabbits like dullness, diarrhea, progressive emaciation/weight loss, and dehydration observed in present study are in consonance with earlier findings reported in different species by Roberson *et al* 2000, Long 1984, Braun *et al* 2000 and Radostits *et al* 2000.

Table 1. Body weight of control and treated rabbits.

Mean body weight (Kg)				
Day	Group A (n=6)	Group B (n=6)	Group C (n=6)	Group D (n=6)

0	3.03 ± 0.15	3.29 ± 0.08	3.07 ± 0.11	3.11 ± 0.12
10	3.03 ± 0.24	3.40 ± 0.11	3.12 ± 0.10	3.11 ± 0.14
20	3.17 ± 0.19	3.47 ± 0.12	3.22 ± 0.11	3.08 ± 0.15
30	3.21 ± 0.21	3.48 ± 0.10	3.21 ± 0.09	3.14 ± 0.08
40	3.26 ± 0.21	3.48 ± 0.08	3.11 ± 0.10	3.08 ± 0.13
50	3.28 ± 0.23	3.46 ± 0.07	3.04 ± 0.07	2.94 ± 0.17
60	3.33 ± 0.23	3.23 ± 0.02	3.03 ± 0.08	2.73 ± 0.17
70 or death	3.34 ± 0.23[a]	3.13 ± 0.04[a]	3.04 ± 0.08[a]	2.60 ± 0.15[b]

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D

Values indicate mean± S.E. mean± S.E. within a column lacking a common superscript differ at P=0.05.

4.2 Mortality pattern

Two of the group B rabbits died on day 68 and 69 of the experiment while four other survived until the end of experiment. Three rabbits of the group D died on day 67,68 and 70.

4.3 Effect on hematological parameters

The detailed results of hematological studies are summarized in Tables 2, 3, 4 and 5 and graphs Fig.1, 2, 3 and 4.

The mean Hb concentration, TLC and DLC had no significant change in group A, B, C and D rabbits on day zero. The mean Hb concentration of group D rabbits decreased significantly to that of group A rabbits on day 28. The mean Hb concentration of group C and D rabbits decreased significantly from group A and B rabbits on day 56 of the experiment. The mean Hb concentration of group A, B, C and D rabbits differed significantly from each other on day 70 of the experiment. Dijk *et al* (2006) reported that decreased hemoglobin levels are associated with an increased risk of coronary mortality and morbidity and increased hemoglobin levels are associated with reduced severity of atherosclerosis. Harusato *et al* (ahead of print) observed negative correlations between hemoglobin concentration and

pulse wave velocity and also between hemoglobin concentration and plaque score. Abdelhalim and Moussa (2010) reported that hyperlipidemia, lipid peroxidation and free radicals promote oxidation of Hb and reduce its concentration. So the present results are in accordance with other studies.

The mean TLC of group C and D rabbits increased significantly from group A and B rabbits on day 56 of the experiment. The mean TLC of group C rabbits increased significantly from that of group B and D rabbits. A higher TLC may reflect the existence of clinical or subclinical harmful inflammatory activity. Many of the non-infectious health problems, such as atherosclerosis and hypertension, associated with a higher TLC are considered risk factor for cardiovascular diseases (Facchini *et al* 1992). Lee *et al* (2001) reported that an elevated TLC is directly associated with increased incidence of coronary heart disease and ischemic stroke and mortality from cardiovascular disease. Several prospective studies have shown a positive and independent association between WBC count and coronary heart disease incidence, ischemic stroke or mortality (Kannel *et al* 1992, Zalokar *et al* 1981, Prentice *et al* 1982, Folsom *et al* 1997, Gillum *et al* 1993 and Lee *et al* 2001). Feldman *et al* (1991) reported increased number of circulating leukocytes within 1 week of a cholesterol-rich diet, prior to the development of atherosclerosis in animal experiments. Facchini *et al* (1992) reported that TLC is significantly correlated with changes in carbohydrate and lipoprotein metabolism and blood pressure that increase the risk of coronary heart disease (CHD).

In the present investigation, group fed cholesterol only showed increase in TLC which is in accordance with other studies. Group B rabbits showed decrease in TLC on day 70 as it was given vitamin D_3 on day 56. This could have been due to the immunosuppressive effect of vitamin D. Group D rabbits showed increase in TLC till day 56 due to cholesterol

feeding but from day 56 to 70, decrease in TLC would have been due to feeding of vitamin D_3 along with cholesterol.

Differential Leukocyte count (DLC)

The heterophil percentage of group C and D rabbits increased significantly from group A and B rabbits on day 56 of the experiment. The mean heterophil percentage of group B and D rabbits increased significantly to that of group A rabbits. The mean heterophil percentage of group D rabbits increased significantly to that of group C rabbits. Hypercholesterolemia can increase the cholesterol content of polymorphonuclear leukocytes thereby acting as a source of oxygen free radicals (Esterbauer *et al* 1992). Wheeler *et al* (2004) reported that the association of coronary heart disease with neutrophil count was somewhat stronger than that with other specific leukocyte components. Epidemiological studies have shown that leukocyte counts in peripheral blood are correlated positively with coronary atherosclerotic risk (Kostis *et al* 1984) and risk of acute myocardial infarction (AMI) (Friedman *et al* 1974). Naruko *et al* (2002) reported that neutrophil activation may be one of the inflammatory components of acute coronary syndromes and that neutrophil contributes to the pathogenesis of plaque destabilization in human atherosclerotic plaques. The results in present study are in accordance with other studies.

The mean lymphocyte percentage of group C and D rabbits decreased significantly from group A and B rabbits on day 56 of the experiment. The mean lymphocyte count of group D rabbits decreased significantly to that of group A, B and C rabbits on day 70. Nunez *et al* (2010) reported that lymphopenia has been associated with atherosclerosis progression and adverse outcomes in cardiovascular diseases. Studies have also reported that the relative lymphocyte count may have prognostic significance in patients with known or suspected coronary artery disease (CAD) (Ommen *et al* 1997). Zouridakis (2000) reported that a low lymphocyte count provides significant prognostic information in patients with unstable

angina. Brown *et al* (1999) reported that vitamin D_3 caused cell cycle arrest and inhibited proliferation of most cell types, including lymphocytes.

Shioi *et al* (2002) observed that immunosuppressant vitamin D has macrophages and lymphocytes as important target cells. Monocyte derived macrophages play a central role in atherogenesis through accumulation of cholesterol and the production of inflammatory mediators and cytokines. But no significant alteration in monocyte percentage was found in the present study. This could be due to a rapid influx of monocytes into the arterial intima after induction of hyperlipidemia, generating chronic inflammation characteristic of the atherosclerotic plaque (Ross, 1999). Eosinophil percentage did not show any significant alteration throughout the experiment.

Table 2. Hematological observations in control and treated rabbits on day 0.

Parameter		**Group A (n=6)**	**Group B (n=6)**	**Group C (n=6)**	**Group D (n=6)**
Hb (%)		11.3 ± 0.15	11 ± 0.45	10.9 ± 0.23	11.1 ± 0.15
TLC (/ µl)		7958.33 ± 196.39	7458.33 ± 498.56	7833.33 ± 199.86	7416.66 ± 604.24
DLC	H (/µl)	2009.67 ± 109.55	1786.91 ± 117.88	2057.83 ± 139.33	1838.33 ± 178.74
	L (/µl)	5802.92 ± 223.35	5516.08 ± 372.89	5627.58 ± 112.28	5341.91 ± 449.79
	M (/µl)	93.25 ± 24.11	60.50 ± 28.66	51.25 ± 26.28	125.41 ± 29.37
	E (/µl)	52.5 ± 25.56	94.83 ± 40.38	96.66 ± 51.91	111.0 ± 66.13

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D

Hb= Hemoglobin; TLC=Total Leukocyte Count; DLC= Differential Leukocyte Count, H= Heterophils; L: Leukocytes; M= Monocytes; E= Eosinophils

Values indicate mean± S.E.
mean± S.E. within a row lacking a common superscript differ at *P*=0.05.

Table 3. Hematology observations in control and treated rabbits on day 28.

Parameter		**Group A (n=6)**	**Group B (n=6)**	**Group C (n=6)**	**Group D (n=6)**
Hb (%)		11.4 ± 0.15[a]	10.73 ± 0.23[ab]	10.80 ± 0.13[ab]	10.43 ± 0.18[b]
TLC (/µl)		7916.67 ± 267.91	7575.0 ± 295.73	8058.0 ± 300.11	7991.66 ± 253.45
DLC	H (/µl)	1974.08 ± 62.65	1814.16 ± 101.74	2015.0 ± 160.97	2044.33 ± 160.45
	L (/µl)	5822.67 ± 227.89	5644.75 ± 258.95	5916.16 ± 207.07	5748.41 ± 484.67
	M (/µl)	67.91 ± 26.61	38.41 ± 17.28	75.33 ± 26.38	108.25 ± 28.74
	E (/µl)	52.0 ± 16.55	77.66 ± 34.71	51.83 ± 24.97	90.66 ± 37.55

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D
Hb= Hemoglobin;
TLC=Total Leukocyte Count; DLC= Differential Leukocyte Count, H= Heterophils; L: Leukocytes; M= Monocytes; E= Eosinophils
Values indicate mean± S.E.
mean± S.E. within a row lacking a common superscript differ at *P*=0.05.

Table 4. Hematology observations in control and treated rabbits on day 56.

Parameter		**Group A (n=6)**	**Group B (n=6)**	**Group C (n=6)**	**Group D (n=6)**
Hb (%)		11.46 ± 0.17[a]	11.06 ± 0.13 [a]	8.20 ± 0.41[b]	8.40 ± 0.30[b]
TLC (/µl)		8141.67±568.83[a]	7733.33± 83.33[a]	10241.67± 453.39[b]	10825.0± 403.26[b]
DLC	H (/µl)	2176.25 ± 218.49[a]	2060.0 ± 93.14[a]	3610.5 ± 252.11[b]	3846.75 ± 207.68[b]
	L (/µl)	5838.75 ± 356.65	5543.33 ± 142.51	6321.16 ± 359.21	6603.75 ± 269.73
	M (/µl)	82.0 ± 31.83[a]	51.83 ± 25.92[a]	244.66 ± 50.39[b]	270.08 ± 45.29[b]
	E (/µl)	44.66 ± 20.26	78.16 ± 45.61	65.33 ± 20.95	104.41 ± 25.86

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D

Hb= Hemoglobin; TLC=Total Leukocyte Count; DLC= Differential Leukocyte Count, H= Heterophils; L: Leukocytes; M= Monocytes; E= Eosinophils
Values indicate mean± S.E.
mean± S.E. within a row lacking a common superscript differ at *P*=0.05.

Table 5. Hematology observations in control and treated rabbits on day 70 or just before death.

Parameter		**Group A (n=6)**	**Group B (n=6)**	**Group C (n=6)**	**Group D (n=6)**
Hb (%)		10.76 ± 0.14^{a}	9.60 ± 0.23^{b}	8.30 ± 0.23^{c}	7.50 ± 0.08^{d}
TLC (/µl)		8600.0 ± 853.62^{ab}	6850.00 ± 425.24^{a}	11008.33 ± 115.77^{b}	7858.33±759.32^{a}
DLC	H (/µl)	2330.75 ± 256.42^{a}	2773.0 ± 255.97^{ab}	3910.58 ± 154.29^{c}	3639.0 ± 309.21^{bc}
	L (/µl)	6053.33 ± 628.45^{a}	3968.5 ± 221.12^{b}	6765.75 ± 90.62^{a}	3996.33 ± 578.83^{b}
	M (/µl)	144.58 ± 34.59^{ab}	88.5 ± 15.75^{a}	276.25 ± 26.98^{b}	184.0 ± 40.36^{ab}
	E (/µl)	70.33 ± 25.47	20.0 ± 20.0	55.75 ± 38.28	39.0 ± 17.85

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D
Hb= Hemoglobin; TLC=Total Leukocyte Count; DLC= Differential Leukocyte Count, H= Heterophils; L: Leukocytes; M= Monocytes; E= Eosinophils
Values indicate mean± S.E.
mean± S.E. within a row lacking a common superscript differ at *P*=0.05.

Thus the hematological studies suggest that hypercholesterolemia elevates TLC and heterophil percentages, however it reduces Hb and lymphocyte percentage. Subsequent vitamin D_3 administration in high cholesterol fed rabbit resulted in decrease in TLC and lymphocyte percentage. Thus, it is concluded that vitamin D_3 is helpful in atherosclerosis by decreasing TLC however, increase in heterophil percentage has detrimental effects.

4.4 Biochemical studies

Results of biochemical parameters are summarized in Table 6, 7, 8, and 9. On day 0, various biochemical parameters in all groups were within normal range and did not show any significant difference between the groups.

On day 28, 56 and 70, the mean total cholesterol concentration in groups C and D rabbits increased significantly (*P*= 0.05) as compared to group A and B rabbits. On day 28 and 56, group C rabbits showed significant increase in mean total cholesterol to that of group D rabbits. On day 56, the mean HDL concentration of group D rabbits increased significantly to that of group A and B rabbits while, group C rabbits mean HDL concentration increased significantly to that of group B rabbits. On day 70, the mean HDL concentration of group D rabbits increased significantly to that of group A, B and C rabbits (Table 7, 8 and 9). These results are consistent with earlier reports (Prasad 1999 and 2005, Vijaimohan *et al* 2006, Kaur and Bansal 2009, Solberg and Strong 1983) which suggest a correlation between dietary lipids and serum lipid profile. Jain *et al* (2007) also reported that serum total cholesterol, LDL-cholesterol, VLDL cholesterol and triglycerides levels increased significantly after cholesterol feeding. Furthermore, the total cholesterol: HDL-cholesterol ratio and LDL-cholesterol: HDL-cholesterol ratios were also increased significantly (Ram 1996). Atherosclerosis is rapidly developed in rabbits fed on excessive amounts of cholesterol (Yamakoshi *et al* 2000, Daley *et al* 1995, Sun *et al* 2000). Table 6. Biochemical observations in rabbits on day zero.

Parameter	**Group A (n=6)**	**Group B (n=6)**	**Group C (n=6)**	**Group D (n=6)**
AST (IU/dl)	35.17 ± 8.93	36.96 ± 7.00	36.56 ± 8.7	36.01 ± 8.7
ALT (IU/dl)	24.91 ± 4.8	27.67 ± 4.41	27.30 ± 3.21	25.78 ± 4.58
AKP (IU/dl)	42.40 ± 8.99	35.64 ± 8.2	37.80 ± 8.28	40.62 ± 7.63
Glucose (g/dl)	156.6 ± 4.39	152.32 ± 5.80	161.24 ± 2.57	158.58 ± 2.7
Ca^{++} (mg/dl)	13.56 ± 0.5	12.96 ± 0.42	14.67 ± 0.97	13.45 ± 1.03
Phosphorus (mg/dl)	5.05 ± 0.33	4.77 ± 0.36	4.96 ± 0.39	4.84 ± 0.37
Total Protein (g/dl)	6.31 ± 0.24	5.81 ± 0.35	6.00 ± 0.15	6.38 ± 0.34
Albumin (g/dl)	3.48 ± 0.09	3.18 ± 0.13	3.31 ± 0.21	3.47 ± 0.22

Globulin (g/dl)	2.83 ± 0.25	2.63 ± 0.25	2.69 ± 0.12	2.92 ± 0.16
Albumin/Globulin	1.3 ± 0.16	1.25 ± 0.10	1.25 ± 0.13	1.19 ± 0.05
T. Cholesterol (mg/dl)	88.93 ± 4.11	86.06 ± 5.48	78.85 ± 2.97	78.65 ± 5.59
Triglycerides (mg/dl)	78.89 ± 2.18	78.43 ± 2.73	76.51 ± 2.12	72.92 ± 1.01
HDL (mg/dl)	34.43 ± 1.7	33.7 ± 3.08	30.36 ± 1.16	30.04 ± 3.29
LDL (mg/dl)	34.78 ± 1.9	34.65 ± 2.36	32.34 ± 1.48	31.30 ± 1.56
VLDL(mg/dl)	19.71 ± 1.08	17.7 ± 0.97	16.14 ± 1.30	17.3 ± 1.14
T. Cholesterol/ HDL	2.59 ± 0.07	2.58 ± 0.07	2.60 ± 0.07	2.67 ± 0.10
LDL/ HDL	1.01 ± 0.04	1.04 ± 0.05	1.06 ± 0.04	1.07 ± 0.06
Creatinine (mg/dl)	1.48 ± 0.12	1.46 ± 0.06	1.19 ± 0.18	1.36 ± 0.09
BUN (mg/dl)	21.37 ± 1.56	20.62 ± 0.56	21.22 ± 2.86	21.33 ± 0.53

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D Values indicate mean± S.E.

Mean± S.E. within a row lacking a common superscript differ at P=0.05.

Table 7. Biochemical observations in rabbits on day 28.

Parameter	**Group A (n=6)**	**Group B (n=6)**	**Group C (n=6)**	**Group D (n=6)**
AST (IU/dl)	31.83 ± 1.98^{ab}	27.04 ± 1.98^{a}	37.17 ± 1.07^{b}	34.09 ± 0.96^{b}
ALT (IU/dl)	26.93 ± 2.11^{a}	26.42 ± 1.30^{a}	47.30 ± 4.45^{b}	52.51 ± 5.17^{b}
AKP (IU/dl)	33.09 ± 1.95	33.39 ± 1.29	41.5 ± 1.78	43.54 ± 4.38
Glucose (g/dl)	159.36 ± 1.96^{a}	154.61 ± 2.84^{a}	165.2 ± 9.0^{ab}	178.99 ± 6.44^{b}
Ca^{++} (mg/dl)	13.77 ± 0.27^{a}	13.43 ± 0.29^{a}	15.45 ± 0.56^{b}	15.64 ± 0.24^{b}
Phosphorus (mg/dl)	3.63 ± 0.22^{a}	4.12 ± 0.36^{a}	7.06 ± 0.25^{b}	6.69 ± 0.21^{b}

Total Protein (g/dl)	6.30 ± 0.25	6.09 ± 0.32	6.33 ± 0.22	6.35 ± 0.19
Albumin (g/dl)	3.40 ± 0.12	3.26 ± 0.17	3.36 ± 0.14	3.29 ± 0.16
Globulin (g/dl)	2.89 ± 0.24	2.83 ± 0.19	2.96 ± 0.09	3.05 ± 0.09
Albumin/Globulin	1.23 ± 0.14	1.18 ± 0.09	1.14 ± 0.03	1.08 ± 0.06
T. Cholesterol (mg/dl)	84.01 ± 5.85[a]	78.97 ± 3.84[a]	623.00 ± 23.91[b]	530.32 ± 20.51[c]
Triglycerides (mg/dl)	102.43 ± 14.70	83.74 ± 4.09	147.15 ± 12.79	182.95 ± 46.95
HDL (mg/dl)	31.87 ± 2.71	30.21 ± 1.6	35.13 ± 2.02	34.32 ± 3.47
LDL (mg/dl)	33.69 ± 2.38[a]	31.32 ± 1.63[a]	495.95 ± 25.08[b]	423.02 ± 21.25[C]
VLDL (mg/dl)	18.45 ± 0.87[a]	17.43 ± 0.83[a]	91.91 ± 9.86[b]	72.96 ± 4.66[b]
T. Cholesterol/ HDL	2.65 ± 0.05[a]	2.62 ± 0.06 [a]	18.13 ± 1.51 [b]	16.28 ± 1.75 [b]
LDL/ HDL	1.06 ± 0.03 [a]	1.04 ± 0.04 [a]	14.52 ± 1.49 [b]	13.02 ± 1.51 [b]
Creatinine (mg/dl)	1.55 ± 0.10	1.56 ± 0.07	1.61 ± 0.12	1.73 ± 0.06
BUN (mg/dl)	21.38 ± 1.19	21.25 ± 0.34	21.31± 0.79	21.78 ± 0.65

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D

Values indicate mean± S.E.

Mean± S.E. within a row lacking a common superscript differ at *P*=0.05.

Table 8. Biochemical observations in rabbits on day 56.

Parameter	**Group A (n=6)**	**Group B (n=6)**	**Group C (n=6)**	**Group D (n=6)**
AST (IU/dl)	34.34 ± 7.56[a]	38.82 ± 10.16[a]	131.59 ± 5.31[b]	165.44 ± 23.45[b]
ALT (IU/dl)	55.94 ± 9.38[a]	49.60 ± 13.24[a]	178.68 ±28.94[b]	291.32 ±26.85[c]
AKP (IU/dl)	24.00 ± 2.72	26.13 ± 5.63	28.85 ± 2.76	27.33 ± 2.55

Glucose (g/dl)	176.05 ± 19.05	214.83 ± 32.3	147.02 ± 8.31	150.29 ± 13.40
Ca^{++} (mg/dl)	13.37 ± 0.25[a]	11.81 ± 0.39[b]	14.36 ± 0.20[a]	14.4 ± 0.33[a]
Phosphorus (mg/dl)	5.19 ± 0.29[a]	5.14 ± 0.17[a]	7.14 ± 0.64[a]	9.94 ± 0.99[b]
Total Protein (g/dl)	6.11 ± 0.05	6.31 ± 0.13	6.54 ± 0.08	6.61 ± 0.17
Albumin (g/dl)	3.42 ± 0.13[a]	3.19 ± 0.02[ab]	2.66 ± 0.10[c]	2.95 ± 0.06[bc]
Globulin (g/dl)	2.67 ± 0.12[a]	3.11 ± 0.12[ab]	3.88 ± 0.13[c]	3.66 ± 0.17[bc]
Albumin/Globulin	1.29 ± 0.11[a]	1.02 ± 0.03[ab]	0.69 ± 0.05[c]	0.81 ± 0.05[bc]
T. Cholesterol (mg/dl)	95.91 ± 6.14[a]	98.60 ± 3.19[a]	598.46 ± 44.59[b]	800.05 ± 92.22[b]
Triglycerides (mg/dl)	96.29 ± 6.57	84.87 ± 5.85	217.17 ± 37.26	224.33 ± 70.87
HDL (mg/dl)	39.45 ± 3.22[a]	39.74 ± 1.9[a]	47.56 ± 2.25[ab]	54.09 ± 4.6[b]
LDL (mg/dl)	37.79 ± 2.19[a]	39.55 ± 1.0[a]	459.44 ± 42.23[b]	643.93 ± 85.38[b]
VLDL (mg/dl)	18.66 ± 1.4[a]	19.3 ± 1.27[a]	91.45 ± 3.73[b]	102.03 ± 3.19[c]
T. Cholesterol/ HDL	2.45 ± 0.09 [a]	2.49 ± 0.08 [a]	12.64 ± 0.83 [b]	14.62 ± 0.56 [b]
LDL/ HDL	0.97 ± 0.06 [a]	1.00 ± 0.04 [a]	9.69 ± 0.77 [b]	11.68 ± 0.65 [b]
Creatinine (mg/dl)	1.47 ± 0.06	1.50 ± 0.02	1.48 ± 0.07	1.33 ± 0.08
BUN (mg/dl)	23.04 ± 2.27	24.63 ± 2.15	42.86 ± 2.1	41.47 ± 3.92

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D

Values indicate mean± S.E.

Mean± S.E. within a row lacking a common superscript differ at *P*=0.05.

Table 9. Biochemical observations in rabbits on day 70 or just before death.

Parameter	**Group A (n=6)**	**Group B (n=6)**	**Group C (n=6)**	**Group D (n=6)**
AST (IU/dl)	48.06 ±	41.19 ± 1.92[a]	163.06 ±	202.58 ±

	6.10[a]		7.01[b]	21.02[b]
ALT (IU/dl)	46.82 ± 12.03[a]	38.85 ± 1.85[a]	198.35 ± 31.02[b]	311.97 ± 27.32[c]
AKP (IU/dl)	50.04 ± 8.61[a]	56.84 ± 3.73[a]	42.37 ± 2.97[a]	79.63 ± 3.65[b]
Glucose (g/dl)	145.96 ± 13.00[ab]	189.93 ± 4.78[b]	107.9 ± 3.14[a]	178.90 ± 16.79[b]
Ca^{++} (mg/dl)	11.37 ± 0.96[a]	17.70 ± 0.64[b]	12.03 ± 0.36[a]	17.61 ± 0.50[b]
Phosphorus (mg/dl)	5.94 ± 0.63[a]	8.60 ± 0.12[ab]	7.18 ± 1.26[a]	12.67 ± 1.34[b]
Total Protein (g/dl)	6.6 ± 0.12[a]	6.83 ± 0.16[ab]	7.11 ± 0.31[ab]	7.85 ± 0.29[b]
Albumin (g/dl)	3.72 ± 0.05[a]	3.37 ± 0.39[a]	2.80 ± 0.10[b]	2.82 ± 0.10[b]
Globulin (g/dl)	2.88 ± 0.10[a]	3.45 ± 0.55[ab]	4.31 ± 0.22[bc]	5.03 ± 0.19[c]
Albumin/Globulin	1.29 ± 0.05[a]	1.05 ± 0.24[a]	0.65 ± 0.02[b]	0.56 ± 0.00[b]
T. Cholesterol (mg/dl)	92.43 ± 3.31[a]	83.38 ± 7.99[a]	939.07 ± 91.03[b]	1082.2 ± 32.54[b]
Triglycerides (mg/dl)	115.45 ± 11.25[a]	76.95 ± 17.81[a]	283.42 ± 81.44[ab]	555.58 ± 144.09[b]
HDL (mg/dl)	36.82 ± 2.24[a]	30.5 ± 3.77[a]	56.28 ± 3.53[a]	54.28 ± 2.55[b]
LDL (mg/dl)	36.29 ± 1.03[a]	33.54 ± 2.82[a]	787.32 ± 87.53[b]	930.38 ± **33**.05[b]
VLDL (mg/dl)	19.31 ± 0.87[a]	19.33 ± 1.89[a]	95.47 ± 2.17[b]	97.54 ± 3.78[b]
T. Cholesterol/ HDL	2.52 ± 0.06[a]	2.76 ± 0.09 [a]	16.53 ± 0.76 [b]	20.15 ± 1.13 [c]
LDL/ HDL	0.99 ± 0.05 [a]	1.12 ± 0.07 [a]	13.8 ± 0.83 [b]	17.33 ± 1.07 [c]
Creatinine (mg/dl)	1.38 ± 0.16[a]	1.68 ± 0.12[a]	1.34 ± 0.13[a]	3.21 ± 0.56[b]

BUN (mg/dl)	22.42 ± 1.72[a]	71.75 ± 2.65 [b]	76.43 ± 3.16 [b]	96.67 ± 6.17 [b]

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D

Values indicate mean± S.E.

Mean± S.E. within a row lacking a common superscript differ at P=0.05.

On lowering of cholesterol level, atherosclerosis lacks progression or is even regressed (Small, 1988). Epidemiological studies have demonstrated that HDL-cholesterol is a strong, independent and inverse predictor of cardiovascular disease risk (Castelli *et al* 1986, Assmann *et al* 1996, Sharrett *et al* 2001, Gordon *et al* 1989, Wierzbicki 2005 and Solberg and Strong 1983) and is found to possess anti-atherogenic activity (Zuliani *et al* 2007).

On day 28, 56 and 70 the mean LDL concentration of groups C and D rabbits increased significantly to that of group A and B rabbits (Table 7, 8 and 9). Oxidative modification of low-density lipoprotein cholesterol (LDL) appears to have an important role in initiation and progression of atherogenic changes in aorta (Esterbauer *et al* 1993). The plasma level of LDL in hyperlipidemic patients and the impairment of endothelial function in arteries are events directly associated with the occurrence of atherosclerosis (Farmer and Gotto 2002, Jayakody *et al* 1988, 1987, 1985 and Dominiczak 1998). Epidemiological studies using a large number of subjects also establish that LDL associated cholesterol levels in serum correlates with atherosclerosis (Kannel *et al* 1979). Elevated plasma LDL levels accelerate atherosclerosis (Solberg and Strong 1983, Tyroler 1987, Goldstein and Brown 1977 and Steinberg 1983) while reduction of total cholesterol or low density lipoprotein cholesterol (LDL) is associated with decreased risk of atherosclerosis and coronary heart disease (Brown *et al* 1998 and Grundy *et al* 2004).

On day 28 and 70 the mean triglyceride concentration of group D rabbits increased significantly from that of groups A and B rabbits. On day 56, the mean triglyceride concentration of group C rabbits increased significantly to that of group B rabbits. On day 28, the mean VLDL concentration of group C rabbits increased significantly from that of group A and B rabbits. On day 56, the mean VLDL concentration of groups C and D rabbits

increased significantly to that of group B rabbits. On day 70, the mean VLDL concentration of group D rabbits increased significantly to that of groups A and B rabbits (Table 7, 8 and 9). Elevated serum triglycerides are considered as independent risk factor for cardiovascular disease (Asia Pacific Cohort Studies Collaboration 2004). More recent case-control studies in patients with premature coronary artery disease have shown that total triglyceride and VLDL levels discriminate better between subjects with and without coronary artery disease. An increased number of VLDL particles are strongly associated with CHD, independently of traditional risk factors or newly recognized atherogenic lipoproteins, such as IDL or small, dense LDL (Koba *et al* 2002).

On day 28, the mean AST concentration of groups C and D rabbits increased significantly to that of group B rabbits. On days 56 and 70, the mean AST concentration of groups C and D rabbits increased significantly to that of groups A and B rabbits. On day 28, the mean ALT concentration of groups C and D rabbits increased significantly to that of groups A and B rabbits. On days 56 and 70, the mean ALT concentration of groups C and D rabbits increased significantly to that of groups A and B rabbits; while group C rabbits differed significantly from that of group D rabbits. On day 70, the mean AKP concentration of group D rabbits increased significantly to that of groups A, B and C rabbits (Table 7, 8 and 9). Obesity and hyperlipidemia are frequent in patients with abnormal serum activities of liver enzymes (Daniel *et al* 1999). The findings show that up to one third of hyperlipidemic patients display hepatic enzyme levels that are superior to the upper limit of normal; and in the multivariate analysis, serum levels of triglyceride and HDL-cholesterol are positively and negatively associated, respectively, with hepatic enzyme activities in women, but not in men (Bruckert *et al* 2002). Serum ALT levels are positively associated with the risk of carotid atherosclerosis in patients with non-alcoholic fatty liver disease (NAFLD), suggesting that

serum ALT levels could serve as a surrogate marker of cardiovascular risk in this special clinical setting.

Patel *et al* (2007) reported that even within the reference range of ALT enzyme, excess prevalence of clinically high-risk conditions of obesity, dyslipidemia, hypertension, and metabolic syndrome were noted among those who were persistently in the highest vs. lowest quintile distribution of this enzyme. Increased serum AST level may be independently associated with CAD (Acikel *et al* 2009), suggesting that serum ALT levels could serve as a surrogate marker of cardiovascular risk (Wang *et al* 2009). Tonelli *et al* (2009) found an independent relation between higher levels of AKP and adverse outcomes among survivors of myocardial infarction and in a general population sample.

On day 28, the mean calcium concentration of groups C and D rabbits increased significantly to that of groups A and B rabbits. On day 56, the mean calcium concentration of groups A, C and D rabbits increased significantly to that of group B rabbits. On day 70, the mean calcium concentration of groups B and D rabbits increased significantly to that of groups A and C rabbits (Table 7, 8 and 9). Elevated serum calcium levels are associated with subclinical atherosclerosis, which is itself predictive of stroke risk. Serum calcium levels were positively associated with carotid plaque thickness, a powerful early predictor of clinical coronary and cerebrovascular events (Rubin *et al* 2007). Excess vitamin D_2 administration followed by treatment with a high-cholesterol diet is characterized by medial calcification and intimal cell proliferation in atherosclerosis model (Kitagawa *et al* 1992 and Kwatra *et al* 1974). Coronary artery disease burden was significant correlated with plasma calcium, but not with plasma phosphorus (Nunes 2005). In vitamin D_3 toxicity, the active metabolites of cholecalciferol have been reported to increase the blood calcium (hypercalcemia) level by increased resorption/mobilization of calcium from bone, increased absorption of calcium from intestine and decreased calcium excretion by kidney. The net

result is high concentration of blood calcium level (hypercalcemia) and death reported due to renal and cardiac failure (Beasley 1999, Radostits *et al* 2000 and Price *et al* 2001).

On day 28, the mean phosphorus concentration of groups C and D rabbits increased significantly to that of groups A and B rabbits. On day 56, the mean phosphorus concentration of group D rabbits increased significantly to that of groups A, B and C rabbits. On day 70, the mean phosphorus concentration of group D rabbits increased significantly to that of groups A and C rabbits (Table 7, 8 and 9). Higher phosphorus levels are associated with peripheral atherosclerosis and arterial stiffness, independent of traditional risk factors for cardiovascular disease (Tonelli *et al* 2005, Dhingra *et al* 2007, Onufrak *et al* 2008, Meng *et al* 2010, Foley *et al* 2009, Block *et al* 2004, Young *et al* 2004, Kestenbaum *et al* 2005, Kalantar-Zadeh *et al* 2006, Menon *et al* 2005 and Kwatra *et al* 1974). Healthy adults with higher levels of phosphate in the blood are more likely to have increased levels of calcium in the coronary arteries—a key indicator of atherosclerosis and future cardiovascular disease risk. Phosphate-lowering drugs—generally used only in patients with end-stage renal disease on dialysis—might help to reduce cardiovascular risk in CKD patients and even in healthy adults with high-normal phosphate levels. Narang *et al* (1997) reported that plasma phosphorus, but not plasma calcium, had an independent positive association with the angiographic severity of coronary disease. Additionally, dietary P restriction ameliorated vascular calcification in mice with hyperphosphatemia and hypervitaminosis D (Stubbs *et al* 2007). Morrow (2001) reported that, in case of acute toxicity of cholecalciferol, there is moderate rise in serum/plasma phosphorus concentration up to 11 mg/dL and it is due to stimulation of transfer of phosphorus along with calcium from bone to plasma.

Although calcium, phosphate, and calcium-phosphate product levels exhibit complex associations with traditional cardiovascular risk factors and outcomes, they may be potentially modifiable risk factors for stroke and death in community-dwelling adults (Foley

et al 2008). Vascular calcification represents an intriguing candidate mechanism connecting phosphate excess with cardiovascular risk (Ketteler *et al* 2003) + phosphorus levels were significantly associated with the category of coronary artery calcium level (Foley *et al* 2009).

On day 70, the mean total protein concentration of group D rabbits increased significantly to that of group A rabbits. On day 56, the mean albumin and globulin concentration and albumin: globulin ratio of group C rabbits differed significantly from that of groups A and B rabbits while group D rabbits differed significantly to that of group A rabbits. On day 70, the mean albumin concentration and albumin: globulin ratio of groups C and D rabbits decreased significantly to that of groups A and B rabbits. On day 70, the mean globulin concentration of group D rabbits increased significantly to that of groups A and B rabbits, while Group C rabbits globulin concentration increased significantly from that of group A rabbits (Table 7, 8 and 9). Extremes of serum albumin levels are linked to vascular dysfunction among healthy individuals. Serum albumin is paradoxically linked to vascular disease under conditions both of over nutrition and of malnutrition and inflammation complex (Kadono *et al* 2010). Serum albumin is a maker of nutritional status and possesses antioxidative properties (Ishizaka *et al* 2007). Low serum albumin condition may increase the prevalence of atherosclerosis through reduced defense oxidative stress, and high serum albumin may link to metabolic syndrome, leading to atherosclerosis (Kadono *et al* 2007). Epidemiological studies have suggested that lower concentrations of serum albumin are associated with an increased risk of cardiovascular disease (CVD) (Richard *et al* 1985, Kuller *et al* 1991, Gillum *et al* 1994 and Weijenberg *et al* 1997). Demireva (1976) reported marked disproteinemia, reduction of albumin and an increase of globulins in the animals with experimental atherosclerosis. Little and Angell (1977) observed that the dietary protein and cholesterol interact in determining the concentration of aorta cholesterol and the atherosclerosis index in pigeon. The significant decrease in albumin levels in present study

could be due to loss of albumin in urine or due to liver dysfunction as confirmed by histopathological observations of damage to kidney and liver in the present study.

The mean creatinine concentration of group D rabbits increased significantly to that of group A, B and C on day 70 (Table 9). Mean BUN concentration in Group B, C and D increased significantly to that of group A on day 70 of the experiment. Renal impairment is associated with an increased risk for mortality in patients with advanced peripheral artery disease suggesting that impaired renal function exerts an unfavorable effect on patient's outcome, independently of these cardiovascular and renal risk factors (Mlekusch *et al* 2004). Matts *et al* (1993) reported that serum creatinine value, obtained in normotensive, nonobese, normoglycemic survivors of a myocardial infarction without preexistent renal disease or heart failure, provides independent prognostic information regarding subsequent overall and atherosclerotic coronary heart disease mortality. Elevated serum creatinine was associated with a higher prevalence of CVD and CVD risk factors at baseline. Hypertension Detection and Follow-up Program (HDFP) found an association between higher serum creatinine and CVD mortality (Shulman *et al* 1989), while the Multiple Risk Factor Intervention trial (Flack *et al* 1993) and the Framingham study (Culleton *et al* 1999) did not.

Thus, the biochemical studies suggest that cholesterol feeding in rabbits elevates levels of lipid profile parameters and impairs liver and kidney functions. Subsequent vitamin D_3 administration in high cholesterol fed rabbits resulted in more intense elevation in lipid profiles. It also caused severe impairment of liver and kidney functions. Deregulation of calcium and phosphorus levels in serum was intensified in hypercholesterolemic animals on subsequent vitamin D_3 administration. Biochemical findings are further supported by tissue mineral assayas, histochemical and lipid observations and histopathological changes. Thus, it is concluded that high vitamin D_3 intake orally is detrimental in atherosclerosis by intensifying the blood cardiovascular risk factors and damage to vital organs.

4.5 Tissue mineral concentration

Various comparative mineral concentration studies in heart, aorta, kidney and liver on day 70 are summarized in table 10, 11, 12 and 13 and graphs Fig. 5-11.

Table 10. Tissue mineral concentration in rabbit heart.

Parameter	Group A (n=6)	Group B (n=6)	Group C (n=6)	Group D (n=6)
Calcium (ppm)	695.25 ± 42.95	870.94 ± 89.55	664.10 ± 69.03	748.38 ± 85.75
Magnesium (ppm)	627.71± 60.97^{a}	470.41 ± 33.22^{ab}	433.61 ± 76.04^{b}	420.01 ± 18.69^{b}
Zinc (mg/g)	0.0335 ± 0.002^{a}	0.0232 ± 0.002^{b}	0.0192 ± 0.002^{b}	0.0232 ± 0.000^{b}
Iron (mg/g)	0.1293 ± 0.013	0.1726 ± 0.022	0.195 ± 0.011	0.285 ± 0.069
Copper (mg/g)	0.0052 ± 0.0007^{a}	0.0024 ± 0.0007^{b}	0.0024 ± 0.0007^{b}	0.0020 ± 0.0004^{b}
Sodium (mg/g)	0.041 ± 0.03	0.067 ± 0.012	0.054 ± 0.006	0.061 ± 0.013
Potassium (mg/g)	0.182 ± 0.015	0.0294 ± 0.006	0.036 ± 0.008	0.039 ± 0.006

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D

Values indicate mean± S.E.

Mean± S.E. within a row lacking a common superscript differ at *P*=0.05.

Table 11. Tissue mineral concentration in rabbit aorta .

Parameter	Group A (n=6)	Group B (n=6)	Group C (n=6)	Group D (n=6)
Calcium (ppm)	646.67 ± 68.54^{a}	3044.10±841.89^{ab}	685.30 ± 88.84^{a}	4134.90±770.48^{b}
Magnesium (ppm)	410.85 ± 67.89	327.78 ± 45.77	262.37 ± 44.55	334.67 ± 49.89
Zinc (mg/g)	0.0253 ± 0.0017^{a}	0.020 ± 0.0011^{ab}	0.0184±0.0016^{b}	0.0208 ± 0.001^{ab}
Iron (mg/g)	0.174 ± 0.011	0.281 ± 0.11	0.159 ± 0.013	0.198 ± 0.022
Copper (mg/g)	0.0028 ± 0.0002^{a}	0.0014 ± 0.0002^{b}	0.0008±0.0002^{b}	0.0007 ± 0.0002^{b}
Sodium (mg/g)	0.0373 ± 0.010	0.0884 ± 0.023	0.0328 ± 0.013	0.0658 ± 0.014
Potassium (mg/g)	0.028 ± 0.006	0.017 ± 0.003	0.0148 ± 0.002	0.0218 ± 0.005

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D

Values indicate mean± S.E.

Mean± S.E. within a row lacking a common superscript differ at *P*=0.05.

Table 12. Tissue mineral concentration in rabbit kidney.

Parameter	Group A (n=6)	Group B (n=6)	Group C (n=6)	Group D (n=6)
Calcium (ppm)	653.45 ±82.56[a]	1737.24±456.42[b]	541.02 ± 74.93[a]	1094.85±135.33[ab]
Magnesium (ppm)	648.76 ± 78.17[a]	502.51 ± 45.98[ab]	452.51 ± 24.91[b]	413.57 ± 30.75[b]
Zinc (mg/g)	0.0645 ± 0.015[a]	0.0328 ± 0.004[b]	0.0263 ± 0.001[b]	0.038 ± 0.002[b]
Iron (mg/g)	0.140 ± 0.009	0.207 ± 0.023	0.252 ± 0.057	0.360 ± 0.092
Copper (mg/g)	0.0028 ± 0.0004[a]	0.0004 ± 0.0002[b]	0.0013 ± 0.0003[b]	0.0012 ± 0.0002[b]
Sodium (mg/g)	0.078 ± 0.009	0.076 ± 0.015	0.083 ± 0.006	0.079 ± 0.014
Potassium (mg/g)	0.059 ± 0.011	0.072 ± 0.030	0.074 ± 0.009	0.057 ± 0.015

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D

Values indicate mean± S.E.

Mean± S.E. within a row lacking a common superscript differ at P=0.05.

Table 13. Tissue mineral concentration in rabbit liver.

Parameter	Group A (n=6)	Group B (n=6)	Group C (n=6)	Group D (n=6)
Calcium (ppm)	547.82 ± 34.65	741.58 ± 98.14	555.43 ± 53.99	777.45 ± 91.49
Magnesium (ppm)	580.63 ± 34.03[a]	518.33 ± 43.16[ab]	410.21 ± 33.37[b]	400.09 ± 33.38[b]
Zinc (mg/g)	0.047 ± 0.001[a]	0.034 ± 0.002[b]	0.033 ± 0.002[b]	0.031 ± 0.003[b]
Iron (mg/g)	0.548 ± 0.148	0.644 ± 0.134	0.689 ± 0.134	0.793 ± 0.126
Copper (mg/g)	0.0040 ± 0.001[a]	0.0016 ±0.0005[b]	0.0017 ± 0.0002[b]	0.001 ± 0.0004[b]
Sodium (mg/g)	0.082 ± 0.007	0.081 ± 0.012	0.069 ± 0.010	0.053 ± 0.007
Potassium (mg/g)	0.062 ± 0.008	0.071 ± 0.018	0.073 ± 0.014	0.041 ± 0.012

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D

Values indicate mean± S.E.

Mean± S.E. within a row lacking a common superscript differ at P=0.05.

Fig. 5 shows the Ca^{++} concentrations in heart, aorta, kidney and liver. The Ca^{++} concentration increased non-significantly in heart of rabbits of group B and D with percentage change of 12.6% and 31% respectively and decreased non-significantly with percentage change of 4.7% in group C rabbits as compared with group A rabbits. The Ca^{++} concentration in aorta of group D rabbits increased significantly with percentage change of 539% as compared to group A rabbits, while non-significant increase in group B and C rabbits with percent change of 370%, and 6% compared to group A rabbits was found. The Ca^{++} concentration significantly increased in kidney of rabbits of group B with percentage increase of 221% and non-significantly in group C and D rabbits with percent increase of 1.5%, and 102% respectively as compared with group A rabbits. Liver of group B, C and D rabbits showed non-significant increase with percentage changes of 35%, 1.5%, and 42% respectively as compared to group A rabbits.

Calcium concentration, an important intracellular signal, is determined by the net effect of all processes that deliver calcium to the cytosol or remove calcium from it. In vascular smooth muscle cells the processes include Ca influx across the plasma membrane, extrusion by a plasma membrane Ca ATPase, binding to and release from intracellular macromolecules, as well as uptake and release of Ca^{++} by sarcoplasmic reticulum and mitochondria (Phair and Hai 1986).

Strickberger *et al* (1988) reported that increased intracellular calcium is a possible mediator of cholesterol-induced atherogenesis. Many studies reported substantial increases in total aortic calcium content in cholesterol fed rabbits. This has led several investigators to suggest that atherogenesis is dependent on calcium movement across the smooth muscle plasma membrane into the cell (Henry *et al* 1981). Gleason *et al* (1991) reported that the smooth muscle cell (SMC) plasma membrane is very sensitive to cholesterol enrichment, which increases cytosolic calcium levels in SMCs. Hypercholesterolemia might promote this

calcium entry by delivering cholesterol to the plasma membrane and thus alter the function of membrane ion channels that are sensitive to their lipid environments (Locher *et al* 1984).

Vitamin D and its active metabolites function to increase the absorption of calcium and phosphorus from the intestines. Active metabolites of vitamin D also stimulate the retention of calcium by increasing its renal distal tubular reabsorption. The active metabolites of cholecalciferol act on bone causing osteoclastic resorption and calcium mobilization from bone (Beasley 1999). This all leads to hypercalcemia causing calcification of various tissues.

In the present investigation group B rabbits showed high calcium concentration due to administration of vitamin D_3 for two weeks. Hypercholesterolemia induced in group C rabbits by feeding high cholesterol diet for 10 weeks led to increased calcium concentration in the tissues which is in accordance with other studies. Group D rabbits were fed cholesterol and vitamin D_3, which showed its additive effect in elevating Ca^{++} concentration in the tissues. This suggest that vitamin D_3 aggravates the atherosclerotic lesions caused by high cholesterol diet in rabbits and also causes liver and kidney calcification causing functional impairment.

Fig. 6 shows the Mg^{++} concentrations in heart, aorta, kidney and liver of group A, B, C and D rabbits. The Mg^{++} concentration was significantly decreased in heart of rabbits of group C and D with percentage changes of 30%, and 33% respectively and non-significantly decreased in group B rabbits with percentage change of 25% as compared with control rabbits. Mg^{++} concentrations in aorta of group B, C and D rabbits decreased non-significantly with percentage changes of 20%, 30% and 18% respectively as compared to group A rabbits. The Mg^{++} concentration was significantly decreased in kidney of rabbits of group C and D with percentage changes of 30%, and 36% respectively and non-significantly in group B rabbits with percentage change of 22% as compared with group A rabbits. In liver Mg^{++} concentration decreased significantly in group C and D rabbits with percentage change of

29%, and 31% respectively while non-significant decrease in group B rabbits with 10% change as compared to group A rabbits.

Chronic subclinical magnesium deficiency is a factor in arteriosclerosis, but has not been usually associated with heart disease and cardiac arrythmia (Seelig 1964). Macintyre and Davidson (1958) reported that magnesium deficiency results in depletion of potassium in the myocardium and so may increase myocardial irritability.

Britton and Stokstad (1970) reported that magnesium deficiency causes aorta and other soft tissue calcification. In aorta, calcification takes place in elastin due to altered metabolism of the elastin. Studies report that magnesium deficiency leads to cardiovascular and kidney calcification in rats, guinea pigs (Maynard *et al* 1958) dogs (Bunce *et al* 1962) and calves (Moore *et al* 1938).

Fig. 7 shows the Fe^{++} concentrations in heart, aorta, kidney and liver of group A, B, C and D rabbits. The Fe^{++} concentration was non-significantly increased in heart of rabbits of group B, C and D with percentage changes of 33%, 51%, and 120% respectively as compared with group A rabbits; and that in aorta in group B and D rabbits with percentage increase of 61% and 14% respectively. Group C rabbits showed decrease in Fe^{++} concentration by 8.5% in aorta. The Fe concentration was increased in kidney of rabbits of group B, C and D rabbits with percentage change of 48%, 80% and 79% respectively as compared with control rabbits and that in liver with percentage increase of 17%, 26% and 44% respectively.

The relationship between iron status and atherosclerosis has long been a topic of debate in the literature. Currently, there is no consensus in the medical literature regarding a causal relationship. Although controversial, several lines of evidence suggest that iron excess may predispose to vascular disease.

Salonen *et al* (1992) reported that elevated body iron stores were associated with an increased risk of myocardial infarction. Thong *et al* (1996), Vlad *et al* (1994), Swain *et al* (1995) and Smith *et al* (1992) reported that iron deposition was disproportionately higher in atherosclerotic lesions compared with controls. Abdelhalim *et al* (2010) reported that Fe^{++} plays a major role in atherogenesis; it may accelerate the process of atherosclerosis probably through the production of free radicals, deposition and absorption of intracellular and extracellular lipids in the intima, connective tissue formation, smooth muscle proliferation, lower matrix degradation capacity and increased plaque stability.

In contrast, Dabbagh *et al* (1997) reported that in rabbit model, Fe^{++} overload decreased atherosclerosis. In addition, they did not support the hypotheses that elevation of Fe^{++} stores increases or that a reduction of Fe^{++} stores decreases the risk of coronary artery disease. Araujo *et al* (1995) also suggested that excessive iron loading in hypercholesterolemic rabbits had a detrimental role. It is proposed that reduction in body iron stores is a possible prophylactic measure in coronary artery disease (Sullivan 1991); but Bari and Rahman (1975) found that iron deficiency anemia did not offer any protection against atherosclerosis in chickens. Gerami *et al* (1970) reported that serum iron levels were lowest and liver iron levels highest in patients with severe versus mild calcific arteriosclerosis.

The observations of Worker and Migicovsky (1961) and of Wasserman (1962) established that vitamin D exerts an effect on the absorption of all of the divalent cations tested. Masuhara and Migicovsky (1963) reported that vitamin D increased the absorption of Fe that appeared in the blood, liver and bone when administered orally to chicks fed a low calcium diet. In the present study increase in Fe concentration in the tissue is possibly due to the consequence of administrating high vitamin D_3 in case of group B, cholesterol induced atherosclerosis and oxidative stress in group C and combined effect of high cholesterol and vitamin D_3 in group D.

Fig. 8 shows the Zn^{++} concentrations in heart, aorta, kidney and liver of group A, B, C and D rabbits. The Zn^{++} concentration was significantly decreased in heart of rabbits of group B, C and D with percentage changes of 31%, 42%, and 31% respectively as compared with control rabbits. Zn^{++} concentration in aorta decreased significantly in group C rabbits with percentage changes of 27% and non-significantly in group B and D rabbits with percent change of 21% and 18% respectively as compared to group A rabbits. The Zn^{++} concentration was significantly decreased in kidney of rabbits of group B, C and D with percentage changes of 49%, 59%, and 41% respectively as compared with control rabbits and that in liver with percentage changes of 27%, 30%, and 34% respectively.

Zinc is indispensable for various physiological functions. One of them is the participation in cholesterol metabolism (Thunus and Lejeune, 1994) and fatty acid metabolism (Eder and Kirchgessner 1997). Vlad *et al* (1994) reported that abdominal aorta of the patients died of heart disease had lower tissue levels of zinc as compared to other causes. Alissa *et al* (2004), Ren *et al* (2006) and Jenner *et al* (2007) reported that atherosclerotic lesions of cholesterol-fed rabbits contained reduced levels of zinc. Zinc supplementation was also shown to reduce accumulation of cholesterol in the aorta, decrease the average aortic lesion cross-sectional area, and reduced number of markers of cholesterol and lipid oxidation (Jenner *et al* 2007). In our study decrease in tissue Zn^{++} concentration is consistent with other studies.

Fig. 9 shows the Cu^{++} concentrations in heart, aorta, kidney and liver of group A, B, C and D rabbits. The Cu^{++} concentration in heart of rabbits of group B, C and D decreased significantly with percentage decrease of 54%, 54% and 61% respectively as compared with group A rabbits; and that in aorta with percentage decrease of 50%, 71%, and 75% respectively. The Cu^{++} concentration was also decreased significantly in kidney of rabbits of group B, C and D with percentage decrease of 86%, 53%, and 57% respectively as compared

with group A rabbits and that in liver with percentage decrease of 60%, 57%, and 75% respectively.

These results suggested that Cu^{++} may catalyze free radicals through the Fenton reaction, and that it plays a minor role in atherogenesis because its percentage change is lower than the percentage change of Ca^{++} in most of the tissues. It is well documented that dietary copper deficiency disturbs liver lipid and lipoprotein synthesis and possibly contributes to the development of atherosclerosis. Additionally, copper deficiency affects endothelium functions depressing nitric oxide production (Lutosławska 2009). There are numerous data indicating that in animals, long term dietary copper deficiency induces cardiac hypertrophy, necrosis of myocytes, fragmentation of elastic fibers in the aorta and altered mechanical properties of the heart (Ferns *et al* 1997). Kazemi-Bajestani *et al* (2007) observed that serum copper levels in patients with coronary artery disease were markedly lower than in healthy ones.

Hamilton *et al* (2000) reported that severe and marginal copper deficit in the diet showed induction of atherosclerotic lesions in rabbits. Lamb *et al* (2005) reported that increased copper intake had a favorable effect on antioxidant protection by increasing superoxide dismutase expression in the rabbit aorta. This reduces the interaction of nitric oxide with superoxide, and hence potentiates a nitric oxide-mediated pathway that protects against atherosclerosis.

But Ford (2000) suggest that, the progress in atherogenesis, the risk of cardiovascular disease and cardiovascular mortality increase with increasing plasma copper levels. Bo *et al* (2008) reported that dietary copper intake and its plasma levels were significantly and inversely associated with total and LDL-cholesterol levels but also significantly and positively correlated with highly-sensitivity C-reactive protein (hCPR) concentrations - the marker of inflammation and with nitrotyrosine-the marker of oxidative stress. Thus, higher

copper intake reduces atherogenic LDL-cholesterol plasma levels but also promotes oxidative stress. At present data concerning the association of copper deficiency and cardiovascular disease are equivocal. However, in the present investigation, low copper level in all the four tissues was observed which is in accordance with most of the investigators.

Fig. 10 shows the Na^{++} concentrations in heart, aorta, kidney and liver of group A, B, C and D rabbits. The Na^{++} concentration increased non-significantly in heart of rabbits of group B, C and D with percentage changes of 63%, 31%, and 49% respectively as compared with group A rabbits; and that in aorta of group B and D rabbits with percentage changes of 136% and 76% respectively. Group C rabbits showed decrease in Na^{++} concentration by 12%. The Na^{++} concentration was non-significantly increased in kidney of rabbits of group C and D with percentage changes of 6.4%, 1.2%, while group B kidney showed decrease by 2.5%. Liver of group B, C and D rabbits showed non-significant decrease in Na^{++} concentration as compared with control rabbits with percentage changes of 1.2%, 16%, and 35% respectively.

Ketonen and Mervaala (2008) reported that the detrimental effects of dietary sodium on endothelial function and progression of atherosclerosis in high-fat diet are mediated by increased reactive oxygen species (ROS) formation mainly through uncoupled nitric oxide synthase (NOS) and NADPH oxidase. They also studied the importance of superoxide and endothelial NOS uncoupling in the pathogenesis of endothelial dysfunction. Jaitovich and Bertorello (2010) reported that sodium-rich diets promote hypertension. Diets rich in sodium resulted in increased intracellular Na(+) and Ca(2+) concentrations in vascular smooth muscle cells, thus increasing the vascular tone, with a corresponding increase in blood pressure. Ketonen *et al* (2005) reported the detrimental role for high salt intake in the development of atherosclerosis and highlighted the importance of increased oxidative stress in the pathogenesis of salt-induced vascular damage.

In the present investigation, increase in Na^{++} level in tissue would have led to the detrimental effect in the cardiovascular system.

Fig. 11 shows the K^+ concentrations in heart, aorta, kidney and liver of group A, B, C and D rabbits. The K^+ concentration was decreased non-significantly in heart of rabbits of group B, C and D with percentage changes of 83%, 80%, and 78% respectively as compared with control rabbits; and that in aorta with percentage changes of 39%, 47% and 22% respectively. The K^+ concentration increased non-significantly in kidney of rabbits of group B and C with percentage changes of 22% and 25% respectively but decreased non-significantly in group D rabbits by 3.4% as compared with group A rabbits. Similarly, liver showed non-significant increase in K concentration in group B and C rabbits with percentage changes of 14% and 18% however group D showed non-significant decrease by 34% as compared to group A rabbits.

Low magnesium leads to low tissue potassium since magnesium is absolutely needed for potassium transport into tissue. Risk for cardiac irritability, irregularity, and hypertension have related to loss of cellular potassium. Potassium ion (K^+) channel activity is a major regulator of vascular muscle cell membrane potential (*E*m) and is therefore an important determinant of vascular tone. There is growing evidence that the function of several types of vascular K^+ channels is altered during major cardiovascular diseases, such as chronic hypertension, diabetes, and atherosclerosis (Sobey 2001).

Faraci and Sobey (1998) reported that large conductance Ca^{++}-activated K^+ (BKCa) channels are activated by intracellular Ca^{++} and also by *E*m depolarization that are particularly abundant in vascular smooth muscle cells. Increased dietary K^+ intake and/or exogenous K^+ administration may afford a protective influence on vascular and tissue-based biology as relates to atheromatous disease. In animal models, K^+ inhibits free radical formation, proliferation of vascular smooth muscle cells, platelet aggregation, and arterial

thrombosis and reduces vessel-wall cholesterol content (Ishimitsu *et al* 1996 and Tobian 1986).

Thus, the tissue mineral concentration studies suggest that increase in Ca^{++}, Fe^{++} and Na^{++} and decrease in Cu^{++}, Zn^{++} and Mg^{++} have more detrimental effect on cardiovascular system in rabbits fed high cholesterol and subsequently given vitamin D_3 per oral. These findings are further supported by biochemical assay, histochemical and lipid observations and histopathological changes.

4.6 Histochemical Studies

Alkaline Phosphatase (AKPase)

The aorta showed no to weak activity in group A, very mild to weak activity in group B, moderate to strong in group D and no to weak activity in group C (Fig. 12a, b, c and d). The heart showed weak AKPase activity in group A, moderate in group B and weak in group C, however the activity was moderate to strong in case of group D (Fig.13a, b, c and d). A moderate AKPase activity was observed in liver of groups A and B. Group C showed focal moderate to strong AKPase activity while group D showed diffused moderate to strong AKPase activity (Fig. 14a, b, c and d).

The expression of AKPase was demonstrated in various vascular-calcified lesions such as atherosclerosis, Mönckeberg-type medial calcification, and cardiac valvular disease (Shanahan *et al* 1999). Tanimura *et al* (1986) also reported that AKPase activity in human atherosclerotic lesions was cytochemically localized in calcified matrix vesicles and intracytoplasmic vesicles of smooth muscle cells. Atherosclerosis and cardiac valvular disease often showed dystrophic calcification (Fitzpatrick *et al* 1994 and Mohler *et al* 2001) and AKPase activity had been implicated in the calcification of aortic valves (Levy *et al* 1991). Shioi *et al* (1995) demonstrated that expression of AKPase was functionally important in bovine vascular smooth muscle cell calcification. The vascular calcifying cells such as

VSMCs, pericyte-like cells, and microvascular pericytes expressed high levels of AKPase (Doherty *et al* 1998) and further, the calcifying capacity of these cells was dependent on the activities of the enzyme. In the present study, cholesterol fed group (group C) would have lead to vascular calcification in advance stages resulting in moderate to strong enzyme activity.

In the present investigations, the AKPase activity was more in the groups administered with vitamin D_3 suggesting that administration of vitamin D_3 in these groups would have stimulated calcification as reported by Shioi *et al* (2002). Shioi *et al* (2002), demonstrated that macrophages may also induce calcifying phenotype, and especially the expression of AKPase in human vascular smooth muscle cells in the presence of IFN-α and 1,25(OH)2D_3. Kim (1976) reported that matrix vesicles play a role in vascular dystrophic calcification of atherosclerotic plaques in association with elevated AKPase activity.

Glucose-6-Phosphate-Dehydrogenase (G6PD)

The G6PD activity was almost absent in aorta of group A, weak to moderate in group B and C and moderate to strong in group D (Fig. 15 a, b, c and d). The G6PD activity was found to be weak to moderate in heart of group A, however group B and C showed moderate G6PD activity and group D showed intense G6PD activity (Fig.16 a, b, c and d). Similarly, in liver, group A and B showed strong G6PD activity while group D showed moderate to intense activity. Leopold *et al* (2003) reported that the vascular endothelium modulates cellular oxidant stress by increasing the activity of enzymes with antioxidant properties such as G6PD, which mediates reactive oxygen species accumulation in vascular endothelial and smooth muscle cells (Leopold *et al* 2001) which contributes to atherosclerosis. Matsui *et al* (2006) reported that mice with G6PD deficiency demonstrated less atherosclerotic lesions, which relates to lower superoxide anion production and nitrotyrosine in the aorta. G6PD overexpression in adipocytes stimulates oxidative stress and inflammatory responses and in

addition promotes the expression of pro-oxidative enzymes (Park *et al* 2006) which may help in progression of atherosclerosis.

Important factor for atherosclerosis progression is the production of superoxide in endothelial cells, which is hindered by the inhibition of G6PD (Guo *et al* 2002). Muntoni *et al* (1992) reported that the serum levels of total cholesterol, low density lipoprotein (LDL) cholesterol, and high-density lipoprotein cholesterol were significantly lower in G6PD deficient men. But Jain *et al* (2003) suggested that G6PD deficiency contributes to cardiac dysfunction through increased susceptibility to oxidative injury and impairment of intracellular calcium transport in cardiomyocytes from rats. Thus in the present study strong to intense G6PD activity in group D is indicative of cellular oxidant stress and inflammatory reaction in cardiovascular system of rabbits administered with cholesterol and vitamin $D_{3,}$ while group B and C showing moderate to strong reaction would have suffered less intense cellular oxidant stress and inflammatory reaction than group D.

NADPH Diphorase (NADPH-d)

The aorta showed weak to moderate activity in group A, moderate to strong in group B and C and strong to intense reaction in group D (Fig. 17a, b, c and d). The NaDPH Diphorase activity in the heart was weak to moderate in group A, while it was moderate to strong in group B, weak to moderate in group C and intense in group D (Fig. 18a, b, c and d). Liver of group A showed weak NADPH-d activity whereas group B and C showed moderate NADPH-d activity. Group D liver showed intense NADPH-d activity (19a, b, c and d). Similar findings by Gjumrakch *et al* (2001) demonstrated that the development and progression of atherosclerotic lesions in rabbits induce increased expression of NADPH-d and noted the enzyme activity in medial layers of thoracic aorta in rabbits with atherosclerotic lesions, but not in nonlesioned aortic wall of control rabbits. They also noted

that subendothelial macrophage foam cells or smooth muscle cells showed the most intense NADPH-d histochemical reactions, particularly in the late stages of atherosclerotic lesion.

NADPH-diaphorase (NADPHd) activity is used widely as a marker for nitric oxide synthase (NOS) (Ott and Elphick 2003). Ozaki *et al* (2002) demonstrated that chronic overexpression of endothelial nitric oxide synthase (eNOS) accelerates atherosclerosis under hypercholesterolemia and that eNOS dysfunction play important roles in the progression of atherosclerosis. NOS itself produces superoxide anions in the absence of its substrate L-arginine or cofactors (Vasquez-vivar *et al* 1998). Laursen *et al* (2001) reported that eNOS expression was maintained or even increased in hypercholesterolemic or atherosclerotic vessels.

Succinate Dehydrogenase (SDH)

The enzyme activity of SDH in heart of group A, B and C was intense while it was strong in group D. Aorta from group A showed moderate to strong activity, while group B and D showed weak activity. The activity of SDH in liver was strong in Group A and strong to intense in group B however the activity was weak to moderate in group D. The level of SDH per se in the smooth muscle cells was found to be fairly high in the wall of normal rabbit aorta (Garbarsch *et al* 1978). Mrhovx *et al* (1963) reported significant decrease in SDH activity in aorta of rabbit as early as 4 weeks and 10 weeks after feeding of cholesterol. Similarly Rosnowski and Kujawa (1977) also reported decrease in SDH activity of the rabbit myocardium in experimental atherosclerosis in the eighth week of the experiment.

Lactate dehydrogenase (LDH)

A strong LDH activity was observed in the heart of all the four groups. Group A showed moderate LDH activity in aorta while the activity in group B was weak. Group C aorta showed moderate to strong LDH activity while group D showed weak to moderate LDH activity (Fig. 20a, b, c and d). The LDH activity was moderate in liver of group A,

however group B, C and D showed moderate to strong LDH activity (Fig. 21a, b, c and d). Schultheiss *et al* (1990) and Markert (1963) reported that myocardial LDH activity and isoenzyme patterns can be considered a sensitive method to analyze changes of the enzyme status in patients with various heart diseases. In the present study, intense LDH activity was observed in the aorta and heart of cholesterol fed rabbits as reported earlier by Zemplenyi *et al* (1984). Doebler *et al* (1984) reported that calcified lesions causes decrease in LDH activity within the involved inner mural zone of the rabbit aorta.

Malate Dehydrogenase (MDH)

The MDH activity in heart was strong and weak to moderate in group A and group B respectively. However, group C and D showed moderate MDH activity in heart. Aorta of group A showed weak to moderate activity which intense activity was observed in group B and D. Similarly in liver, MDH activity was weak to moderate in group A, whereas it was strong to intense in group B and D.

Acetylcholine esterase (AChE)

The AChE activity in heart of group A, B and C was weak to moderate however group D showed moderate to strong activity. In aorta, AChE the activity was weak in group A and B, weak to moderate in group C and moderate in group D. The AChE activity in liver of group A was weak, strong in group B, while it was moderate to strong in group D.

NADH Diphorase (NADH-d)

The aorta of group A showed weak to moderate NADH-d activity. The NADH-d activity was intense in group B and D; however, the activity was strong in group C. The liver of group A and C showed strong NADH-d activity. The activity was strong to intense in group C and intense in group D. Conversely, Adams and Bayliss (1969) observed loss of NADH-d during diffuse intimal thickening of human aorta.

Thus, the enzymehistochemical studies suggest that increase in AKPase, G6PD, LDH MDH, NADPH-diphorase and NADH-diphorase and decrease in SDH activity have more detrimental effect on cardiovascular system in rabbits fed high cholesterol and subsequently given vitamin D_3 per os. These findings are further supported by biochemical assay, tissue mineral assay and lipid observations and histopathological changes.

4.7 Lipid Demonstration

Aorta

Frozen sections of aorta from group A rabbits did not show any accumulation of fat (sudanophilia), while group B rabbits showed weak to moderate fat deposition. Group C rabbits aorta showed moderate to strong fat deposition in T. intima and T. media of the aorta, while group D rabbits aorta showed intense sudaniphilia (fat deposition) in T. intima and T. media of the aorta (Fig. 22a, b, c and d).

Heart

Frozen section of heart from group A rabbits (control feed) did not show any accumulation of fat (sudanophilia), while group B rabbits (control feed + vitamin D) and C rabbits (control feed + cholesterol) heart showed weak to moderate fat deposition in and around mural coronary artery. Group D rabbits (cholesterol + vitamin D) heart showed moderate fat deposition in and around mural coronary artery (Fig. 23a, b, c and d).

Liver

Frozen section of liver from group A (Fig. 24a) and B (Fig. 24b) rabbits did not show any accumulation of fat (sudanophilia), while group C rabbits showed moderate to strong fat deposition in hepatocytes (Fig. 24c and d).. Group D rabbits liver showed strong to intense sudaniphilia (fat deposition) within hepatocytes of the liver (Fig. 25a, b, c, and d).

4.8 Gross post-mortem lesions

The gross lesions observed in the rabbits are as follows:

Aorta

Group A (control feed) rabbits aorta did not show any gross lesion. The aorta was thin, with smooth luminal surface (Fig.26 and 27). Group B (control feed + vitamin D) rabbit's aortas were firm and inelastic because of the calcium deposits in T. intima and T. media (Fig.29). The lumen surface was rough with small atheromatous plaques and white chalky deposits of calcium (Fig.30). Group C (control feed + cholesterol) rabbit's aorta showed numerous small and large yellowish white atheromatous plaques on the intimal surface (Fig.33). The plaques were smooth surfaced, irregular in shape but had well defined borders (Fig.34). Thickening of the aorta was also appreciable. Group D (cholesterol + vitamin D) rabbits showed numerous ruptured atheromatous plaques along with multiple white chalky mineralized foci calcium making it firm and inelastic (Fig.40). Aortic wall was also thickened. Kunitomo et al (1981) and Togashi et al (2003) observed similar gross changes in aorta of rats supplemented with cholesterol and vitamin D. Mackey *et al* (2007) and Kwatra *et al* (1974) observed that medial calcification of elastic fibers is associated with arteriosclerosis and increased arterial stiffness. Fillois *et al*., (1956) found gross lesion of atherosclerosis in the intimal surface of the vessels, heart valves, and aortic arch.

Heart

No apparent changes were seen group A rabbits heart involving the myocardium and endocardium. (Fig.26). Cardiac valves showed smooth elastic surface (Fig.28). Group B rabbits showed cardiac valves and endocardium mineralization (Fig.31). Group C rabbits showed thickening and plaque formation on the valves (Fig.35). No apparent changes were seen grossly involving the myocardium and endocardium. Group D rabbits showed severe atherosclerotic lesions and white chalky mineralization on the valves causing stiffness of the valves (Fig.41).

Liver

The group C (Fig.36) and group D (Fig.42) rabbits showed moderate to severe fatty changes with yellow to white discoloration. The liver was large in size and had rounded borders. One of the group C rabbits did not show fatty change grossly in the liver but the gall bladder was whitish in colour (Fig.39). Marked fatty changes and hypertrophy of liver of the group D rabbits was noted as compared to group A rabbits liver (Fig.43). Two group B rabbits showed mild fatty changes.

Kidneys

Kidneys from the group A (control feed) and group C (control feed + cholesterol) rabbits showed no gross changes. Kidney of the group B (control feed + vitamin D) (Fig.32) rabbits and group D (cholesterol + vitamin D) showed white chalky deposits on renal capsule and on corticomedullary junction.

Spleen

Four of the group B rabbit's spleen showed white petechae on the surface while the remaining two did not show any change. Two of the group C rabbits showed increase in size of spleen with mild chalky white deposition on the external surface while four rabbits of the group showed only increase in the size of spleen. The group D rabbits showed marked hypertrophy of spleen compared to group A rabbits (Fig.44). Lipid and calcium deposition in the spleen with pin point hemorrhages was seen on the surface of group D animals.

Adrenals

Group A and group B rabbit's adrenal did not show any gross changes. Group C (Fig.38) and group D (Fig.45) rabbit's adrenals showed marked hypertrophy as compared to group A. The adrenals were white in colour with soft consistency.

Lungs

Group A and group C rabbits lung did not show any gross changes. Gross examination of the lungs of group B and group D rabbits showed petechial to ecchymotic hemorrhages. Group D rabbits also showed severe edematous changes (Fig.42).

Brain

No change was evident in brains of group A and group B rabbits. Grossly visible congested meninges were evident in group C and Group D rabbits.

Stomach and Intestine

Group D rabbits showed thickened walls of stomach and intestine and fat like deposits on the luminal surface and white chalky petechial deposits on the serosal surface (Fig. 46, 47 and 48). In addition the luminal surface was corrugated with white deposits (Fig.47). Group B rabbits showed white chalky petechial deposits on the serosal surface of the stomach and intestine. Group C (Fig.37) rabbits had no gross changes and resembled to Group A rabbits.

Trachea

Mild tracheal congestion and hemorrhages were found on the luminal surface of the trachea of the group B and group D rabbits (Fig.49). Tracheas from the other two groups were normal grossly.

Thyroids

Group C (cholesterol + cholesterol) and Group D (cholesterol + vitamin D) rabbits showed increase in size of thyroid gland with yellowish discoloration. Thyroids of other two groups were normal.

Skin and Hair

Group B and Group D rabbits showed mild to severe emaciation and ruffled hair coat. While other two groups did not show any change.

Skull Bones

Group B and Group D rabbits had soft and easy to cut skull bones then the group A and Group C (rabbits.

No gross changes were evident in tongue, esophagus, spinal cord, sciatic nerve, long bones,

4.6 Histopathological observations

Comparative histopathology of severity of the lesions of most affected organs is summarized in Table 14 and 15. The microscopic lesions in various organs were scored according to the severity as compared to the group A rabbits.

Table 14. Comparative score of microscopic lesions in various groups.

Sr. No.	Parameter	Group A	Group B	Group C	Group D
Aorta					
1	Atheroma formation	-	- to +	+ to +++	+ to +++
2	Aortic calcification	-	+++ to ++++	-	++ to ++++
3	Smooth Muscle cell proliferation	-	-	- to +	- to ++
Heart					
1	Cardiomyocyte calcification	-	- to ++	-	- to ++
2	Mural artery calcification	-	- to ++	-	- to ++
3	Mural artery occlusion	-	-	+ to +++	+ to +++
4	Fat dysplasia	-	-	+ to +++	+ to +++
5	Lymphomononuclear cell infiltration	-	- to +	- to ++	- to ++
Liver					
1	Fatty changes	-	- to +	+ to +++	++ to ++++
2	Hepatic sinusoidal reduction	-	-	++ to +++	++ to +++
3	Von Kuffer cell proliferation	-	- to +	+	+
Kidney					
1	Glomerular calcification	-	++ to +++	-	+ to ++
2	PCT calcification	-	+ to +++	- to +	- to ++
3	DCT calcification	-	+ to +++	- to +	- to ++

4	Inter-tubular calcification	-	++ to +++	-	++ to +++
5	CT calcification	-	- to +	-	- to ++
Spleen					
1	Trabecular calcification	-	- to +	-	++ to +++
2	Decrease in white pulp area	-	- to +	+ to ++	++ to +++
3	Spleenic foam cells	-	-	+++ to ++++	+++ to ++++
Lung					
1	Bronchial calcification	-	+ to +++	-	+ to +++
2	Edema	-	+ to +++	-	+ to +++
Adrenal					
1	Adrenal nodular hyperplasia	-	-	++ to ++++	+++ to ++++
2	Cholesterol clefts	-	-	- to +++	- to +++
Stomach and Intestine					
1	Villous degeneration	-	- to +	- to +	+

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D

- = No Change, + = Mild; ++ = moderate; +++ = severe; ++++ = intense.

Table 15. Statastical analysis of comparative score of microscopic lesions.

Sr. No.	**Parameter**	**Group A & Group B**	**Group A & Group C**	**Group A & Group D**	**Group B & Group C**	**Group B & Group D**	**Group C & Group D**
				***P*-value**			
Aorta							
1	Atheroma formation	0.002	0.002	0.002	NS	NS	NS
2	Aortic calcification	0.002	NS	0.002	0.003	NS	0.003
3	Smooth Muscle cell proliferation	NS	NS	0.022	NS	0.022	NS
Heart							
1	Cardiomyocyte calcification	0.022	NS	NS	NS	NS	NS
2	Mural artery calcification	0.022	NS	NS	NS	NS	NS
3	Mural artery occlusion	NS	0.002	0.002	0.002	0.002	NS
4	Fat dysplasia	NS	0.002	0.002	0.002	0.002	NS
5	Lymphomononuclear cell infiltration	NS	NS	0.015	NS	NS	NS
Liver							
1	Fatty changes	NS	0.002	0.002	0.008	0.003	NS
2	Hepatic sinusoidal reduction	NS	0.002	0.002	0.002	0.002S	NS
3	Von Kuffer cell proliferation	NS	0.002	0.002	NS	NS	NS
Kidney							
1	Glomerular	0.002	NS	0.002	0.002	0.019	0.002

	calcification						
2	PCT calcification	0.002	NS	0.007	0.008	NS	NS
3	DCT calcification	0.002	NS	0.007	0.005	NS	NS
4	Inter-tubular calcification	0.002	NS	0.002	0.002	NS	0.002
5	CT calcification	NS	NS	0.022	NS	NS	0.002
Spleen							
1	Trabecular calcification	0.002	NS	0.002	0.002	0.003	0.002
2	Decrease in white pulp	NS	0.002	0.002	0.003	0.003	NS
3	Spleenic foam cells	NS	0.002	0.002	0.002	0.002	NS
Lung							
1	Bronchial calcification	0.002	NS	0.001	0.001	NS	0.001
2	Edema	0.002	NS	0.002	0.007	NS	0.007
Adrenal							
1	Adrenal nodular hyperplasia	NS	0.002	0.002	0.002	0.002	NS
2	Cholesterol clefts	NS	0.007	0.007	0.007	0.002	NS
Stomach and Intestine							
1	Villous degeneration	NS	NS	0.001	NS	NS	NS

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D

Statistical difference between group A, B, C and D was determined by Mann-Whitney *U* test.

NS: non-significant

Significance assumed at $P<0.05$.

The detailed microscopic changes in various groups are as below:

Aorta

Aorta from group A (control feed) did not show any microscopic lesions (Fig. 50 and 51). Group D (cholesterol + vitamin D) rabbits showed variation in the lesions in different parts of the aorta. Microscopic examination of aorta revealed calcification to be the most prominent feature of tunica media. Calcification created a space in T. media separating the T. intima from T. adventitia (Fig.56).

In initial stage first grade of atheroma formation in the wall of the T. intima was seen. Small elevation of the T. intima protruding into the aortic lumen was evident. The atheroma was composed of small amount of lipid with intact endothelial lining. Lipid deposits were not seen in the T. media or T. adventitia, however calcification was evident in T. media (Fig.56). In second grade of lesion atheroma were larger than the first grade atheromas. It was composed of lipid laden macrophages (foam cells) and connective tissue fibers. The

endothelial lining was intact and mild lipid infiltration in the T. media was evident. In some lesions the lipid-laden macrophages, necrotic debris, calcium deposits and the inflammatory cells formed the core of the lesion. Endothelial lining of the T. intima was not intact. Cap of the atheromatous lesion formed of fibrous tissue, smooth muscle cell and inflammatory cell infiltration. Hemorrhages in the core of the atheroma were seen which could be due to rupture of new delicate blood vessels (Fig.58). The atheromas increased in size to partially occlude the aortic lumen in later stages. Calcification in these atheromas was also a prominent feature. Ultimately these atheromas ruptured.

Microscopic examination of the aortic valves revealed development of fibroatheromas. Smooth muscle cell proliferation in T. intima with inflammatory cell infiltration and fibrous cap formation was evident. Foam cell accumulation and hemorrhages within the atheromatous lesion were more prominent. Calcification was confined to the T. media layer only (Fig.59).

The Group C (control feed + cholesterol) rabbits showed similar changes, but the lesions were less intense as compared to group D. Aorta showed initial stage of atheromatous lesion with elevation of T. intima due to lipid deposition underneath (Fig.53). In advanced atheroma the endothelial lining of the T. intima was ruptured. Foam cells and mild smooth muscle cell proliferation formed the core of the plaque (Fig.54). Intact endothelium was also found in few atheroma lesions (Fig.55). Calcification was not the characteristic of these lesions. The Group B (control feed + vitamin D) rabbits showed mineralization in the T. intima and T. media (Fig.52). Space formation in T. media caused elevation in the T. intima layer.

Kunitomo *et al* (1983) observed similar findings in guinea pigs fed high cholesterol and vitamin D. Kunitomo *et al* (1981) suggested significance of vitamin D supplementation in deposition of cholesterol and calcium in rat aortas who found similar microscopic lesions.

However Tang *et al* (2006) attributed oxidative stress to be a potential factor for vascular calcification in hypercholesterolic rats. Rats given high cholesterol and vitamin D showed significantly enhanced vessel calcium deposits and activity of alkaline phosphate compared with vitaminD alone. Togashi *et al* (2003) credited the histopathological changes in valves to the shear stress, intramural pressure and mechanical stress and noted that the difference in the microscopic changes in aorta and valves is due to absence of medial smooth muscle in valves. Iwasaki *et al* (1988) observed monckeberg's type of atherosclerosis in aorta of dogs fed cholesterol and vitamin D. Madhumati *et al* (2006) observed accumulation of foam cells, atheoromatous plaque formation, and replacement fibrosis in experimentally induced atherosclerosis in rabbits. Fillois *et al* (1956) found vascular lesion charecterised by medial and intimal lipid infiltration and cellular intimal plaque formation. Histological changes like, plaque formation, fatty streaks, lipid and calcium deposition, internal elastic membrane duplication and disruption and smooth muscle cell in intima, aortic sudanophilia of wild and domestic ruminants were quite similar to that of human and other animals (Wiggers *et al* 1971). The intact endothelial lining represented a structural barrier against the excessive influx of cholesterol and the integrity of this barrier is decreased during atherogenesis (Bondjers and Bjorkerud 1973). Sudan IV and gross calcium staining in aortas revealed both lipid and mineral deposition in young goats supplemented with dietary vitamin D and calcium (Thomas *et al* 1985). Kwatra *et al* (1974) reported a natural case of arteriosclerosis with metastatic calcification in corriedale sheep in Punjab due to ingestion of some poisonous plants.

The histopathological changes observed within aorta in group B (control feed + vitamin D) are in agreement with earlier changes reported by Price *et al* (2001) in vitamin D_3 toxicity in rats. Price *et al* (2001) also reported the calcification of tunica media of aorta causing wide separation of elastic lamellae.

Heart

Microscopic examination of the heart of group A showed normal histology (Fig.60 and 61). Group B rabbit's heart showed calcification in T. intima of the blood vessel (Fig.62) and calcification in cardiac muscle (Fig.63). Group C rabbit's heart showed occlusion of the lumen of the mural coronary arteries (Fig.64) and fat dysplasia of heart with very few remnants of cardiac muscle (Fig.65). Group D rabbit's heart showed occlusion and calcification within T. intima of the mural coronary artery (Fig.66). Few mural coronary arteries were occluded to a stage that blood did not flow through them (Fig.67). Fat dysplasia with very few remnants of cardiac muscle was apparent in the heart section (Fig.68). Heart showing lymphocytic infiltration and fibrous tissue proliferation was also apparent (Fig.69) around the occluded blood vessels suggestive of ischemia. Fibrous intimal thickening without lipid the coronary arteries in monkeys was earlier found by Chakravarti *et al* (1976). Lesions were seen as early as 7 weeks in the aorta and pulmonary vessels and by 11 weeks in the small intramyocardial arteries and arterioles, while myocardial fibrosis and infarction were evident by 15 weeks (Lee *et al* 1978). Taura *et al* (1978) reported calcification of T. media and internal elastic lamina in coronary artery in hypervitaminosis D. Valvular calcification and stenosis in advace stages of disease is seen (Togashi *et al* 2003). Kunitomo *et al* (1981) suggested significance of vitamin D supplementation in deposotion of cholesterol and calcium in heart and aorta of rats. The histopathological changes observed within heart in group B are in agreement with earlier changes reported by Price *et al* (2001) in vitamin D_3 toxicity in rats. Increase in LDL, VLDL, total cholesterol and tissue iron ultimately caused atherosclerostic lesion formation in heart. Fillois *et al* (1956) found microscopic coronary artery lesion accompanied by massive myocardial infarction.

Liver

Liver of group A was normal histologically (Fig.70). Microscopic examination of liver of group D rabbits revealed severe fatty changes with small to large lipid droplet accumulation in the hepatocytes. Hepatic necrosis and reduction in sinusoidal space was evident (Fig.74, 76 and 77). Cholesterol crystals were seen in hepatocytes. Liver of two rabbit's showed mononuclear cell infiltration and fibrous tissue proliferation around central vein (Fig.75) with activation and proliferation of Von-kupffer cells. Group C rabbits manifested similar changes, but of mild nature and with the exception of infiltration of inflammatory cells and fibrous tissue proliferation (Fig.72 and 73). Liver of two rabbits from group B showed mild fatty changes (Fig.71) while the four others illustrated similar picture to that of the group A. Buja *et al* (1983) earlier reported prominent lipid accumulation in hepatocytes and macrophages of liver. Wouters *et al* (2008), earlier reported foamy Kupffer cells in high cholesterol fed mice. Severity of liver histopathology among nonalcoholic fatty liver disease patients is strongly associated with early carotid atherosclerosis, independent of classical risk factors, insulin resistance, and the presence of metabolic syndrome (Targher *et al* 2006).

Kidney

Group A rabbits did not show any microscopic change (Fig.78 and 79). Microscopic examination of kidney of group D rabbits showed degeneration and coagulative necrosis of the tubules. Increase in thickness of basement membrane and mineralization within the glomerular tufts was evident (Fig.86). Few kidney sections revealed atrophy of glomerular tuft creating a large glomerular space (Fig.87). Mineralization of the glomerular basement membrane, PCT, DCT, CT, interstitial space and glomerular capillaries was apparent (Fig.88). Glomerulosclerosis was manifested by hypocellularity and hyalinization due to an increase in fibrous connective tissue and mesengial matrix and almost complete loss of glomerular capillaries. Basement membrane of the glomeruli and matrix within glomeruli

were mineralized (Fig.89). Complete loss of glomeruli was seen. Glomerular hyperplasia causing union of glomerular tuft and basement membrane leading to occlusion of the glomerular space was also evident along with renal artery calcification.

Group C showed similar changes, but of mild nature and without calcification (Fig. 82, 83, 84 and 85). Group B rabbits showed mineralization in basement membrane of glomeruli and interstitial space (Fig.80) and within the T. intima of renal artery (Fig.81). Hess *et al* (2006) found significant association between atherosclerosis and glomerulopathy in dogs. The lesions observed in kidney of vitamin D fed animals are in consonance with lesions reported by Morita *et al* (1995) in cholecalciferol toxicity in different animal species. Hunt *et al* (1972) studied toxicity of cholecalciferol in Rhesus monkeys (*Macaca mulatta*). They reported the mineralization of cortical tubular epithelium, their basement membrane, within interstitium, in walls of renal blood vessels and within epithelium of collecting ducts in medulla.

Adrenal

Group A (Fig.90 and 91) and group B rabbits did not show any microscopic changes (Fig. 92). Microscopic examination of the adrenals from the group C and group D rabbits revealed adrenocortical nodular hyperplasia (Fig. 93 and 95). Zona fascilulata and zona reticularis were replaced by the nodules of cholesterol fed foam cells. Needle shaped cholesterol clefts were also evident in and out of these foam cells (Fig. 94 & 97). Rupture of the foam cell membrane caused two to four cells to coalesce together to form a single cell (Fig.94 and 96).

A marked increase in lipid droplet content and increase in adrenal cholesterol content is earlier reported by Civen *et al* (1984). Adrenocortical hypertrophy and lipid deposition in adrenals was also reported by Albrecht *et al* (1965). Long term vitamin D administration

resulted in elevated adrenal weights, medullary hyperplasia and pheochromocytoma (Ikezaki *et al* 1999).

Spleen

Microscopic examination of the spleen of group D rabbits demonstrated appreciable decrease in white pulp as compared to the group A rabbits (Fig.104 & 106). Subcapsular and cortical area revealed noticeable accumulation of spleenic foam cells (Fig. 104 and 105) and hemosiderin laden macrophages (Fig.107). Medullary region was constricted to a very small area. Calcification was seen in the trabaculae, spleenic arteries and the capsule (Fig.105, 106 & 107). Similar changes were evident in the spleen of the Group C (control feed + cholesterol) rabbits with exception of calcification (Fig.102 & 103). Group B rabbits spleen revealed calcification in artery (Fig.100), trabaculae, and capsule. Group A rabbits showed normal histology of spleen (Fig.98 & 99). Prominent lipid accumulation in macrophages of spleen is earlier reported by Buja *et al* (1983). The histopathological lesions observed in spleen of vitamin D fed animals are similar as reported by Stevenson *et al* (1976) in rabbits due to vitamin D_3 toxicity.

Lung

No changes were seen in the group A (control feed) rabbits (Fig.108). Microscopic examination of lung from group D rabbits revealed edema, hemorrhages, congestion. Lymphomononuclear cell, heterophils and cellular debris accumulate within the edematous fluid (Fig.112 and 113). Section of lung showed hypertrophy of T. media and periarteritis (Fig.113). Periarterial cuff of chronic inflammatory cells and some heterophils and the lipid vacuoles in and around the blood vessels were evident (Fig.113). Loss of villi of the bronchiolar epithelium and mineralization in the basement membrane was apparent (Fig.115). Lungs of the group C rabbits showed severe occlusion of pulmonary arteries by atheroma formation. Lipid infiltration in the T. media of the arteries and infiltration of

mononuclear cell in the perivascular area was seen (Fig.111). Group B (control feed + vitamin D) rabbits showed calcification in the pulmonary arteries and basement membrane of the bronchioles (fig.110). Edema, lymphomononuclear cell and cellular debris accumulated within the edematous fluid (Fig.109).

Hunt *et al* (1972), Stevenson *et al* (1976), Long (1984), Kwatra *et al* (1974) and Morita *et al* (1995) reported the same type of lesions in bronchi and lungs in different animal species. Hunt *et al* (1972) studied toxicity of cholecalciferol in Rhesus monkeys (*Macaca mulatta*) and reported the presence of brown dirty colour deposits of calcification within alveolar septa, thickening of alveolar septa with infiltration of macrophages, lymphocytes, few multinucleated giant cells and neutrophil.

Trachea

Group A and C did not show any histological alteration (Fig.116 and 119). Microscopic examination of the trachea of group D showed mineralization in the submucosal layer and in the cartilage (Fig.120 and 121). In group B rabbits trachea showed mineralization in the submucosal layer (Fig.117).

Brain

No changes were observed in group A (Fig.122) and group C (Fig.125) rabbits. Microscopic examination of the brain of group D rabbits showed perineuronal satellitosis (Fig.127). Cerebellar Purkinje cells showed shrunken cell bodies and nuclear pyknosis (Fig.126). Group B rabbit's brain showed focal degeneration of neurons surrounded by glia cells (123). Cerebullum showed shrunken purkinje cell bodies and nuclear pyknosis (124). Several pathogenic mechanisms promoting atherosclerosis are involved in neurodegenerative diseases (Napolia and Palinski, 2005). In addition to causing pathological changes in cells directly involved in atherosclerosis, cholesterol oxides may induce toxicity in neurons of the central nervous system (Chang and Liu, 1998).

Stomach and Intestine

No microscopic changes were observed in group A rabbits (Fig.128 and 129). Microscopic examination of the stomach and intestine of group D rabbits showed necrosis and sluffing of the villi in the mucosal layer (Fig.136). Calcification of the muscular layer and arteries of the stomach and intestine was evident (Fig.134 and 135). There was heterophilic cell infiltration in the muscular layers (Fig.137). In Group B rabbits, stomach and intestinal muscular layer and villi showed degeneration, necrosis and calcification (Fig.130 and 131). No microscopic change was observed in the group C (Fig. 132 and 133) but few rabbits showed villous degeneration.

The histopathological lesions, calcification of mucosa, muscularis mucosa and muscularis externa layers of stomach observed in present study are in agreement with lesions reported by Long (1984) and Morita *et al* (1995). In stomach lesions reported by Long (1984) in pigs include necrosis of glandular epithelium, hemorrhages and inflammatory infiltrate in mucosa with calcification of mucosa, submucosa, in walls of sub-mucosal blood vessels. Morita *et al* (1995) reported same lesions in stomach except calcification of muscle layer in vitamin D_3 toxicity in cats.

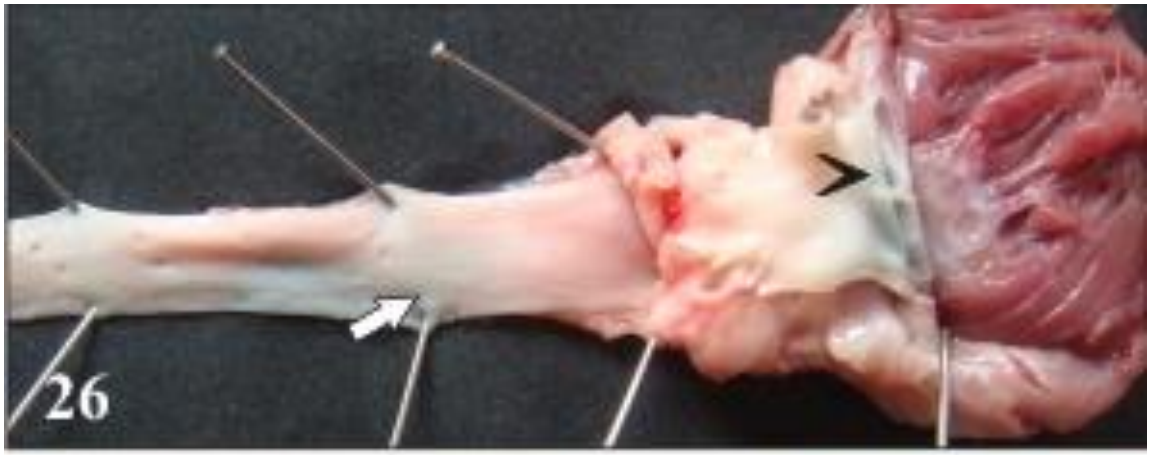
Pancreas

Well-demarcated patchy lesions composed of degenerating acinar cells showing deeply eosinophilic cytoplasm and pyknotic nuclei along with patchy fibrotic foci, remnant intralobular ductules and the patchy retraction was seen in pancreas. Similar changes were earlier reported by Matsukuma *et al* (1998).

Von Kossa staining for calcium demonstation in tissue sections

Calcium depostion within tissue section was demonstrated using Von-Kossa staining. An aorta from group B rabbits showed black deposits of calcification in the T. media (Fig. 138) and also in the mural artery of the heart (Fig. 139). In group D rabbits calcification was

also demonstrated in the T. intima of the occluded mural artery of heart (Fig.140) and T. media of the atheromatous plaque (Fig. 141). Black deposits of calcification were also seen in T. media of aorta (Fig. 142) and the cardiac muscles (Fig. 143). Kidneys showed calcification in basement membrane of the glomerulus and tubules (Fig. 144) and T. intima of renal artery (Fig. 145). Trachea showed calcification in basement membrane of the epithelial lining (Fig. 146) while adrenal showed diffuse calcification (Fig. 147).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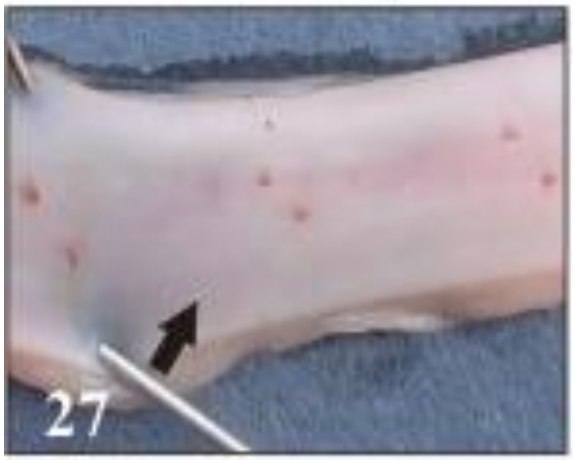
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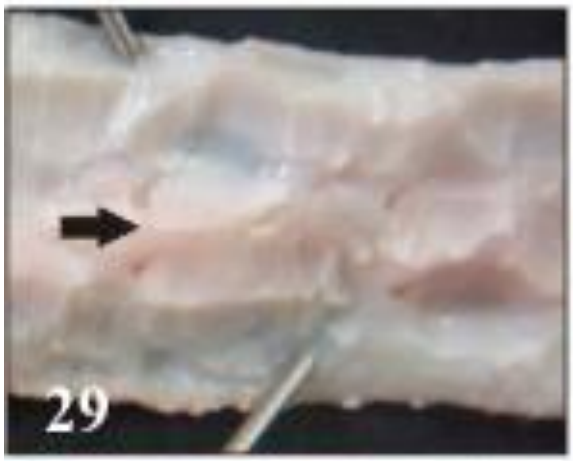
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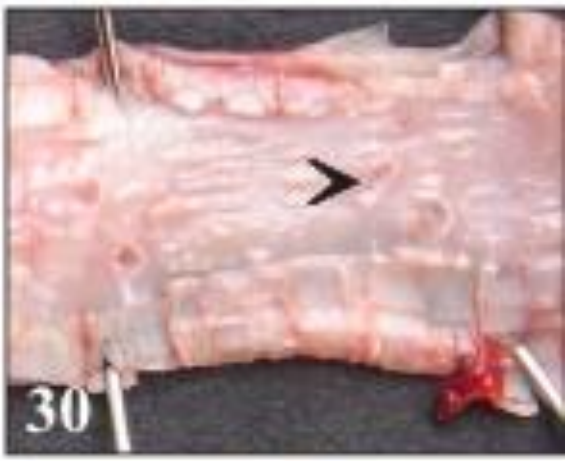
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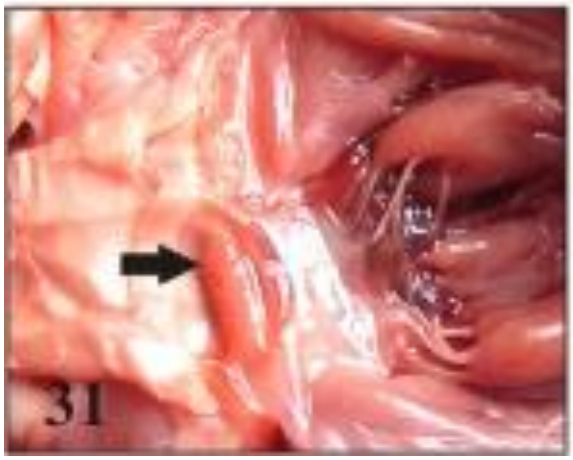
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32

Legends to figures

Fig.1. Hemoglobin concentration in rabbits of group A, B, C and D on Day 0, 28, 56 and 70.

Fig.2. TLC in rabbits of group A, B, C and D on Day 0, 28, 56 and 70.

Fig.3. Heterophil counts in rabbits of group A, B, C and D on Day 0, 28, 56 and 70.

Fig.4. Lymphocyte counts in rabbits of group A, B, C and D on Day 0, 28, 56 and 70.

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D

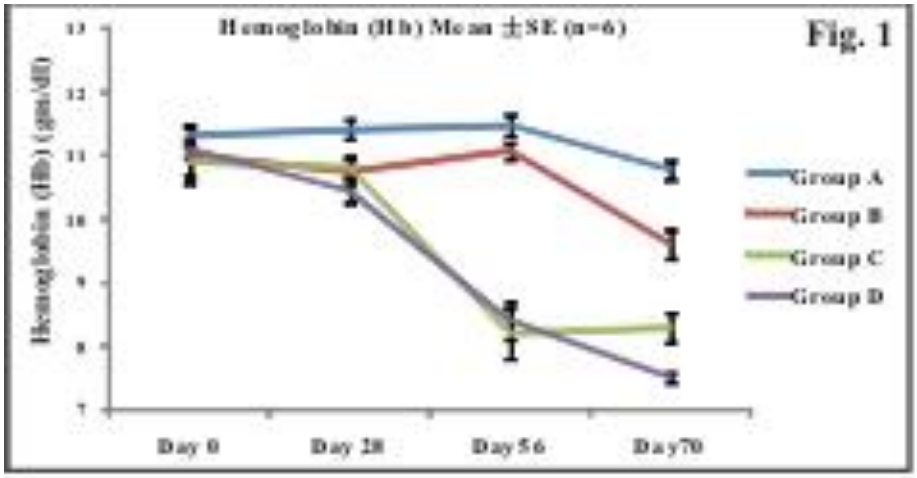


Fig. 1

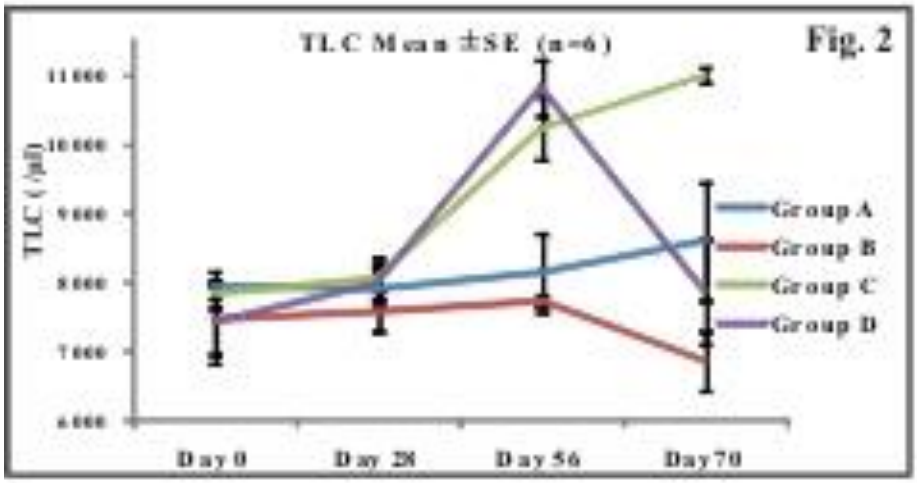


Fig. 2

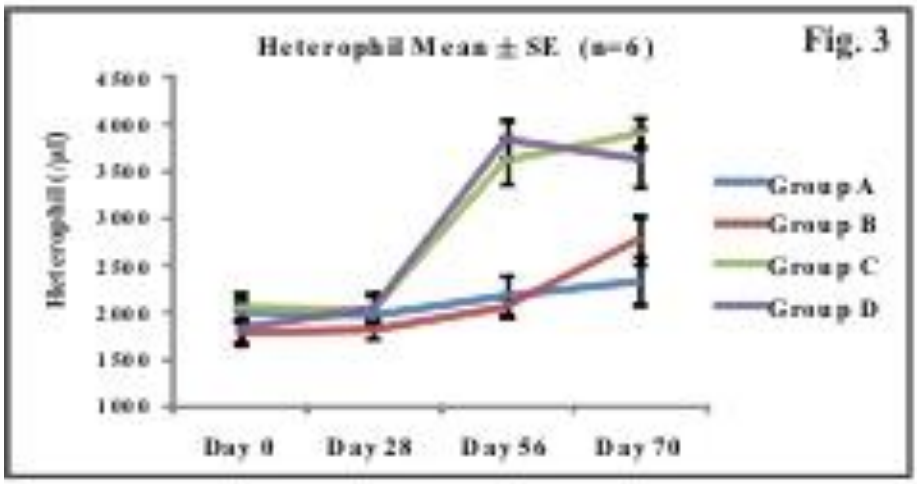


Fig. 3

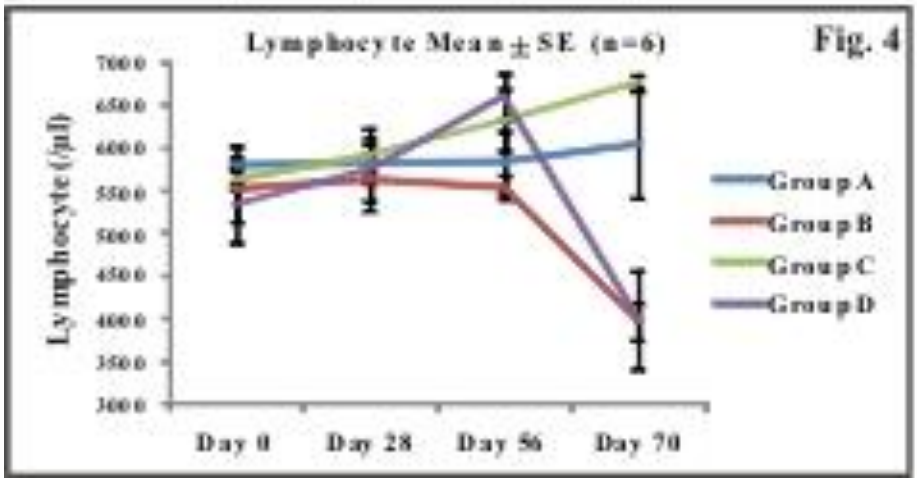


Fig. 4

Legends to figures

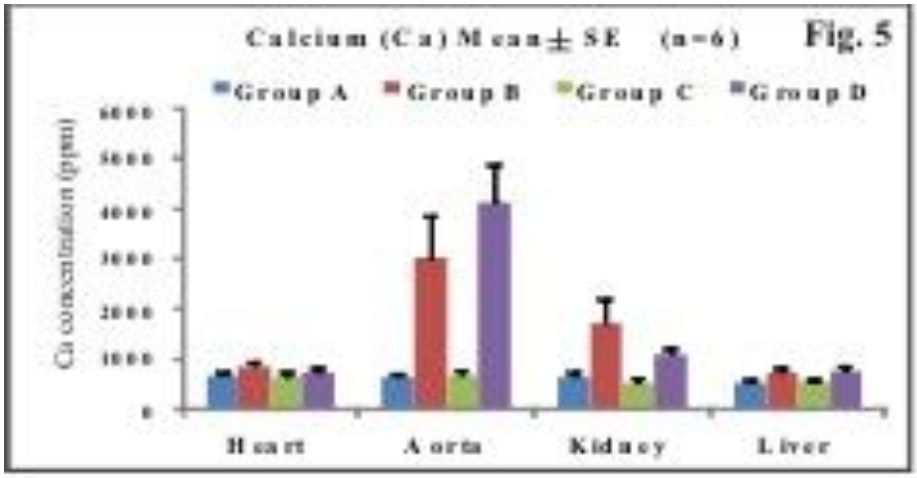
Fig.5. Calcium concentrations in heart, aorta, kidney and liver tissues of group A, B, C and D rabbits.

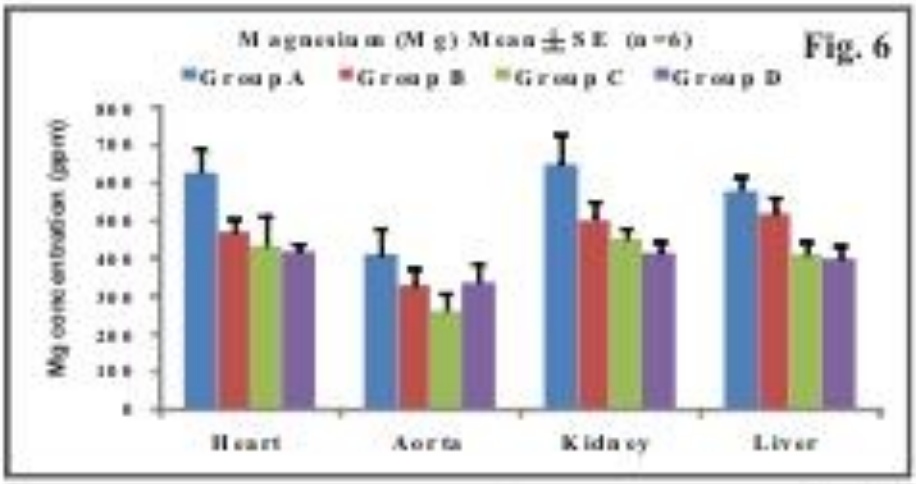
Fig.6. Magnesium concentrations in heart, aorta, kidney and liver tissues of group A, B, C and D rabbits.

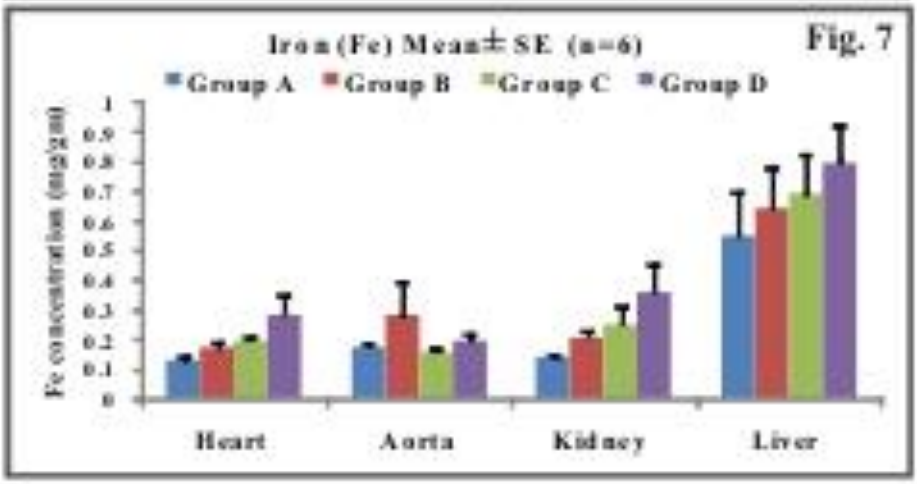
Fig.7. Iron concentrations in heart, aorta, kidney and liver tissues of group A, B, C and D rabbits.

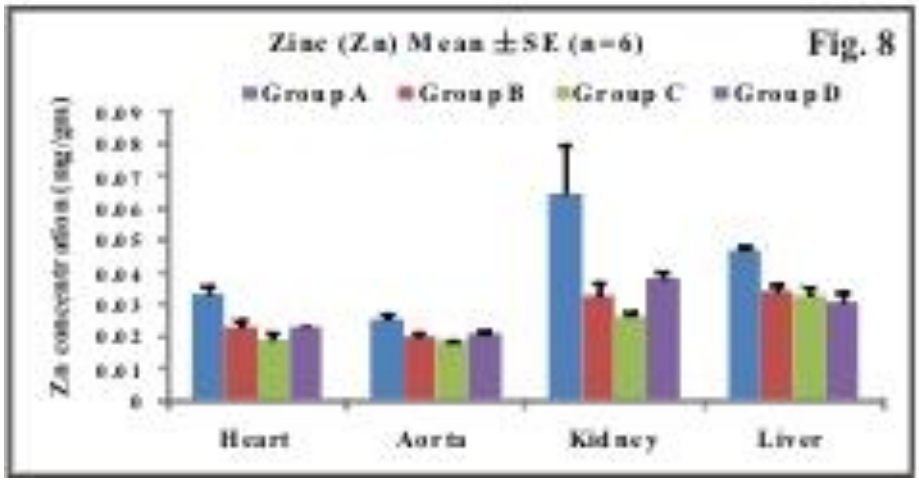
Fig.8. Zinc concentrations in heart, aorta, kidney and liver tissues of group A, B, C and D rabbits.

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D


Calcium (Ca) Mean ± SE (n=6)
Fig. 5
Group A
Group B
Group C
Group D
Ca concentration (ppm)
Heart
Aorta
Kidney
Liver


Magnesium (Mg) Mean ± SE (n=6)
Fig. 6
Group A
Group B
Group C
Group D
Mg concentration (ppm)
Heart
Aorta
Kidney
Liver


Iron (Fe) Mean ± SE (n=6)
Fig. 7
Group A
Group B
Group C
Group D
Fe concentration (mg/gm)
Heart
Aorta
Kidney
Liver


Zinc (Zn) Mean ± SE (n=6)
Fig. 8
Group A
Group B
Group C
Group D
Heart
Aorta
Kidney
Liver

Legends to figures

Fig.9. Copper concentrations in heart, aorta, kidney and liver tissues of group A, B, C and D rabbits.

Fig.10. Sodium concentrations in heart, aorta, kidney and liver tissues of group A, B, C and D rabbits.

Fig.11. Potassium concentrations in heart, aorta, kidney and liver tissues of group A, B, C and D rabbits.

Group A: control feed; Group B: control feed + vitamin D; Group C: control feed + cholesterol; Group D: control feed + cholesterol + vitamin D

Legends to figures

Fig.12a. Group A (control feed). Section of an aorta showing no AKPase activity. X40.

Fig.12b. Group B (control feed + vitamin D). Section of an aorta showing very mild AKPase activity. X40.

Fig.12c. Group C (control feed + cholesterol). Section of an aorta showing no AKPase activity. X40.

Fig.12d. Group D (cholesterol + vitamin D). Section of an aorta showing moderate AKPase activity. X40.

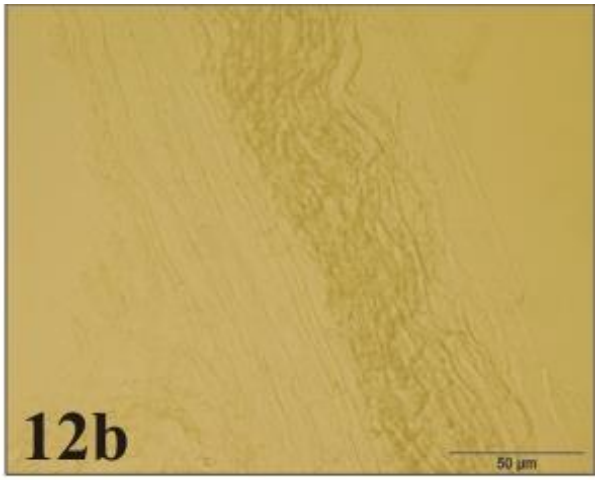
Fig.13a. Group A. Section of heart showing weak AKPase activity. X40.

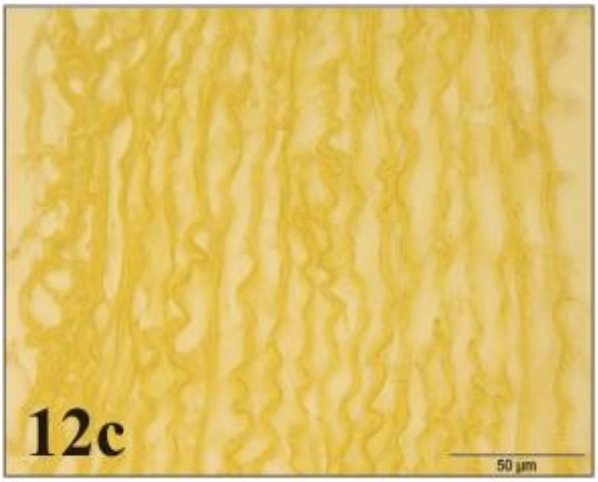
Fig.13b. Group B. Section of heart showing weak to moderate AKPase activity. X40.

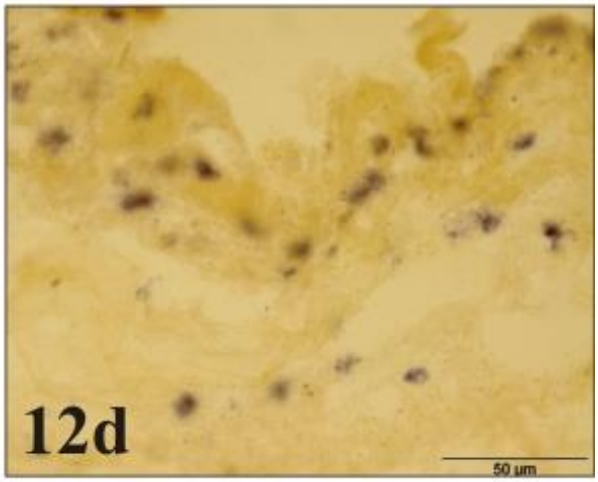
Fig.13c. Group C. Section of heart showing weak AKPase activity. X40.

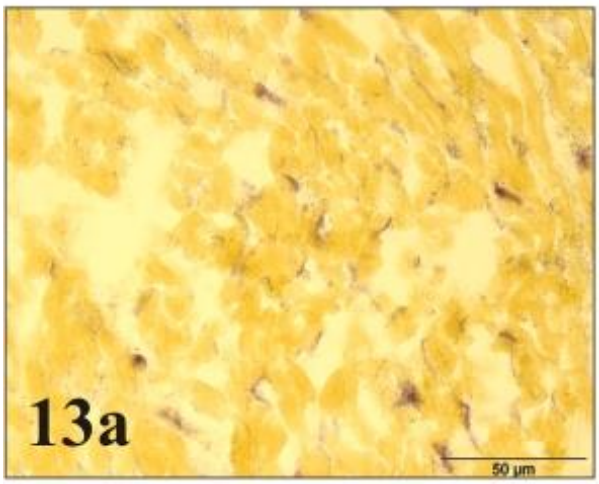
Fig.13d. Group D. Section of heart showing moderate AKPase activity. X40.

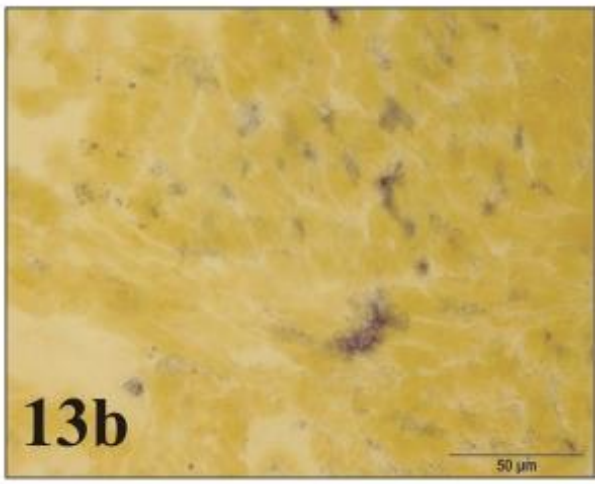

12a
50 µm


12b
50 µm


12c
50 µm


12d
50 µm


13a
50 µm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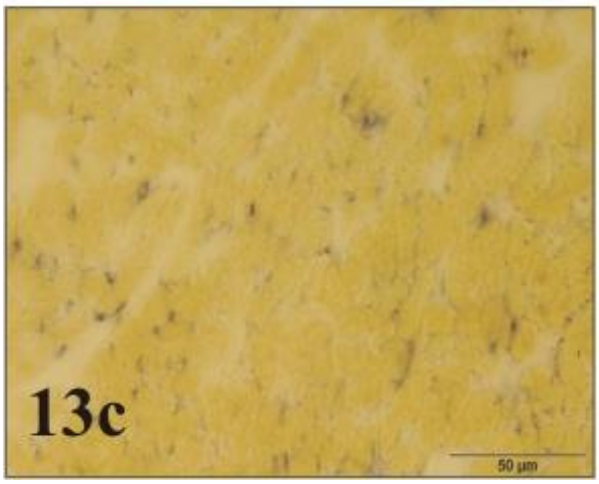
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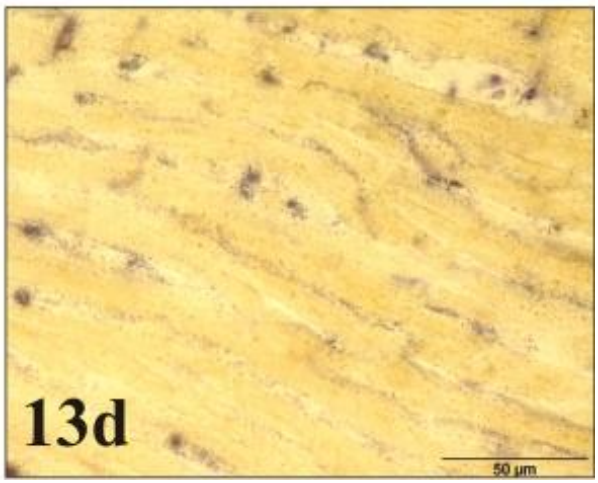
50 µm


13d
50 µm

Legends to figures

Fig.14a. Group A (control feed). Section of liver showing moderate AKPase activity. X40.

Fig.14b. Group B (control feed + vitamin D). Section of liver showing moderate AKPase activity. X40.

Fig.14c. Group C (control feed + cholesterol). Section of liver showing focal moderate to strong AKPase activity. X40.

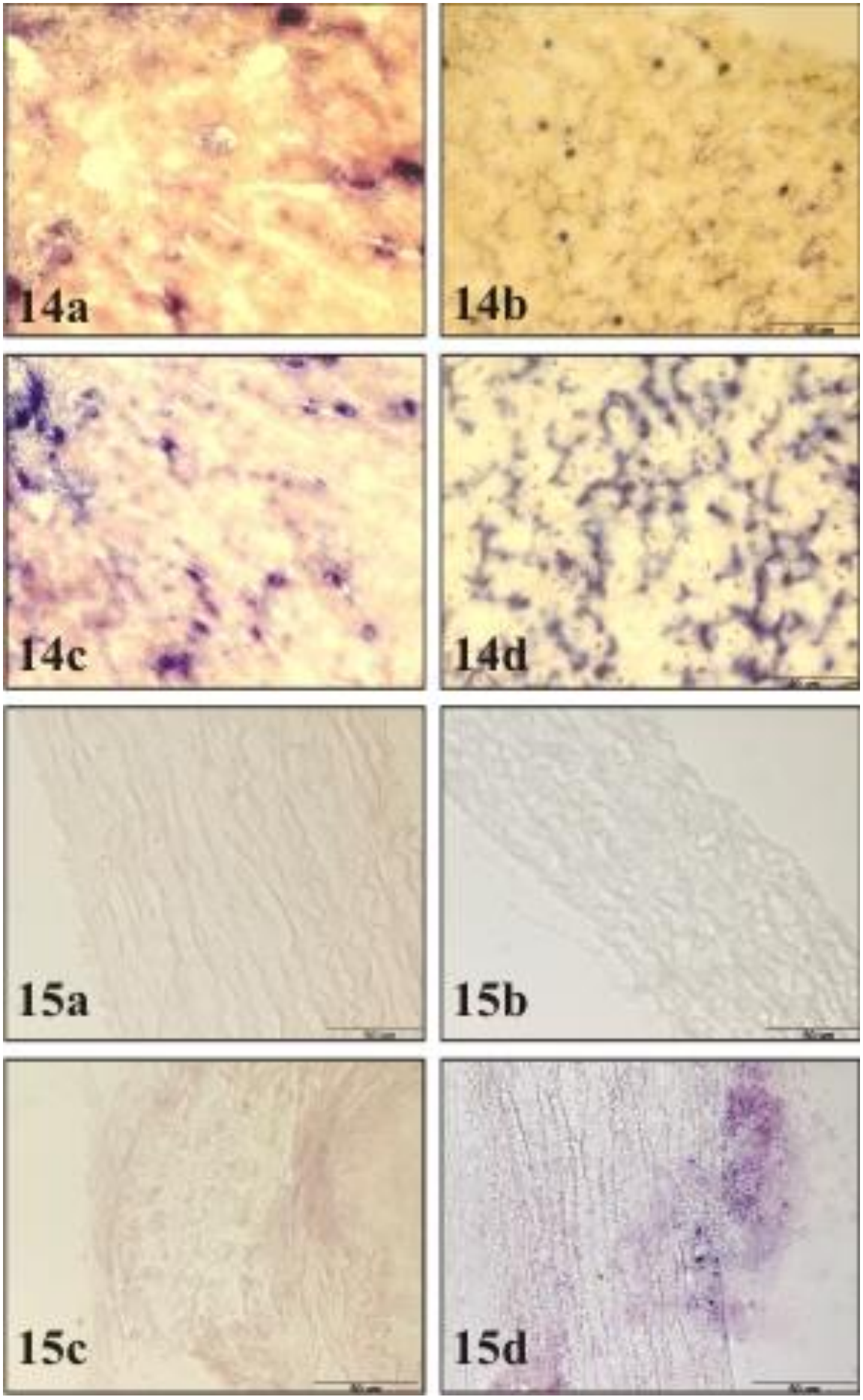
Fig.14d. Group D (cholesterol + vitamin D). Section of liver showing diffused moderate to strong AKPase activity. X40.

Fig.15a. Group A. Section of an aorta showing no G6PD activity. X40.

Fig.15b. Group B. Section of an aorta showing weak G6PD activity. X40.

Fig.15c. Group C. Section of an aorta showing weak to moderate G6PD activity. X40.

Fig.15d. Group D. Section of an aorta showing moderate to strong G6PD activity. X40.


14a
14b
14c
14d
15a
15b
15c
15d

Legends to figures

Fig.16a. Group A (control feed). Section of heart showing weak G6PD activity. X40.

Fig.16b. Group B (control feed + vitamin D). Section of heart showing moderate G6PD activity. X40.

Fig16c. Group C (control feed + cholesterol). Section of heart showing moderate G6PD activity. X40.

Fig.16d. Group D (cholesterol + vitamin D). Section of heart showing intense G6PD activity. X40.

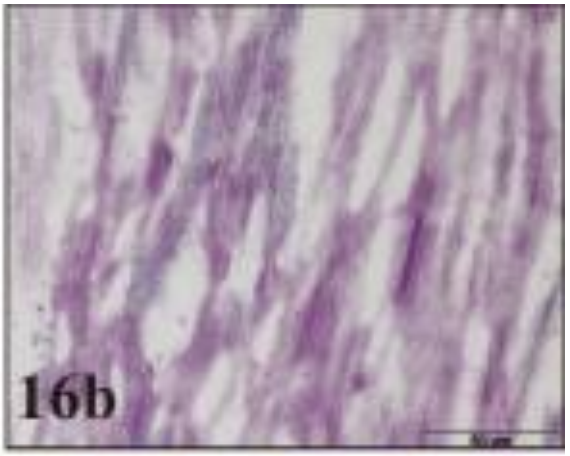
Fig.17a. Group A. Section of an aorta showing weak NADPH Diphorase activity. X40.

Fig.17b. Group B. Section of an aorta showing moderate NADPH Diphorase activity. X40.

Fig.17c. Group C. Section of an aorta showing moderate NADPH Diphorase activity. X40.

Fig.17d. Group D. Section of an aorta showing strong to intense NADPH Diphorase activity. X40.


16a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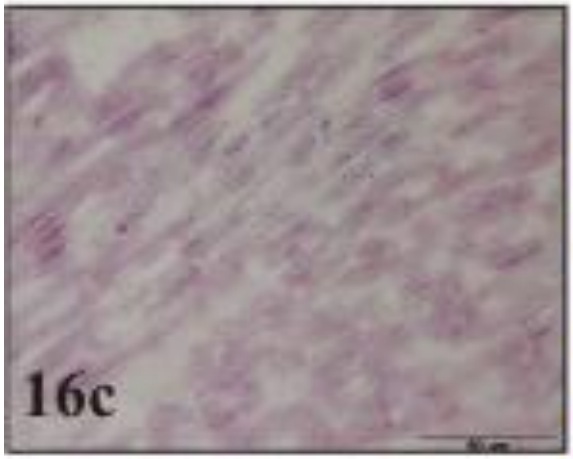
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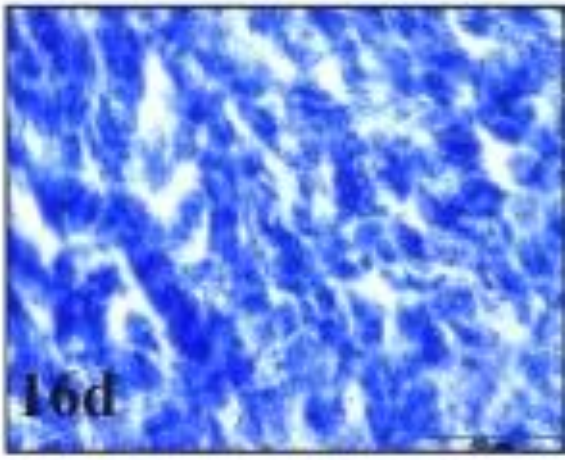
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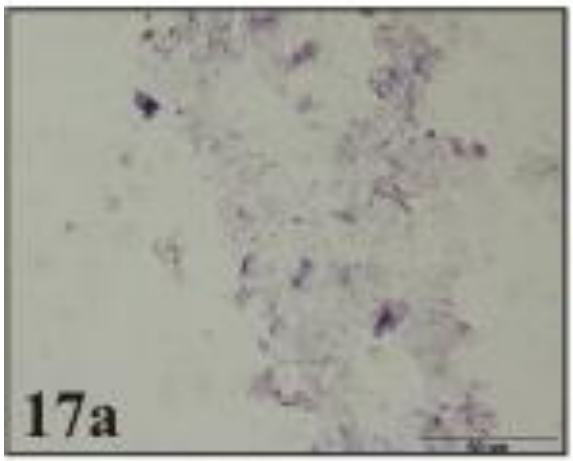
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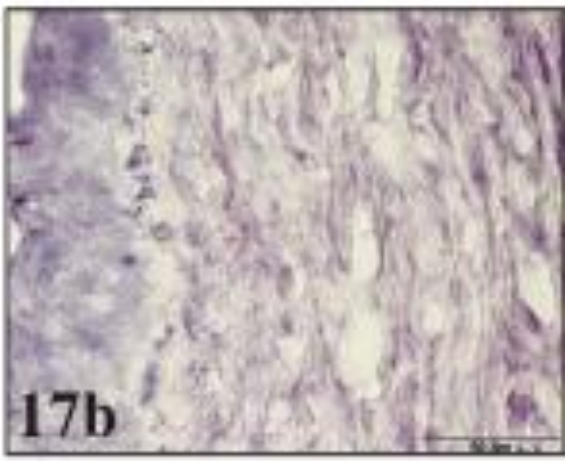
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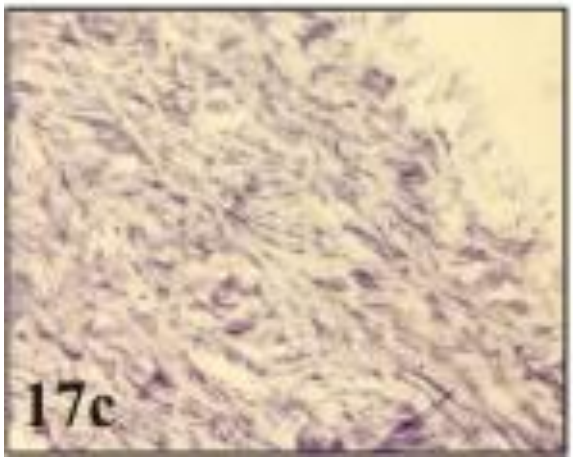
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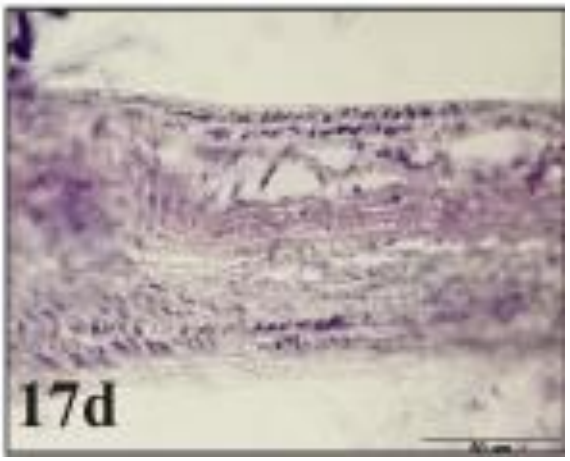

17d

Legends to figures

Fig.18a. Group A (control feed). Section of heart showing weak NADPH Diphorase activity. X40.

Fig.18b. Group B (control feed + vitamin D). Section of heart showing moderate to strong NADPH Diphorase activity. X40.

Fig.18c. Group C (control feed + cholesterol). Section of heart showing weak to moderate to strong NADPH Diphorase activity. X40.

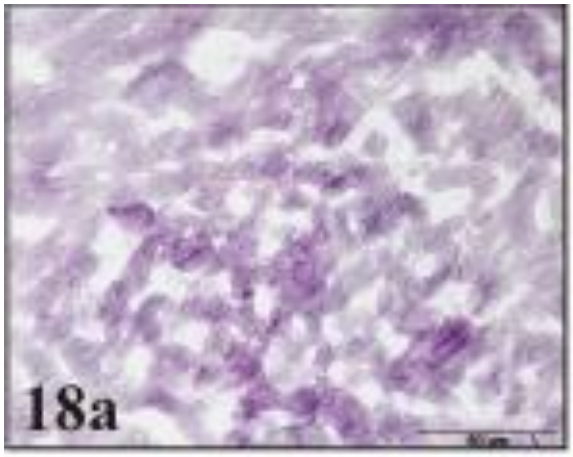
Fig.18d. Group D (cholesterol + vitamin D). Section of heart showing intense NADPH Diphorase activity. X40.

Fig.19a. Group A. Section of liver showing weak NADPH Diphorase activity. X40.

Fig.19b. Group B. Section of liver showing moderate NADPH Diphorase activity. X40.

Fig.19c. Group C. Section of liver showing moderate NADPH Diphorase activity. X40.

Fig.19d. Group D. Section of liver showing intense NADPH Diphorase activity. X40.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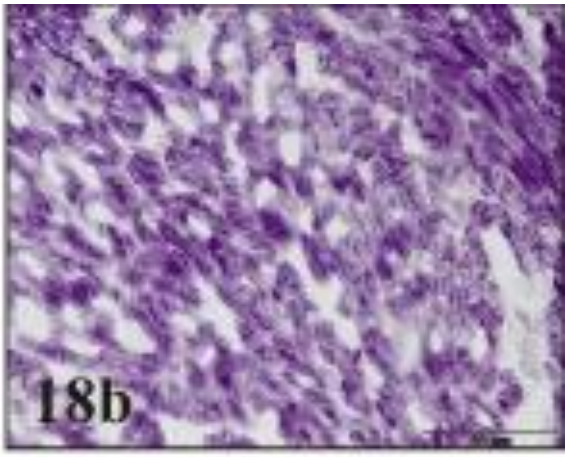
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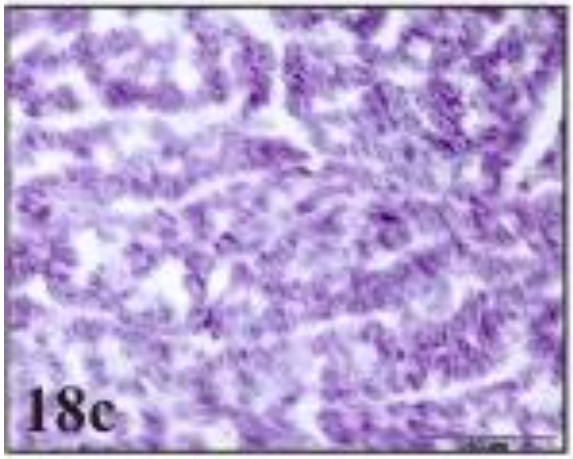
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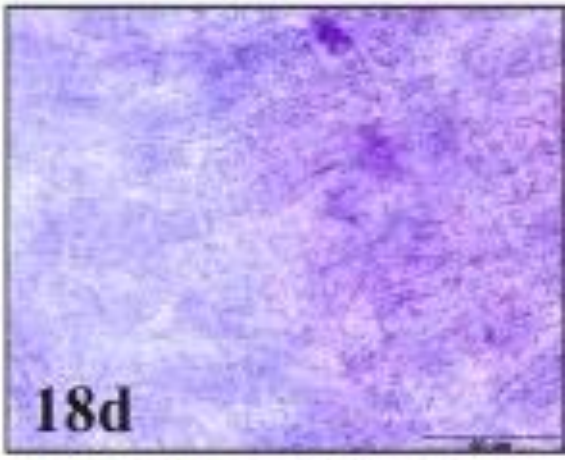
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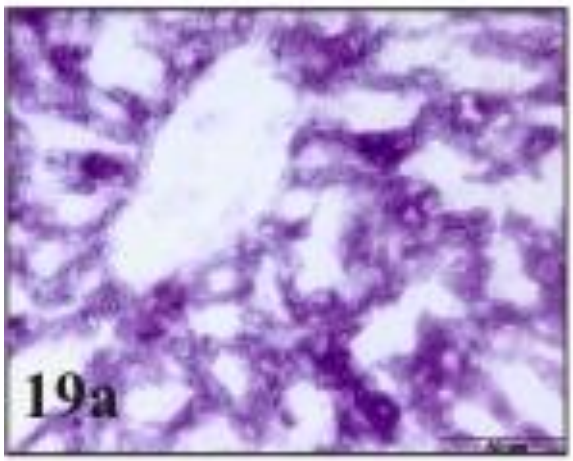
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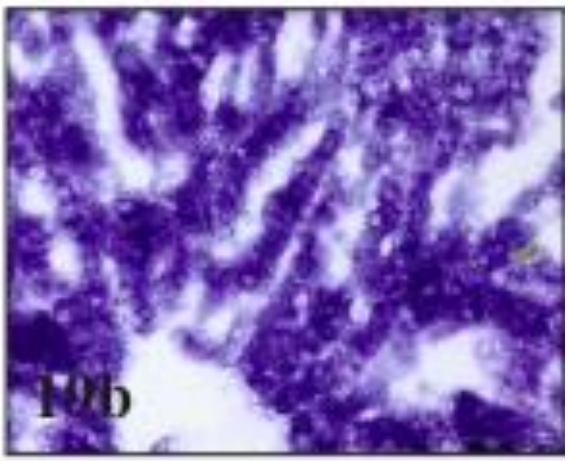
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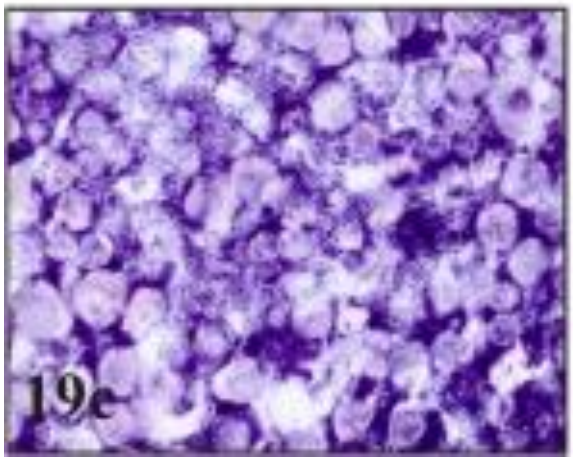
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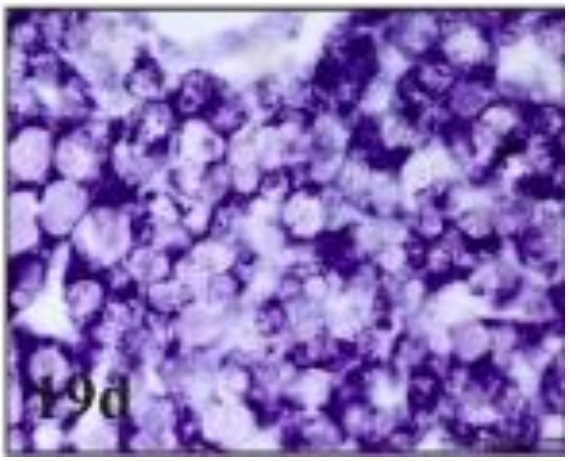

19d

Legends to figures

Fig.20a. Group A (control feed). Section of an aorta showing moderate LDH activity. X40.

Fig.20b. Group B (control feed + vitamin D). Section of an aorta showing weak LDH activity. X40.

Fig.20c. Group C (control feed + cholesterol). Section of an aorta showing moderate to strong LDH activity. X40.

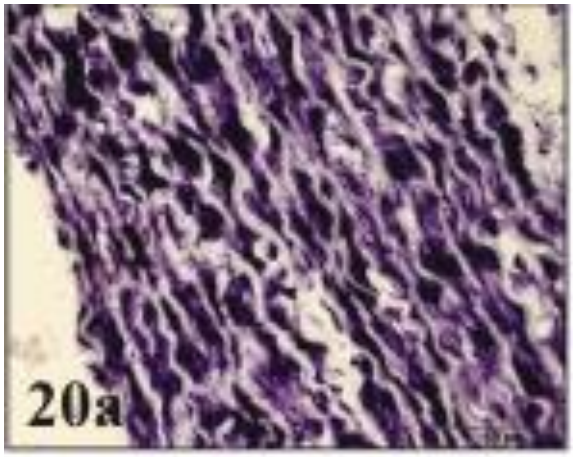
Fig.20d. Group D (cholesterol + vitamin D). Section of an aorta showing weak to moderate LDH activity. X40.

Fig.21a. Group A. Section of liver showing moderate LDH activity. X40.

Fig.21b. Group B. Section of liver showing moderate to strong LDH activity. X40.

Fig.21c. Group C. Section of liver showing moderate to strong LDH activity. X40.

Fig.21d. Group D. Section of liver showing moderate to strong LDH activity. X40.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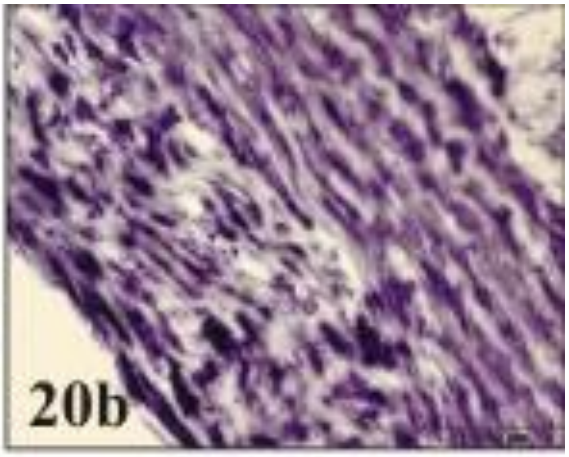
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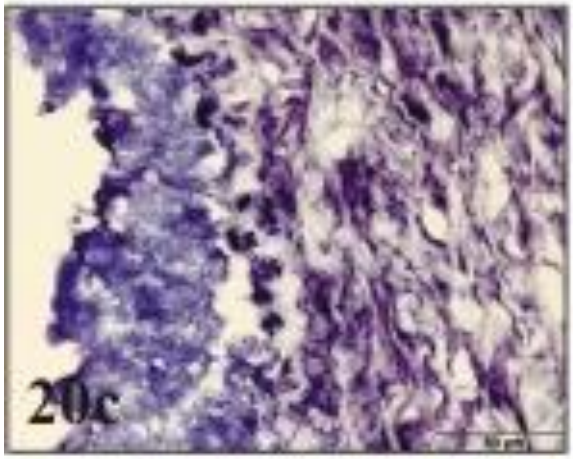
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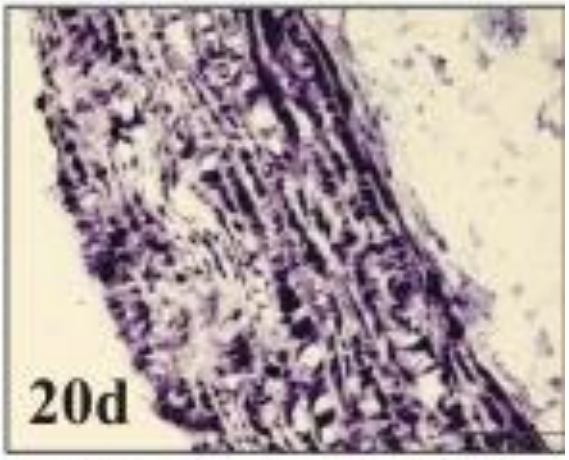
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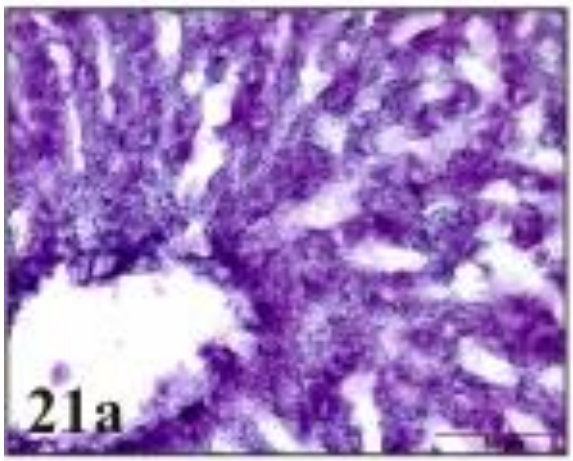
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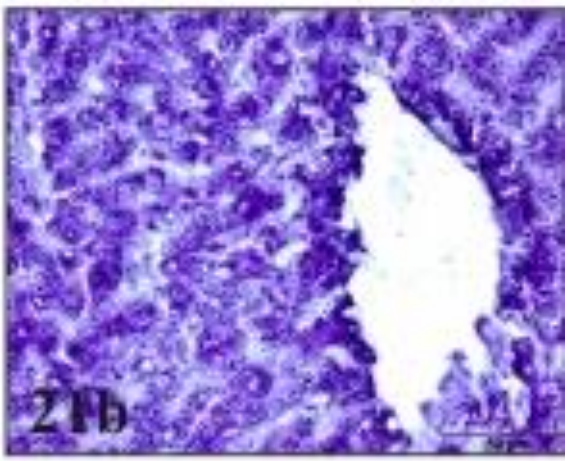
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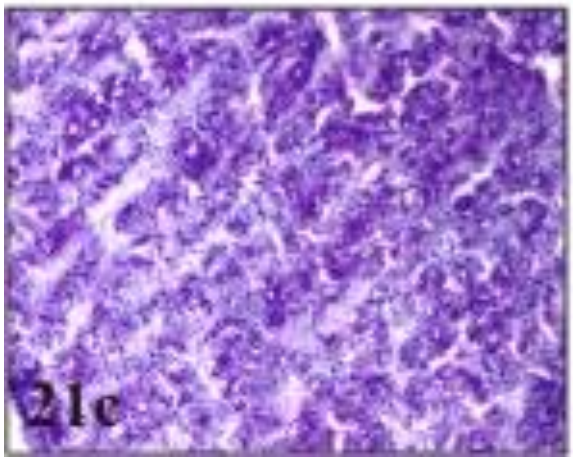
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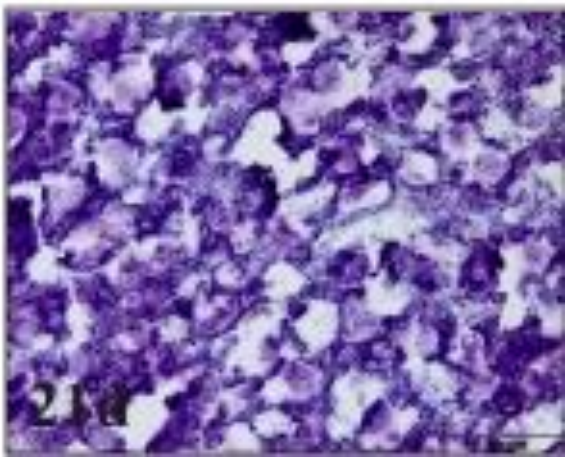
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Fig.22a. Group A (control feed). Section of an aorta showing no sudanophilia. Sudan III. X40.

Fig.22b. Group B (control feed + vitamin D). Section of an aorta showing mild sudanophilia. Sudan III. X40.

Fig.22c. Group C (control feed + cholesterol). Section of an aorta showing strong sudanophilia. Sudan III. X40.

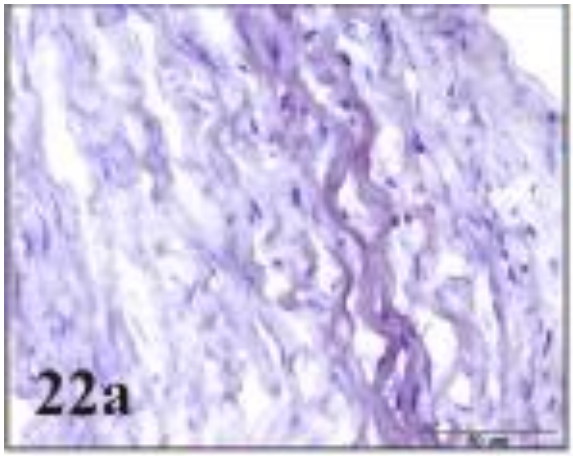
Fig.22d. Group D (cholesterol + vitamin D). Section of an aorta showing strong to intense sudanophilia. Sudan III. X40.

Fig. 23a. Group A. Section of heart showing no sudanophilia. Sudan III. X20.

Fig. 23b. Group B. Section of heart showing mild sudanophilia. Sudan III. X20.

Fig.23c. Group C. Section of heart showing mild to moderate sudanophilia. Sudan III. X20.

Fig.23d. Group D. Section of heart showing moderate sudanophilia. Sudan III. X20.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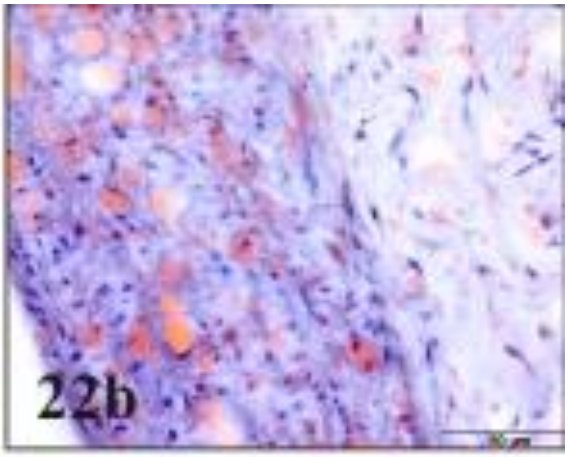
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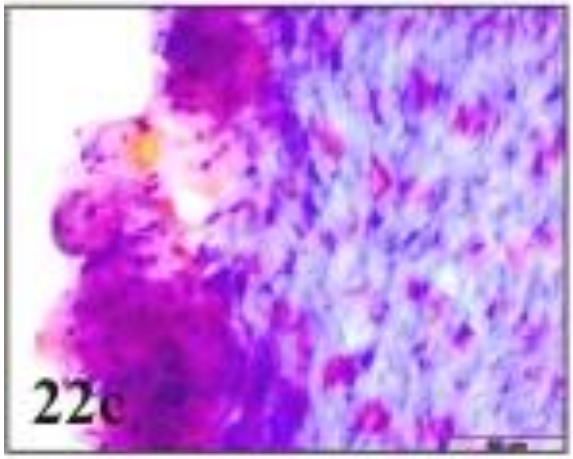
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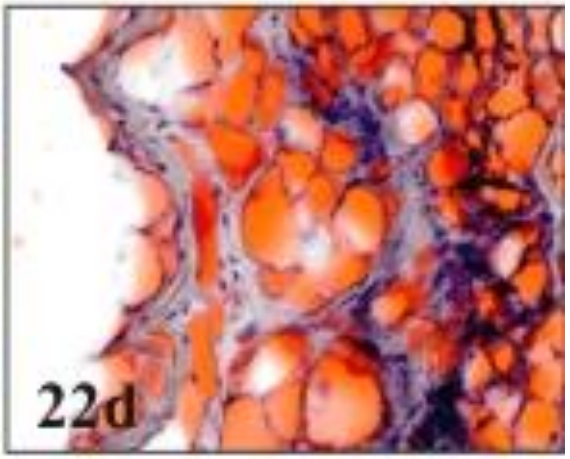
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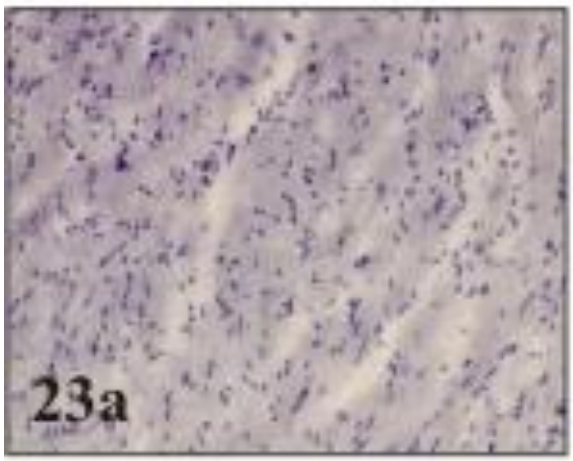
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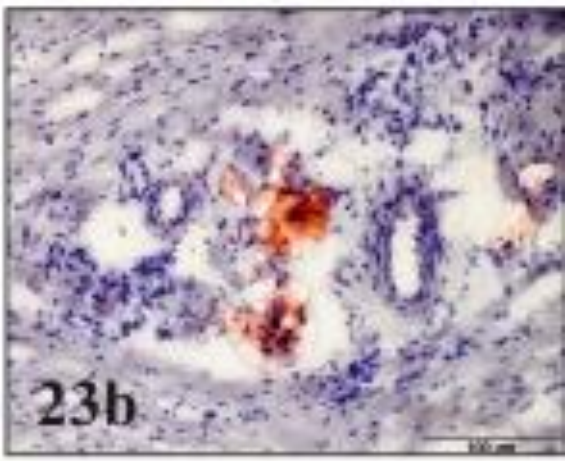
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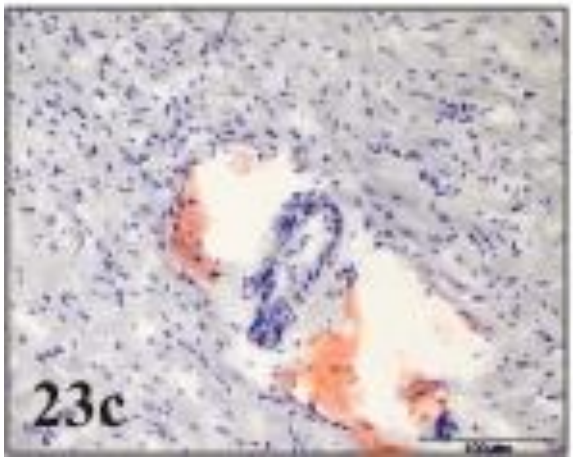
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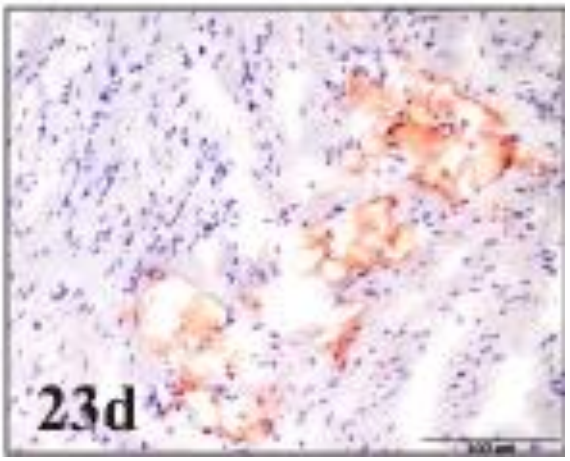
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Fig.24a. Group A (control feed). Section of liver showing no sudanophilia. Sudan III. X40.

Fig.24b. Group B (control feed + vitamin D). Section of liver showing no sudanophilia. Sudan III. X40.

Fig.24c. Group C(control feed + cholesterol).. Section of liver showing strong to intense sudanophilia. Sudan III. X20.

Fig.24d. Group C Section of liver showing strong sudanophilia. Sudan III. X10.

Fig.25a. Group D (cholesterol + vitamin D). Section of liver showing moderate sudanophilia. Sudan III. X20.

Fig.25b. Group D. Section of liver showing intense sudanophilia. Sudan III. X20.

Fig.25c. Group D. Section of liver showing intense sudanophilia. Higher magnification of fig. 25b. Sudan III. X40.

Fig.25d. Group D. Section of liver showing strong to intense sudanophilia. Sudan III. X100.

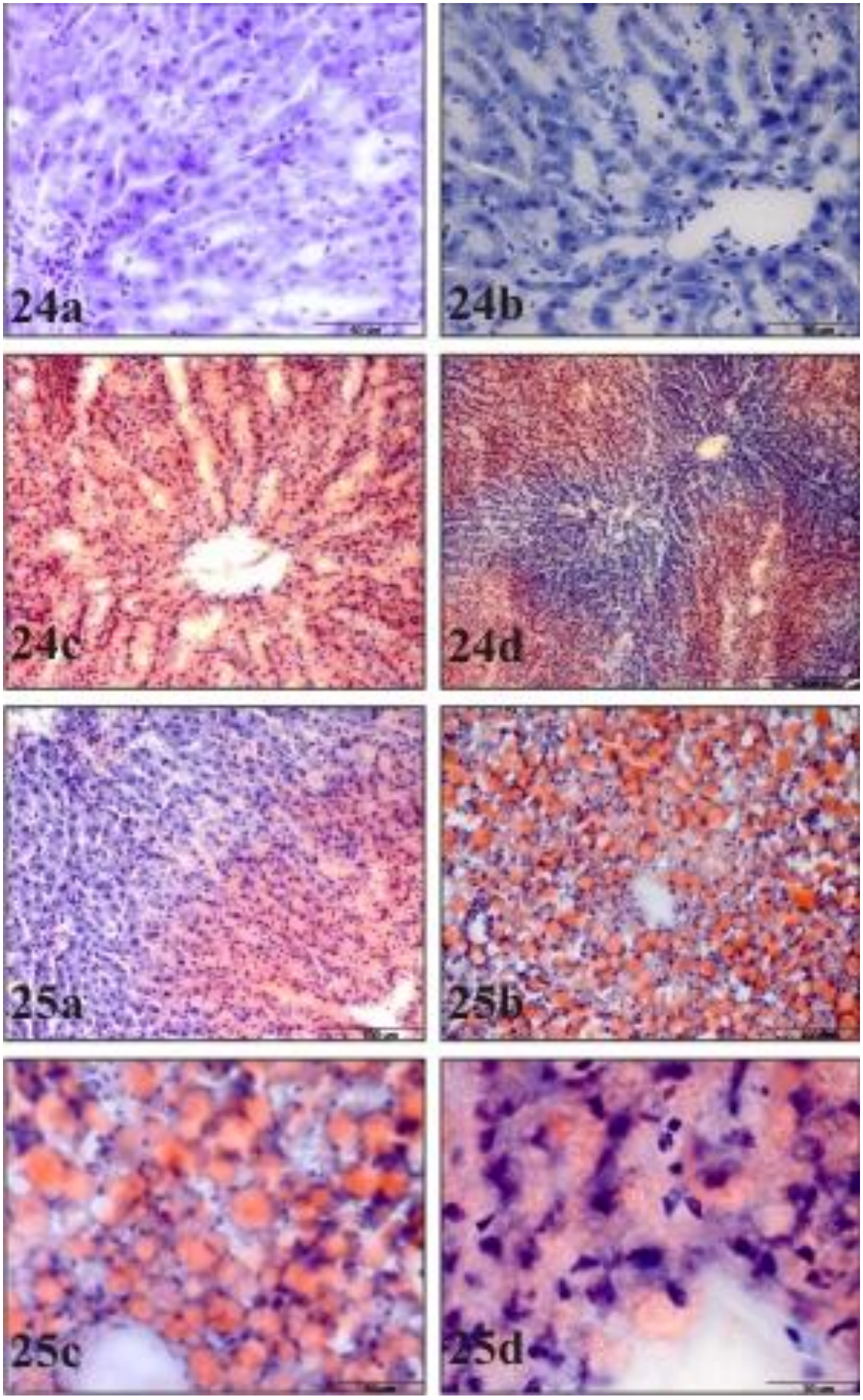
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Fig.26. Group A (control feed). Smooth, thin, and elastic cardiac valves and luminal surface of aorta. No apparent changes are se

en involving the myocardium and endocardium.

Fig.27. Group A: Aorta showing thin smooth luminal surface.

Fig.28. Group A: Cardiac valves showing smooth elastic surface.

Fig.29. Group B (control feed + vitamin D). The aorta is firm and inelastic because of the calcium deposits in T. intima and T. media.

Fig.30. Group B. The aorta showing small atheromatous plaque.

Fig.31. Group B. Cardiac valves and endocardium showing mineralization.

Fig.32. Group B. Kidney showing pin point chalky white calcification in cortex and at the corticomedullary junction.

Legends to figures

Fig.33. Group C (control feed + cholesterol). Plaque formation and thickening of the cardiac valves and luminal surface of the aorta. No apparent changes are seen involving the myocardium and endocardium.

Fig.34. Group C. Aorta showing small and large yellowish white atheromatous plaques on the intimal surface.

Fig.35. Group C. Cardiac valves showing thickening and small atherosclerotic plaques.

Fig.36. Group C. Heart showing normal coronary arteries. Liver is swollen and yellowish brown (fatty liver).

Fig.37. Group C. Stomach showing normal mucosal surface.

Fig.38. Comparison between the size of adrenals from group C (left) and group A (right) rabbits. Note the marked hypertrophied adrenals of the group C rabbits.

Fig.39. Group C. Whitish coloured gall bladder.

Legends to figures

Fig.40. Group D (cholesterol + vitamin D). Heart valves and aorta showing severe atherosclerotic changes. The aorta is firm and inelastic showing multiple white mineralized foci.

Fig.41. Group D. Cardiac valves showing severe atherosclerotic changes. The valves are firm showing white mineralized deposits.

Fig.42. Group D. Heart showing prominent coronary arteries. Lungs parenchyma is red because of congestion of pulmonary vasculature and alveolar capillaries.. Liver is swollen and yellow.

Fig.43. Comparison of colour and size of the liver from the group D (left) and group A (right) rabbit. Note the marked fatty change and hypertrophy of liver of the group D rabbit.

Fig.44. Comparison of colour and size of the spleen from the group D (right) and group A (left) rabbits. Note the marked hypertrophy and whitish discolouration of the spleen of group D rabbit.

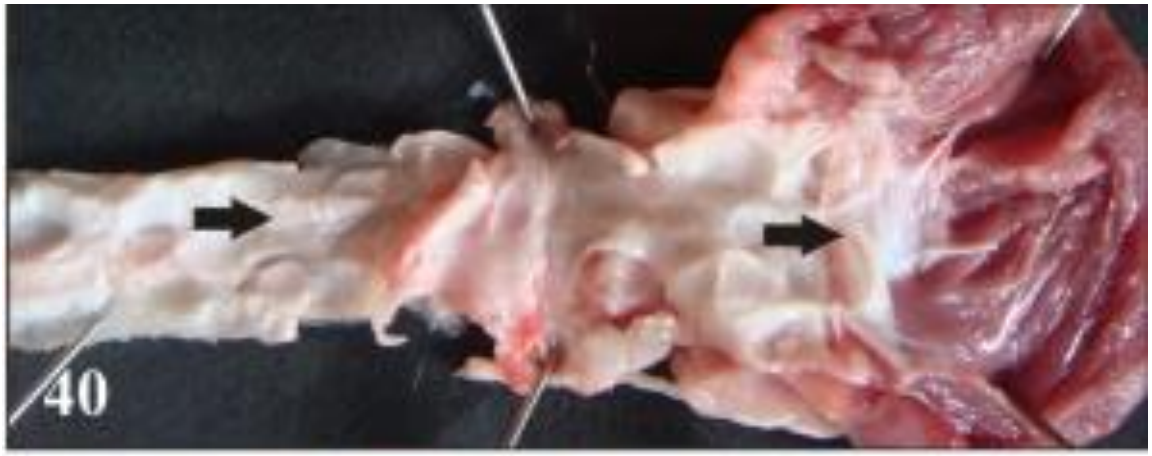
Fig.45. Comparison between the size of adrenals from group D (top) and group A (bottom) rabbit. Note the marked hypertrophy of adrenals of the group D rabbit.

Fig.46. Group D. Note the presence of chalky white deposits on the serosal surface of the stomach.

Fig.47. Group D. Mucosal surface of stomach showing thickening and corrugation. Marked deposition of chalky white material seen.

Fig.48. Group D. Intestine showing presence of white deposits of calcium on the serosal surface.

Fig.49. Group D. Trachea showing hemorrhages and white deposits on the mucosal surface.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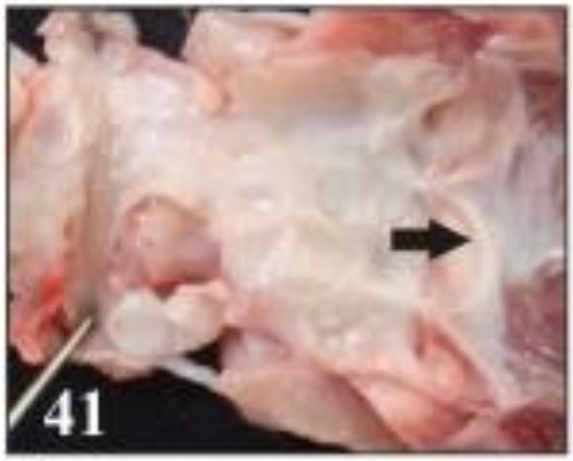
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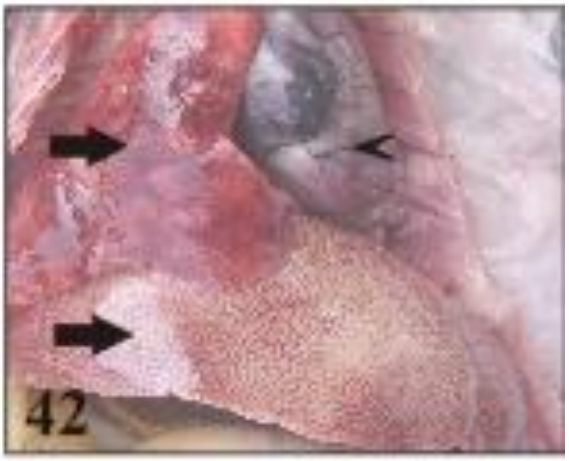
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Fig.50. Group A (control feed). Section of an aorta showing normal histology. H&E X20.

Fig.51. Group A. Section of an aorta showing normal histology. H&E X40.

Fig.52. Group B (control feed + vitamin D). Section of an aorta showing mineralization in the T. intima and T. media. H&E X40.

Fig.53. Group C (control feed + cholesterol). Section of an aorta showing initial stage of atheromatous lesion. Note the elevation of T. intima due to lipid deposition underneath (arrowhead). H&E X40.

Fig.54. Group C. Section of an aorta showing advanced atheroma. Note the ruptured endothelial lining of the T. intima. Foam cells and mild smooth muscle cell proliferation form the core. Lipid vacoules are seen in the T. media. H&E X20.

Fig.55. Group C. Section of an aorta showing atheroma. Note the intact endothelial lining of the plaque (arrowhead). Core is formed of the lipid-laden macrophages (foam cells) (arrow). Lipid infiltration in the tunica media is also evident. H&E X40.

Fig.56. Group D (cholesterol + vitamin D). Section of an aorta showing medial calcification and adjacent atheroma formation. Note the layer of mineralization in the tunica media (arrow) on the left half region seperating the layers. Note the lipid laden macrophages in the sub-intima of the aorta on the right half region. H&E X20.

Fig.57. Group D. Section of an aorta showing atheromatous lesion and calcification in T. media. H&E X4.

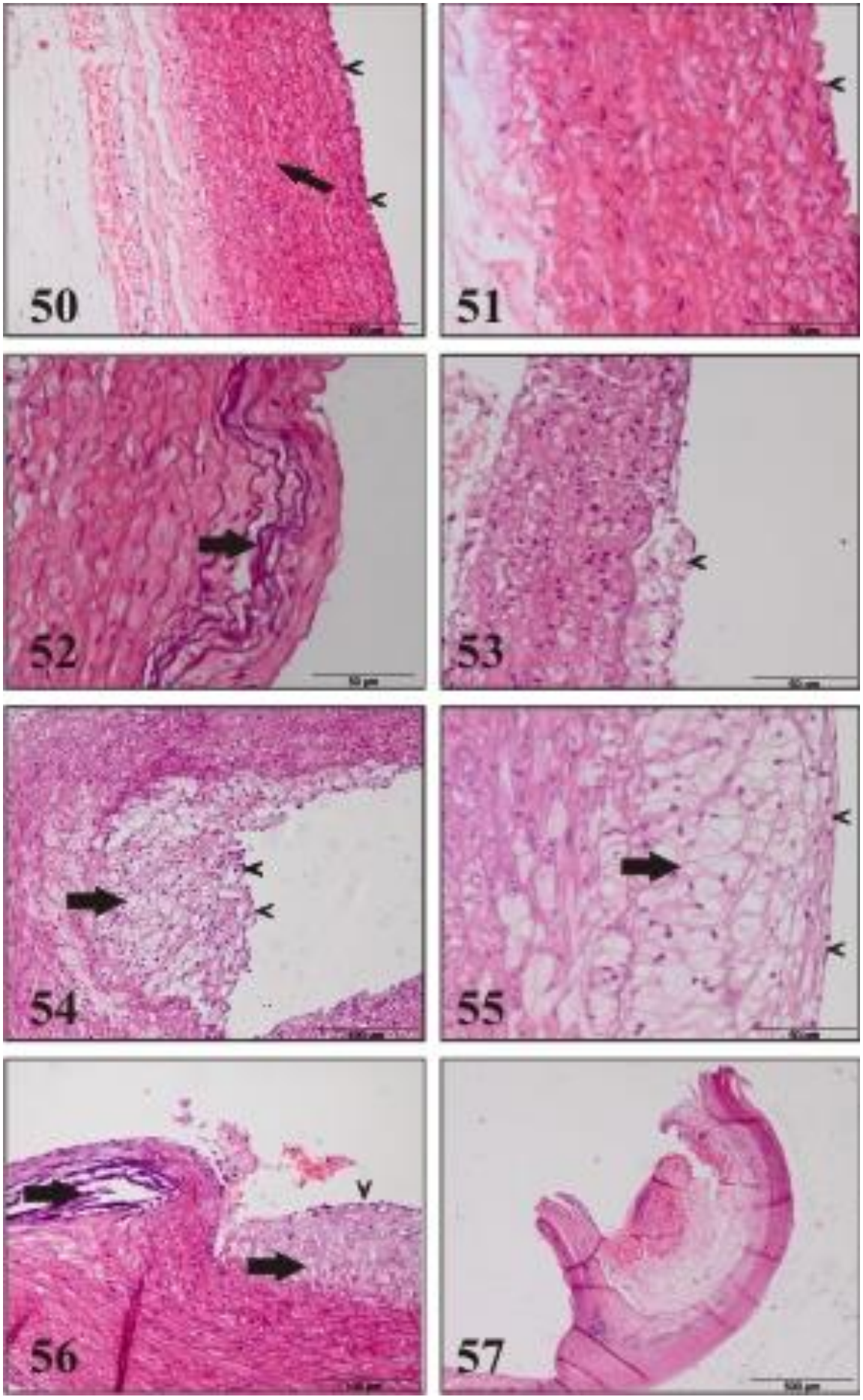
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Fig.58. Group D. Higher magnification of Fig. 57. The Cap of the atheromatous lesion is formed by fibrous tissue, smooth muscle cell and inflammatory cell infiltration (arrow). Note the hemorrhages in the core of the atheroma which is due to rupture of new delicate blood vessels. Mineralization in the T. media is evident (arrowhead). H&E X10.

Fig.59. Group D. Section of an aorta showing ruptured atheromatous lesion. Note the fibrous tissue cap formation and smooth muscle cell proliferation in the tunica intima (arrow). Core of the atheroma is composed of cellular debris, foam cells, connective tissue fibers and calcium deposition. H&E X10.

Fig.60. Group A (control feed). Section of heart showing normal histology. H&E X40.

Fig.61. Group A. Section of heart showing normal histology. H&E X100.

Fig.62. Group B (control feed + vitamin D). Section of heart showing calcification of T. intima of the blood vessel. H&E X40.

Fig.63. Group B. Section of heart showing Calcification in cardiac muscle. H&E X40.

Fig.64. Group C (control feed + cholesterol). Section of mural coronary artery showing occlusion of the lumen. H&E X40.

Fig.65. Group C. Section of heart showing fat dysplasia with very few remanent of cardiac muscle. H&E X40.

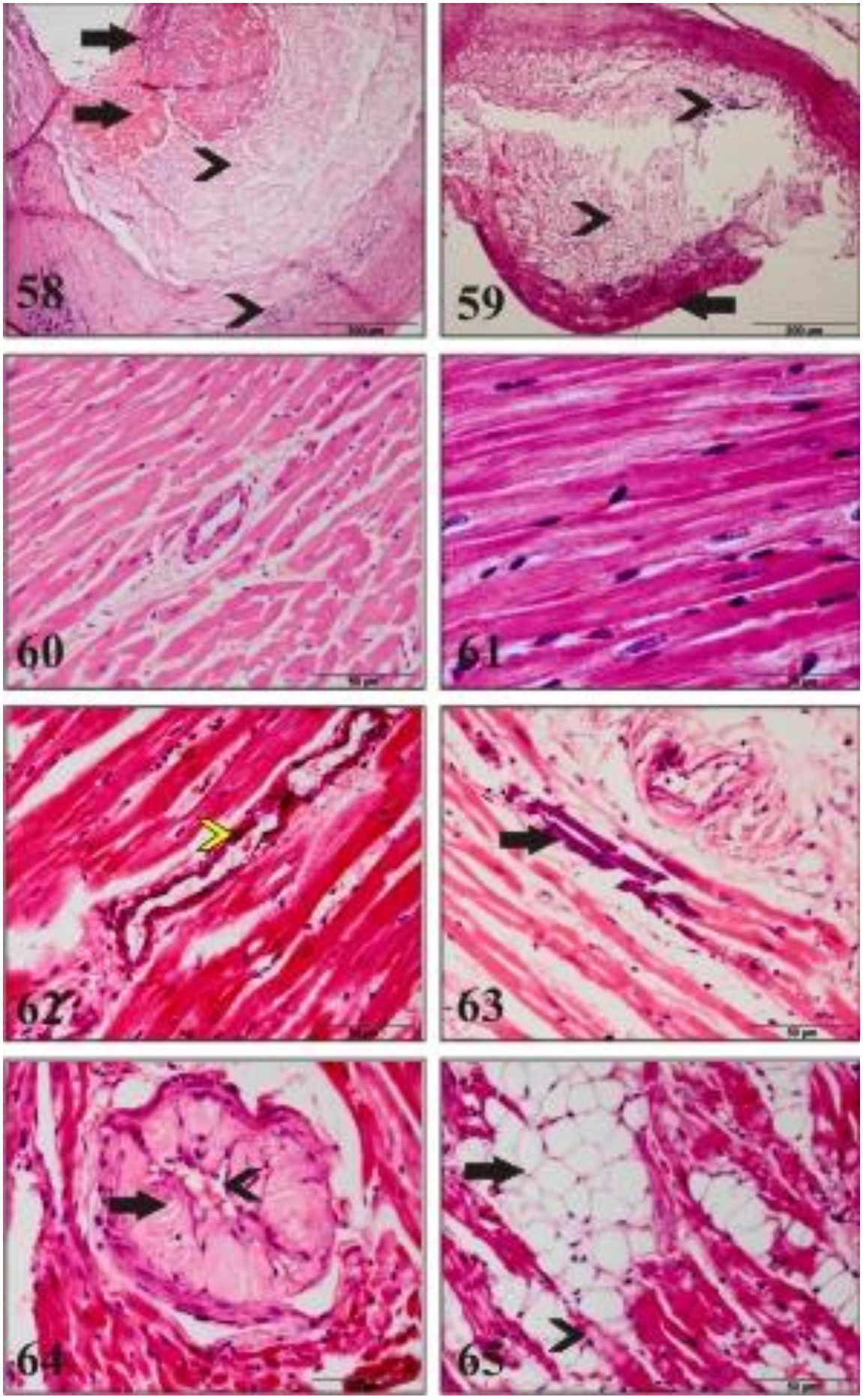
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Fig.66. Group D (cholesterol + vitamin D). Section of mural coronary artery showing occlusion and calcification in T. intima (arrow). H&E X40.

Fig.67. Group D. Section of mural coronary artery showing complete occlusion of the lumen (arrow). H&E X100.

Fig.68. Group D. Section of heart showing fat dysplasia (arrow) with very few remanent of cardiac muscle (arrowhead). H&E X40.

Fig.69. Group D. Section of heart showing lymphomononuclear infiltration in cardiac muscle (arrow) in the ischemic area. H&E X40.

Fig.70. Group A (control feed). Section of liver showing normal histology. H&E X40.

Fig.71. Group B (control feed + vitamin D). Section of liver showing mild fatty changes. Note the vacoulation in the hepatocytes (arrowhead). H&E X20.

Fig.72. Group C (control feed + cholesterol). Section of liver showing fatty changes (arrowhead). Note the decrease in sinusoidal space. H&E X40.

Fig.73. Group C. Section of liver showing fatty changes. Diffuse cytoplasmic accumulation of lipid is evident within hepatocytes throughout the liver (arrowhead). Note the decrease in sinusoidal space. H&E X40.

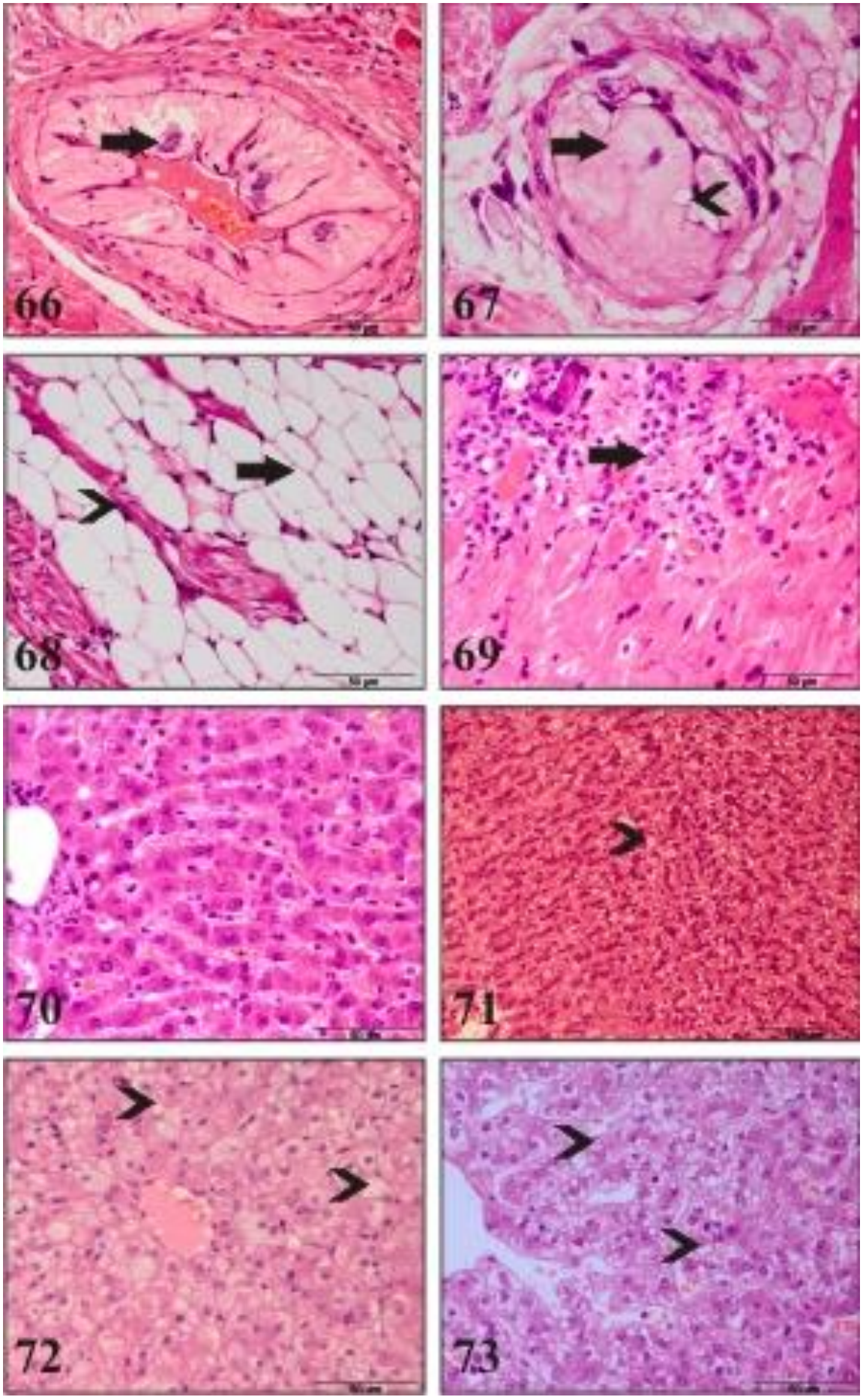
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Fig.74. Group D. Section of liver showing fibrous tissue proliferation (arrow). H&E X40.

Fig.75. Group D (cholesterol + vitamin D). Section of liver showing fatty changes. Diffuse cytoplasmic accumulation of lipid is evident within hepatocytes throughout the liver (arrowhead). Note the decrease in sinusoidal space. H&E X20.

Fig.76. Group D. Section of liver showing severe fatty changes. Note the small and large vacuoles formed in the hepatocytes (arrowhead). Sinusoidal space is obliterated by the lipid vacuoles. H&E X40.

Fig.77. Group D. Higher magnification of the sction of liver in fig.76. Note that the hepatocytes coalesce together because of the rupture of membranes (arrowhead). H&E X100.

Fig.78. Group A (control feed). Section of kidney showing normal histology. H&E X40.

Fig.79. Group A (control feed). Section of kidney showing normal histology. H&E X20.

Fig.80. Group B (control feed + vitamin D). Section of kidney showing mineralization in basement membrane of glomeruli and interstitial space (arrowhead). H&E X40.

Fig.81. Group B. Section of kidney showing mineralization within the T. intima of renal artery (arrowhead). H&E X20.

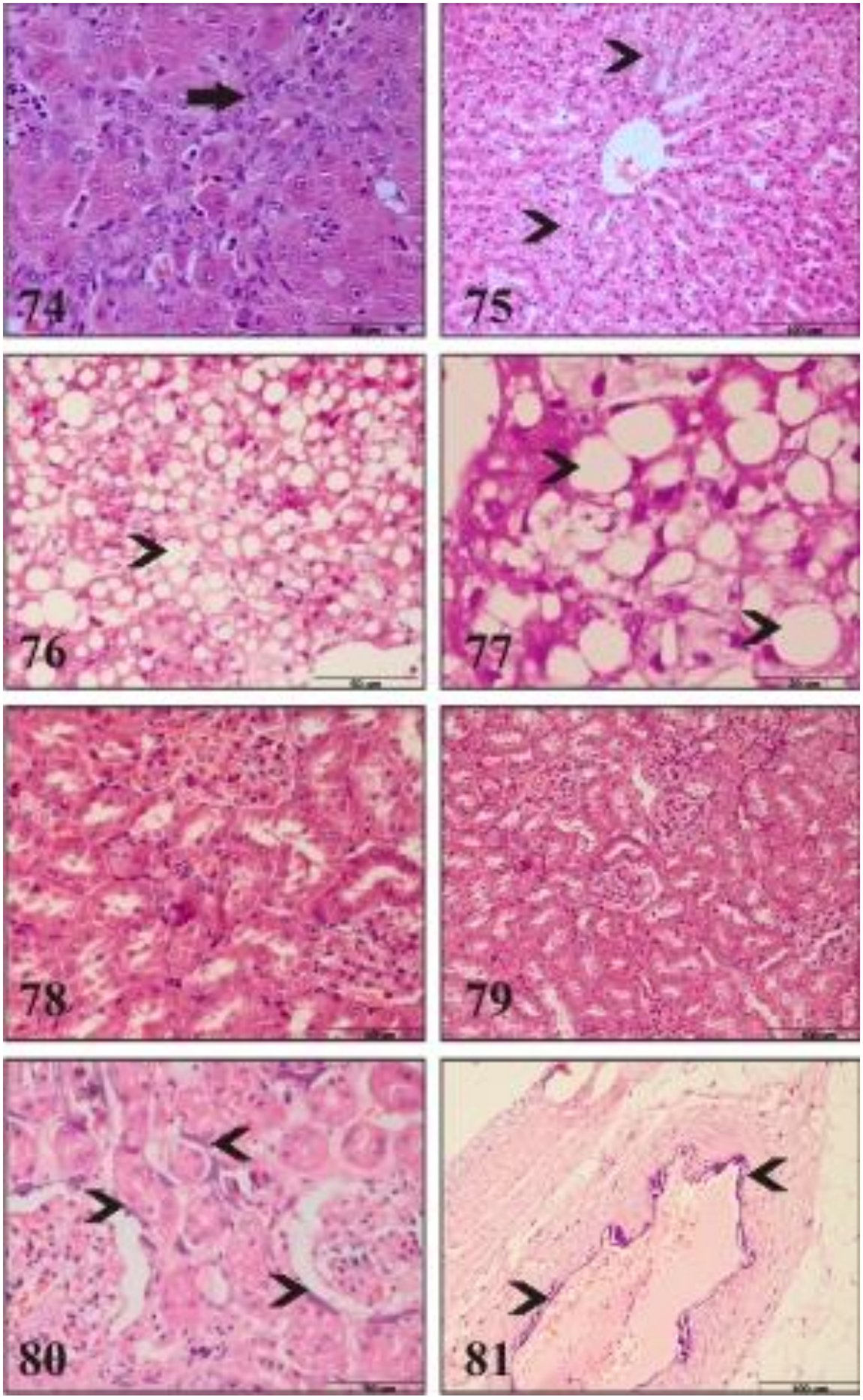
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Fig.82. Group C (control feed + cholesterol). Section of kidney showing partial to complete loss of glomeruli which is replaced by pink proteinecious material within glomerular space (arrow). H&E X40.

Fig. 83. Group C. Section of kidney showing obliterated glomerular space due to hypertrophy of glomerular tuft (arrow). H&E X40.

Fig.84. Group C. Section of kidney showing lymphomononuclear cell infiltration in the medullary region (arrow). H&E X40.

Fig.85. Group C. Section of kidney showing atrophy of glomerular tuft creating a large glomerular space. Glomerular tuft is diffusely and is filled with pink proteinecious material (arrow). Note the thickening of the glomerular basement membrane (arrowhead). H&E X40.

Fig.86. Group D (cholesterol + vitamin D). Section of kidney showing degeneration and necrosis of the tubules. Also note mineralization within the glomerular tufts (arrowhead). H&E X40.

Fig.87. Group D. Section of kidney showing atrophy of glomerular tuft creating a large glomerular space (arrow). H&E X100.

Fig.88. Group D. Section of kidney showing atrophy of glomerular tuft creating a large glomerular space. Note the mineralization of basement membrane of the glomeruli (arrowhead). H&E X40.

Fig.89. Group D. Section of kidney showing glomerulosclerosis (end stage of chronic glomerulonephritis). Note the hypocellularity and hyalinization due to an increase in fibrous connective tissue and mesengial matrix and almost complete loss of glomerular capillaries (arrow). Basement membrane of the glomeruli and matrix within glomeruli are mineralized (arrowhead). H&E X40.

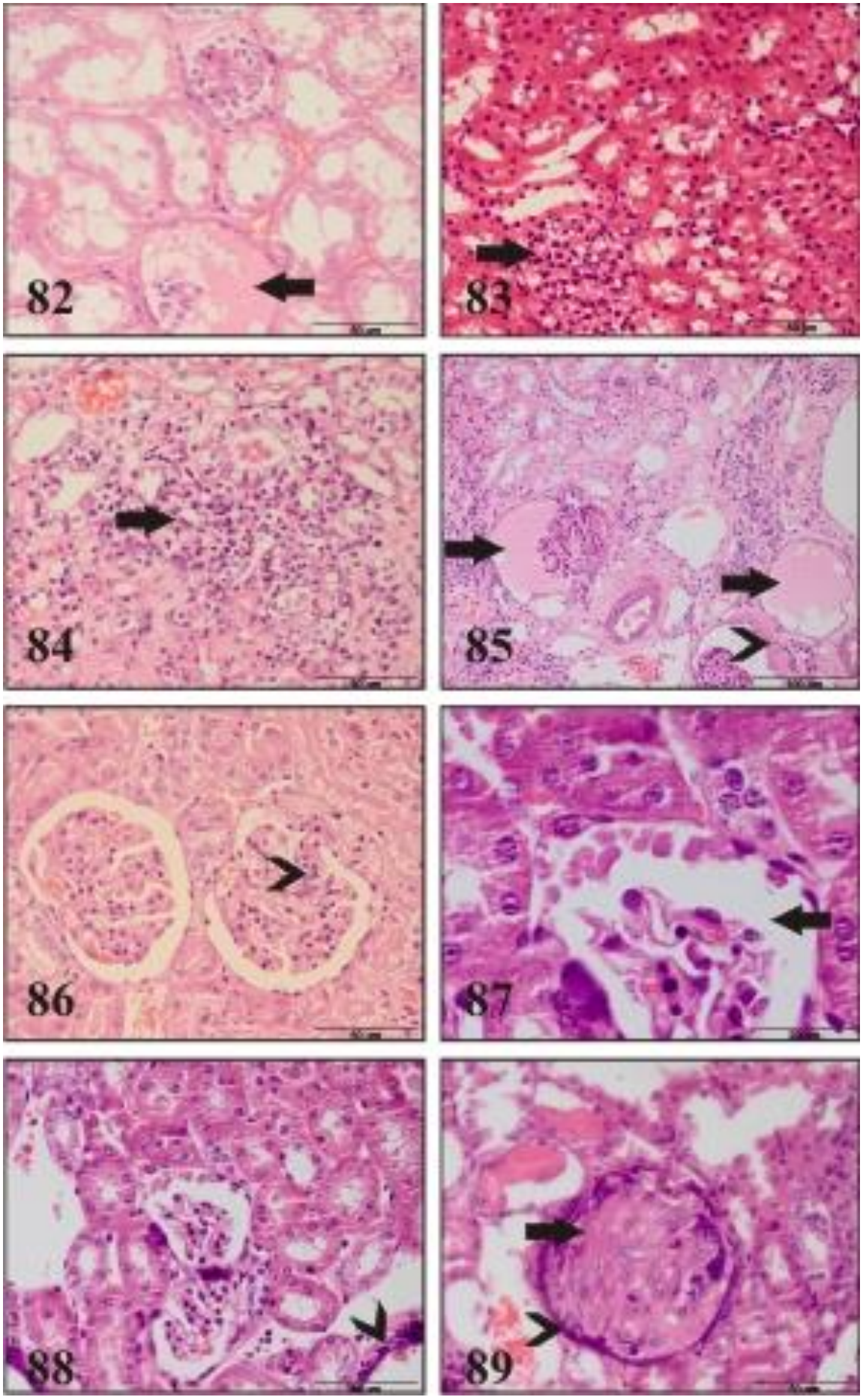
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Fig.90. Group A (control feed). Section of an adrenal gland showing normal histology. H&E X20.

Fig.91. Group A. Section of an adrenal gland showing normal histology. H&E X100.

Fig.92. Group B (control feed + vitamin D). Section of an adrenal gland showing normal histology. H&E X20.

Fig.93. Group C (control feed + cholesterol). Section of an adrenal gland showing adrenocortical nodular hyperplasia. Nodules of the lipid laden cells are seen within zona fasciculate and zona reticularis (arrow). H&E X10.

Fig.94. Group C. Section of an adrenal gland showing rupture of cell membrane resulting in coalesce of two or more cells. Note the cholesterol crystals within cells of the nodular region (arrowhead). H&E X100.

Fig.95. Group D (cholesterol + Vitamin D). Section of an adrenal gland showing adrenocortical nodular hyperplasia. Nodules of the lipid laden cells are formed within zona fasciculate and zona reticularis (arrow). H&E X10.

Fig.96. Group D. Section of an adrenal gland showing rupture of cell membrane resulting in coalesce of two or more cells (arrowhead). H&E X100.

Fig.97. Group D. Section of an adrenal gland showing cholesterol crystals within the nodular region in zona reticularis (arrowhead). H&E X40.

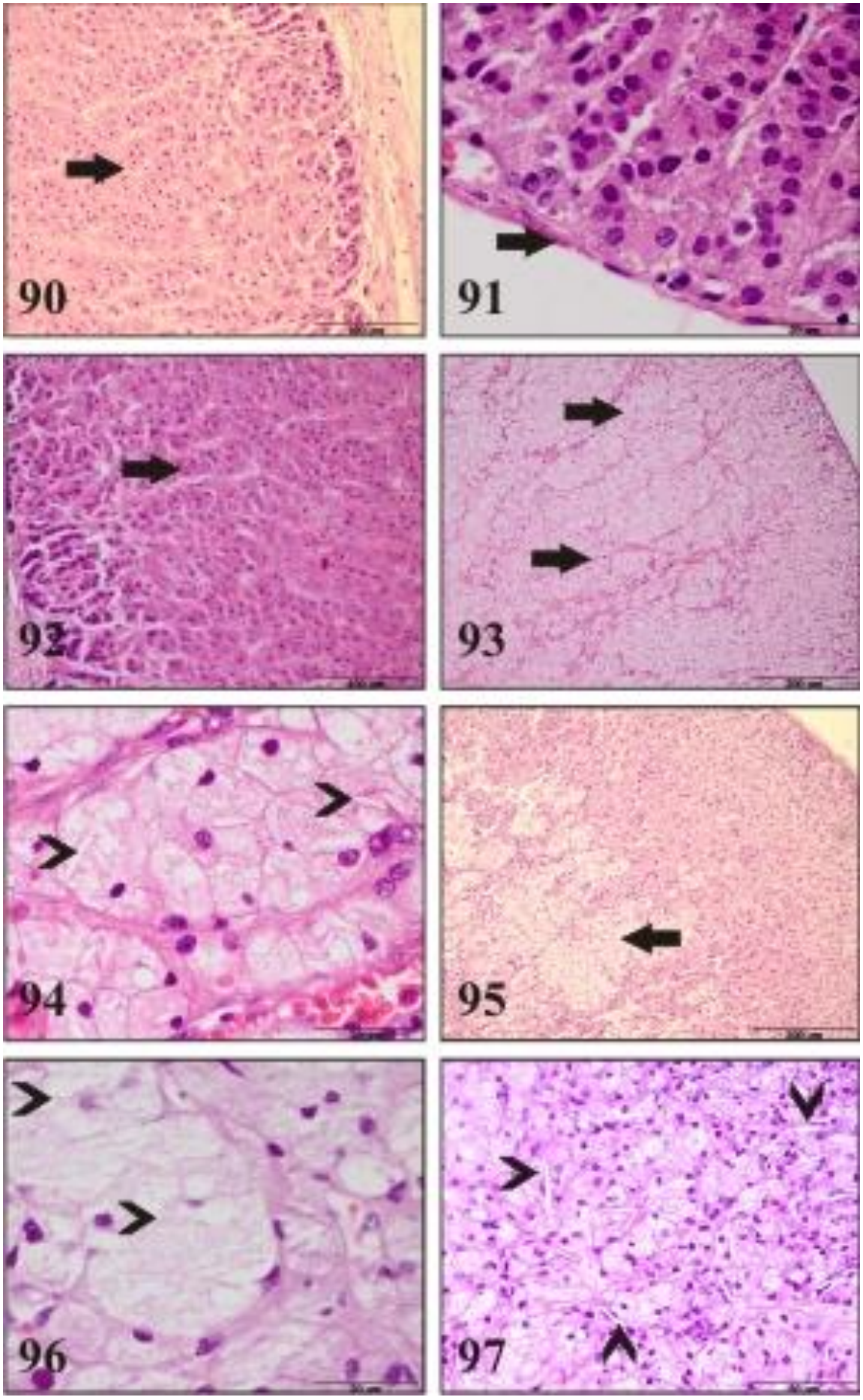
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Fig.98. Group A (control feed). Section of spleen showing normal histology. H&E X40.

Fig.99. Group A (control feed). Section of spleen showing normal histology. H&E X100.

Fig.100. Group B (control feed + vitamin D). Section of spleen showing mineralization of T. intima of the spleenic artery (arrowhead). H&E X40.

Fig.101. Group B. Section of spleen showing mineralization of T. intima of the spleenic artery (arrowhead). H&E X100.

Fig.102. Group C (control feed + cholesterol). Section of spleen showing accumulation of spleenic foam cells (arrowhead). Note the rarefaction of white pulp. H&E X20.

Fig.103. Group C. Section of spleen showing accumulation of spleenic foam cells (arrowhead). H&E X100.

Fig.104. Group D (cholesterol + Vitamin D). Section of a spleen showing decrease in white pulp (arrow) and accumulation of spleenic foam cells (arrow). Note the mineralization within trabaculae. H&E X20.

Fig.105. Group D. Section of a spleen showing accumulation of spleenic foam cells (arrowhead). Note the mineralization in the capsule. H&E X100.

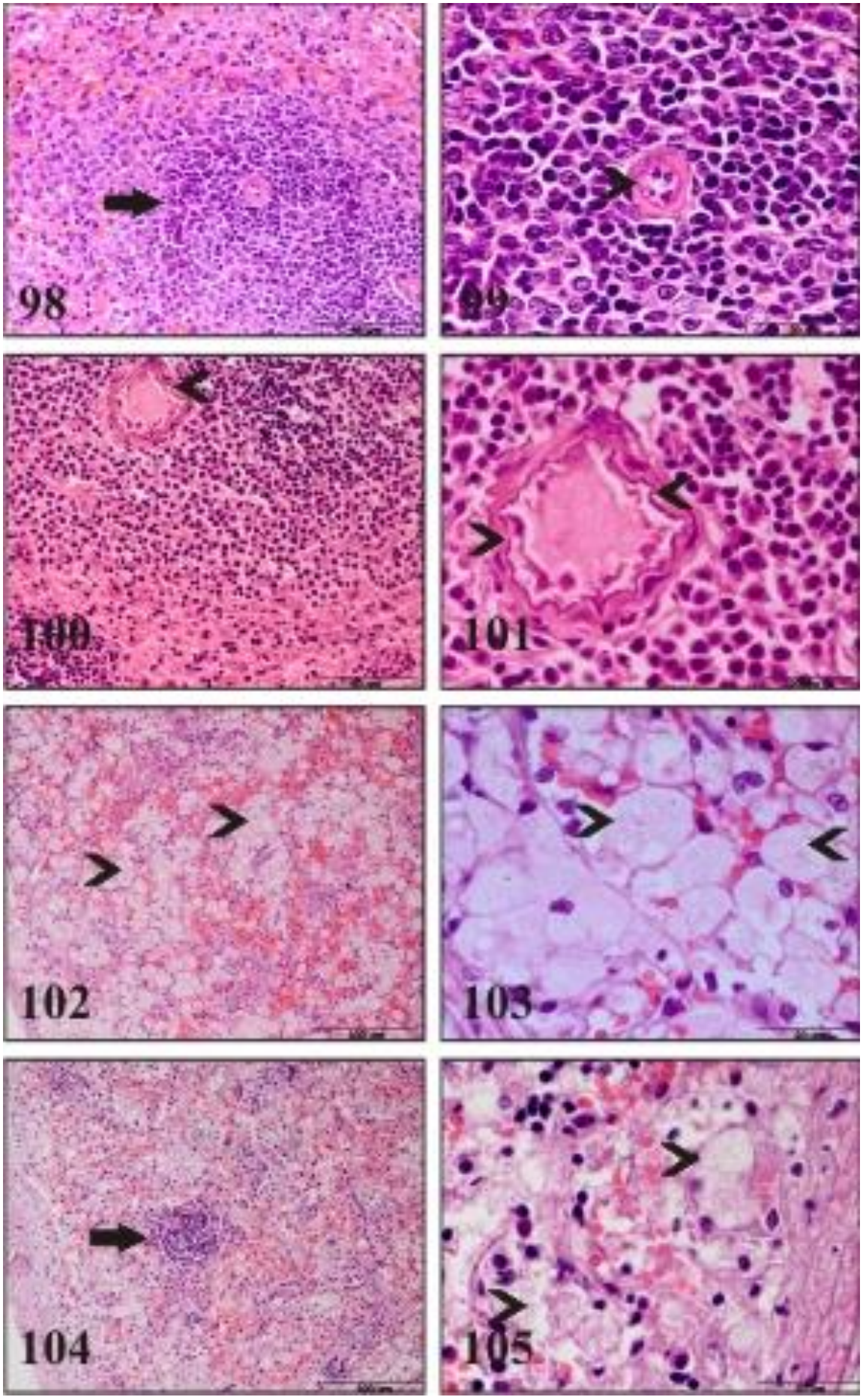
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Fig.106. Group D. Section of a spleen showing decrease in white pulp and accumulation of spleenic foam cells. Note the mineralization within trabaculae (arrowhead). H&E X40.

Fig.107. Group D. Section of a spleen showing hemosiderin laden macrophages (arrow). Note the mineralization of spleenic trabaculae (arrowhead). H&E X100.

Fig.108. Group A (control feed). Section of lung showing normal histology. H&E X20.

Fig.109. Group B (control feed + vitamin D). Section of lung showing edema. Lymphomononuclear cell and cellular debris accumulate within the pink edematous fluid (arrow). Note the mineralization of the vessels (arrowhead). H&E X40.

Fig.110. Group B. Section of lung showing loss of villi of the bronchoilar epithelium and mineralization in the basement membrane (arrowhead). H&E X40.

Fig.111. Group C (control feed + cholesterol). Section of lung showing occlusion of pulmonary artery. Note the atheroma formation by hypertrophy of T. media and intimal foam cell accumulation (arrow). Note the luminal narrowing of the blood vessel (arrowhead). H&E X10.

Fig.112. Group D (cholesterol + vitamin D). Section of lung showing edema. Lymphomononuclear cell, heterophils and cellular debris accumulate within the pink edematous fluid (arrow). Note the mineralization of the vessels (arrowhead). H&E X40.

Fig.113. Group D. Section of lung showing hypertrophy of T. media and periarteritis. Note the thickened T. media of the small branches of the pulmonary artery and their periarterial cuff of chronic inflammatory cells and some heterophils (arrow). H&E X40.

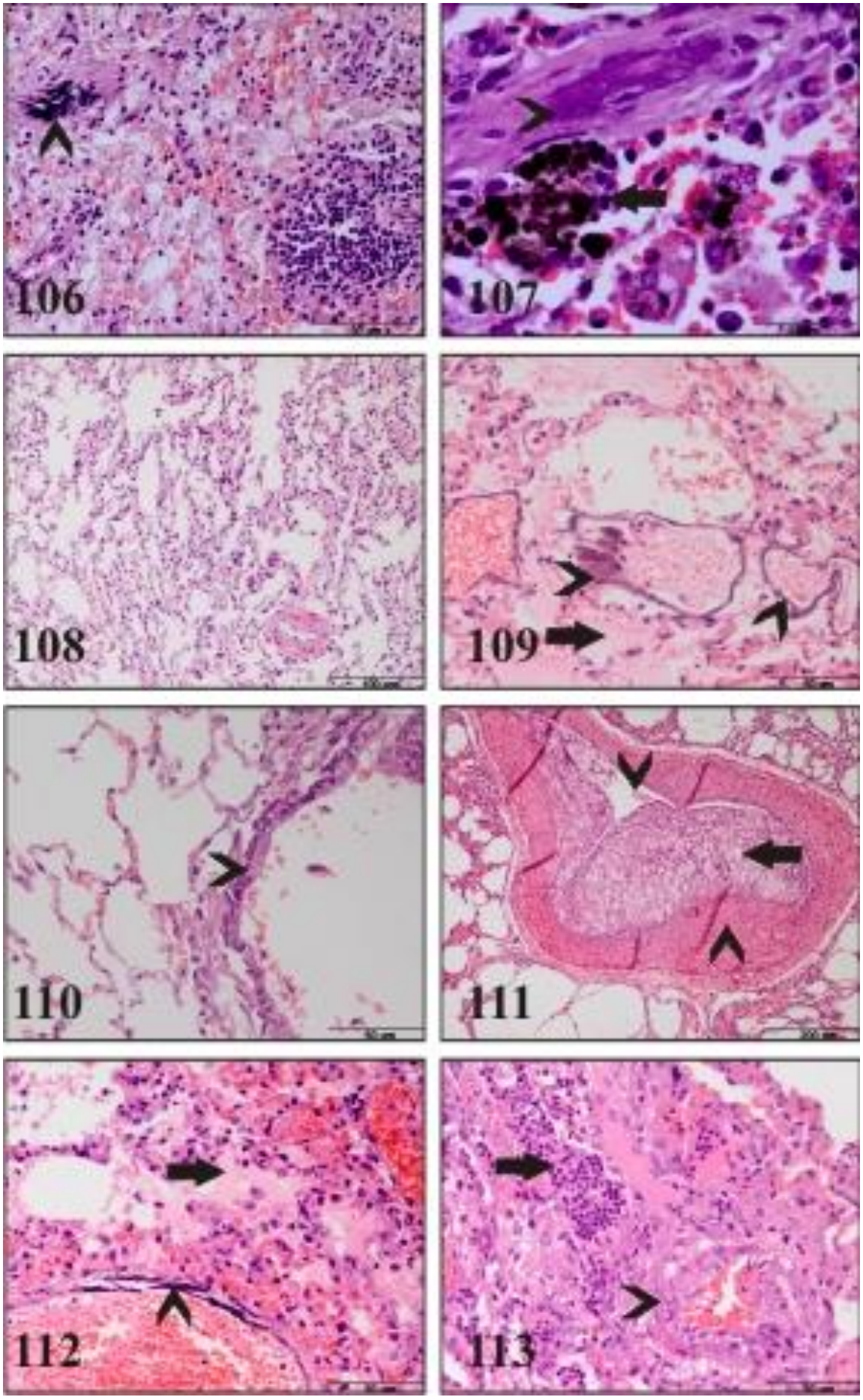
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Fig114. Group D. Section of lung showing edema, lymphomononuclear and heterophil cells infiltration (arrow). H&E X40.

Fig.115. Group D. Section of lung showing loss of villi of the bronchiolar epithelium and mineralization in the basement membrane (arrowhead). H&E X100.

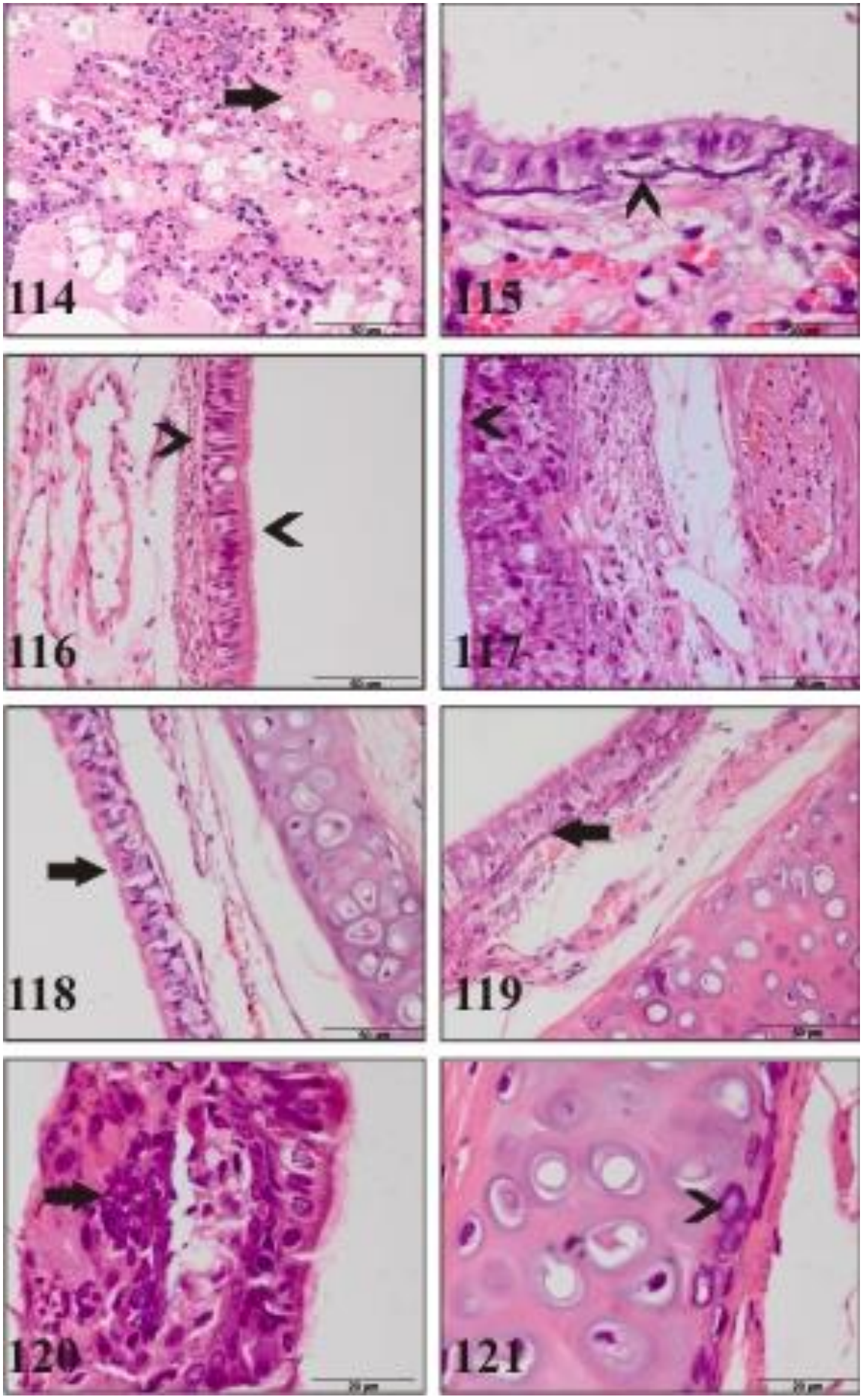
Fig.116. Group A (control feed). Section of trachea showing normal histology. H&E X40.

Fig.117. Group B (control feed + vitamin D). Section of trachea showing mineralization in the submucosal layer (arrowhead). H&E X40.

Fig.119. Group C (control feed + cholesterol). Section of trachea showing normal histology. H&E X40.

Fig.120. Group D (cholesterol + vitamin D). Section of trachea showing mineralization in the submucosal layer (arrow). H&E X100.

Fig.121. Group D. Section of trachea showing mineralization in the cartilage (arrowhead). H&E X100.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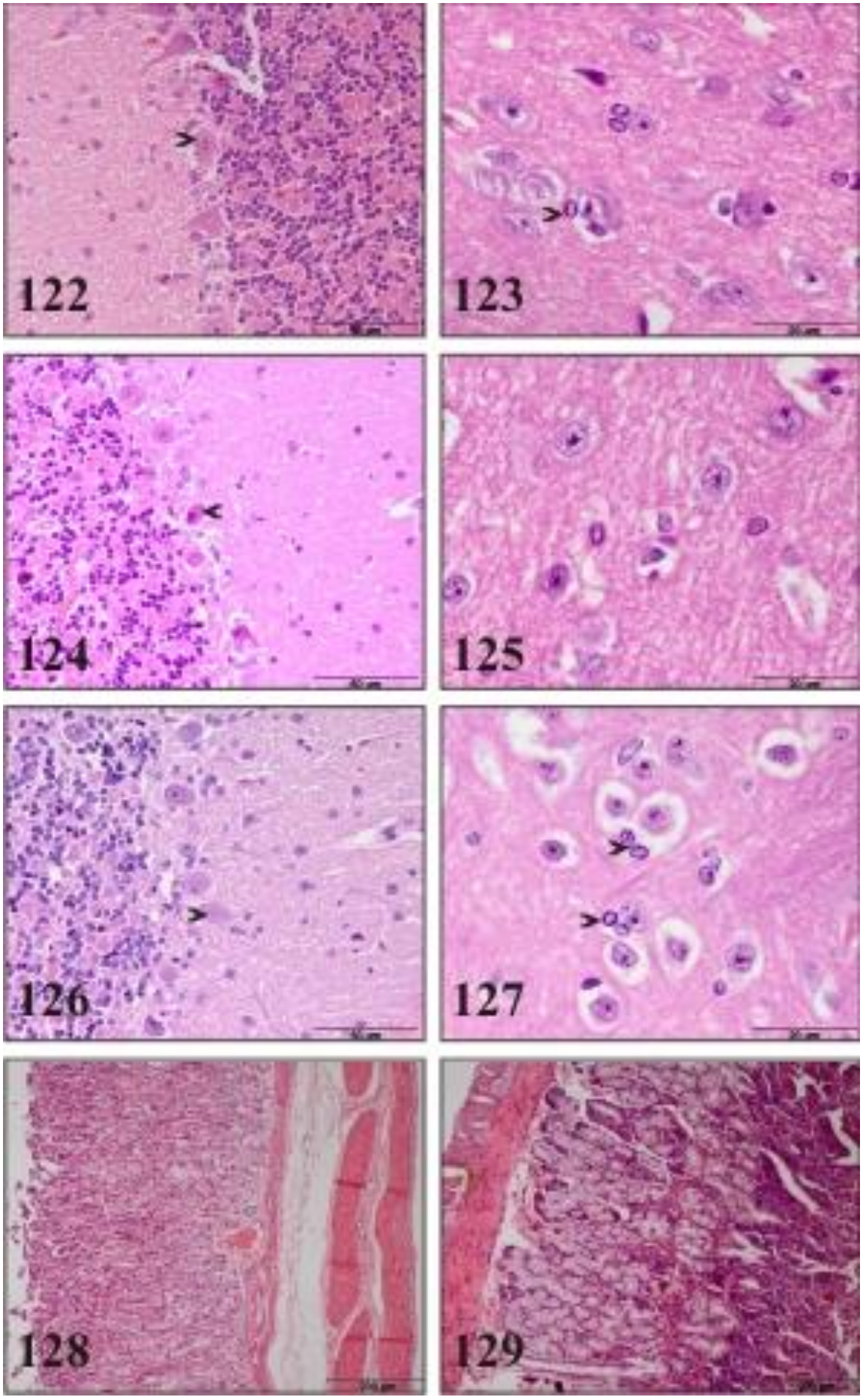
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Legends to figures

Fig.130. Group B (control feed + vitamin D). Section of intestine showing calcification in the muscular layer (arrowhead). H&E X10.

Fig.131. Group B (control feed + vitamin D). Section of stomach showing calcification in the villi of the mucosa (arrowhead). H&E X10.

Fig.132. Group C (control feed + cholesterol). Section of stomach showing no apparent changes. H&E X20.

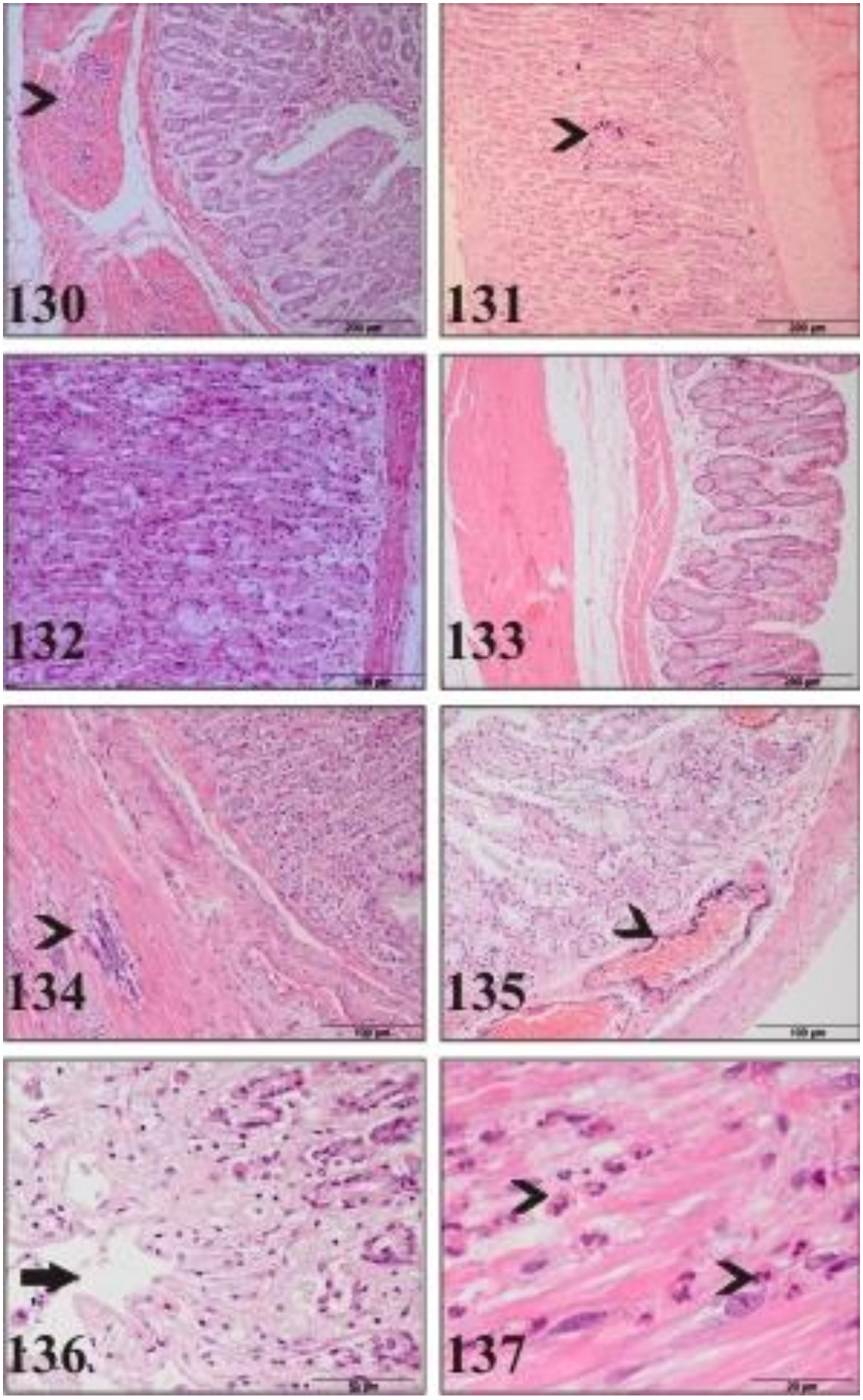
Fig.133. Group C. Section of intestine showing no apparent changes. H&E X10.

Fig.134. Group D (cholesterol + vitamin D). Section of stomach showing calcification in the muscular layer (arrowhead). H&E X20.

Fig.135. Group D. Section of intestine showing calcification in the blood vessels in the submucosal layer (arrowhead). H&E X20.

Fig.136. Group D. Section of stomach showing necrosis and sluffing of the villi in the mucosal layer (arrow). H&E X40.

Fig.137. Group D. Section of intestine showing heterophil cell infiltration in the muscular layer (arrowhead). H&E X100.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Legends to figures

Fig.138. Group B (control feed + vitamin D). Section of an aorta showing black deposits of calcification in T. media (arrow). Von Kossa stain. X20.

Fig.139. Group B. Section of heart showing calcification in the mural artery (arrow). Von Kossa stain. X40.

Fig.140. Group D. Section of heart showing calcification in the T. intima of the occluded mural artery (arrow). Von Kossa stain. X40.

Fig.141. Group D. Section of an aorta with atheromatous plaque showing calcification in T. media (arrow). Von Kossa stain. X20.

Fig.142. Group D (cholesterol + vitamin D). Section of an aorta showing black deposits of calcification in T. media of aorta (arrow). Von Kossa stain. X40.

Fig.143. Group D. Section of heart showing calcification in the cardiac muscle (arrow). Von Kossa stain. X20.

Fig.144. Group D. Section of kidney showing calcification in basement membrane of the glomerulus and tubules (arrow). Von Kossa stain. X10.

Fig.145. Group D. Section of kidney showing calcification in T. intima of renal artery (arrow). Von Kossa stain. X10.

Fig.146. Group D. Section of trachea showing calcification in basement membrane of the epithelial lining (arrow). Von Kossa stain. X20.

Fig.147. Group D. Section of adrenal showing diffused calcification (arrow). Von Kossa stain. X20.


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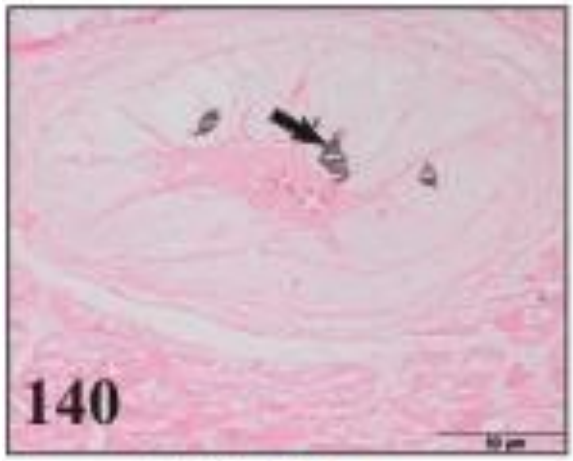
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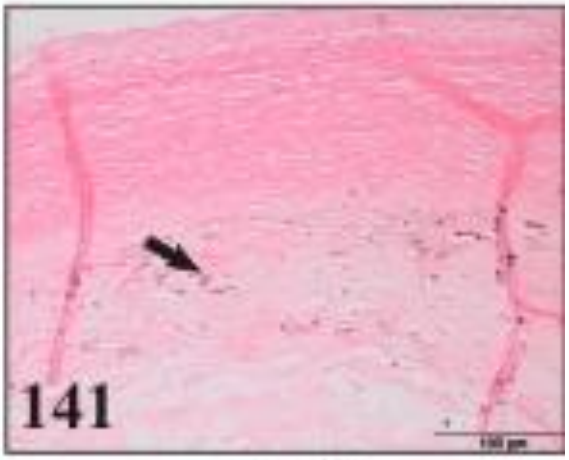
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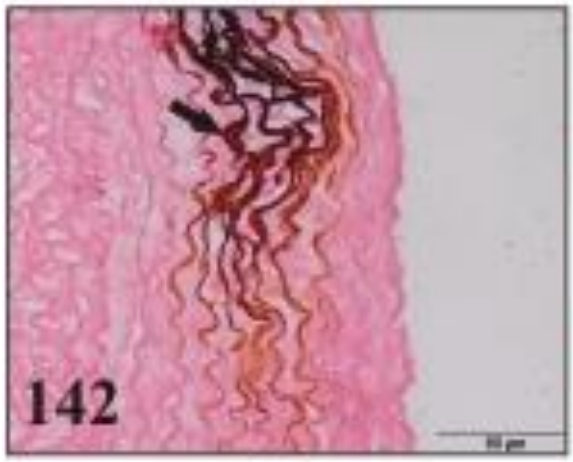
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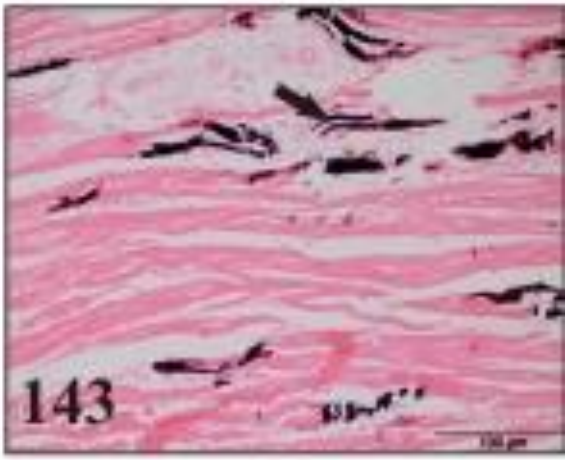
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CHAPTER-V

SUMMARY AND CONCLUSION

The present study entitled "Studies on effects of high cholesterol and vitamin D_3 on cardiovascular system" was carried out at Department of Veterinary Pathology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana. The programme of work was duly approved by IAEC. The objectives of the present study included:

1. To study the clinicopathology in high cholesterol fed rabbits and subsequently given vitamin D_3 per oral.
2. To study the enzymehistochemistry of aorta in high cholesterol fed rabbits and subsequently given vitamin D_3 per oral.

To achieve the above said objectives, an experiment was conducted on rabbits. The experimental design was as follows:

Group	**No. of rabbits**	**Treatment**
A	6	Control group with normal diet and was administered groundnut oil daily for 70 days.
B	6	Control group given vitamin D_3 @ 0.9 mg/Kg BW dissolved in groundnut oil from day 56 of experiment until death or maximum for 2 weeks.
C	6	Control group given cholesterol @ 400 mg/Kg BW dissolved in 4% groundnut oil in feed daily for 70 days.
D	6	Group given cholesterol @ 400 mg/Kg BW dissolved in 4% groundnut oil daily in feed daily + vitamin D_3 @ 0.9 mg/Kg BW dissolved in groundnut oil from day 56 of experiment until death or maximum for 14 days.

The summary of the findings observed in the experiment is presented below:

Clinical Signs

Group B (control feed + vitamin D_3) and group D (cholesterol + vitamin D_3) rabbits showed ruffled body coat, cachexia and diarrhea on day 65 onwards of the experiment.

Mortality pattern

Two rabbits from group B died on day 68 and 69 of the experiment, while three rabbits from group D died on day 67, 68 and 70.

Hematological findings

Hemoglobin (Hb) percent of group A rabbits did not differ significantly on day 0, 28, 56 and 70. Group B rabbits showed significant decrease in Hb percent from day 56 onwards. The mean Hb concentration of group C and D rabbits decreased significantly from group A and B rabbits on day 56 of the experiment. The mean TLC of group C and D rabbits increased significantly from group A and B rabbits on day 56 of the experiment. The heterophil percentage of group C and D rabbits increased significantly from group A and B rabbits on day 56 of the experiment. The mean heterophil percentage of group B and D rabbits increased significantly to that of group A rabbits. The mean heterophil percentage of group D rabbits increased significantly to that of group C rabbits.

Feeding of high cholesterol diet, elevated TLC and heterophil count; however, it reduced Hb and lymphocyte percentage. Subsequently vitamin D_3 administration in high cholesterol fed rabbit resulted in decrease in TLC and lymphocyte percentage.

Biochemical findings

No significant difference between the four groups was found on day 0. On day 28, the mean ALT, Ca, P, total cholesterol, LDL, VLDL concentration and T. Cholesterol: HDL, LDL: HDL ratios increased significantly in group C and D rabbits as compared to group A rabbits. On day 56, rabbits of group C and D showed significant increase in ALT, AST,

globulin, total cholesterol, HDL, LDL, VLDL concentration and T. Cholesterol: HDL, LDL: HDL ratios as compared to rabbits of group A. Decrease in albumin, albumin: globulin ratio was seen in rabbits of group C and D as compared to group A rabbits on day 56. On day 70 or just before death, group D rabbits showed significant difference in almost all the biochemical parameters as compared to group A rabbits while group C rabbits showed significant difference in ALT, AST, globulin, albumin, albumin:globulin ratio and lipid profile parameters as compared to group A rabbits. Group B rabbits showed significant increase in Ca^{++} concentration on day 70 or just before death.

Tissue Mineral Concentration

Ca^{++} concentration in heart, aorta, kidney and liver showed increase in group B and D rabbits and decrease in group C rabbits as compared to group A rabbits. Mg^{++} concentration in heart, aorta, kidney and liver decreased in rabbits of group B, C and D as compared to group A.

Fe^{++} concentration in heart, aorta, kidney and liver increased in group B, C and D rabbits as compared to group A rabbits. Zn^{++} and Cu^{++} concentration in heart, aorta, kidney and liver decreased in rabbits of group B, C and D as compared to group A rabbits.

Na^{++} concentration in heart of group B, C and D rabbits increased as compared to group A rabbits. Na^{++} concentration in aorta of group B and D rabbits increased while that in group C rabbits decreased as compared to group A rabbits. Na^{++} concentration in liver of group B, C and D rabbits decreased as compared to group A rabbits.

K^{+} concentration in heart and aorta decreased in group B, C and D rabbits as compared to group A rabbits. Group B and C rabbits showed increase of K^{+} concentration while group D showed decrease in kidney and liver as compared to group A rabbits.

Enzymehistochemical observations

Increase in AKPase, G6PD, LDH, MDH, NADH-diphorase and NADPH-diphorase was observed while decrease in SDH was observed in heart, aorta and liver.

Lipid demonstration

Sudan III stain was used to demonstrate fat in cryostat section of heart, aorta and liver. Group A rabbits did not show any deposition, while group B showed mild deposition. Group C and D showed strong to intense depots of fat.

Gross Lesions

Gross lesions seen in group B rabbits showed firm and inelastic aorta with small atheromatous plaques and mineralization. Mineralization was also seen in kidney. Group C rabbits showed plaque formation and thickening of the cardiac valves and luminal surface of aorta. Fatty liver, hypertrophied adrenal, and distended gall bladder were apparent.

Grossly, group D rabbits showed plaques and mineralization in cardiac valves and aorta. The coronary arteries were prominent and the liver was fatty. Intestine and stomach showed white deposits of mineralization, while trachea showed hemorrhages and calcification on the luminal surface. There was marked hypertrophy of adrenals and spleen in rabbits of group D as compared to group A rabbits.

Histopathological observations

Group B rabbits showed calcification in the T. media of the aorta and T. intima of the spleenic artery. Lung showed edema with inflammatory cell and cellular debris and mineralization of the vessels. Loss of villi of the bronchiolar epithelium and mineralization in the basement membrane was also seen. Kidney showed mineralization in basement membrane of glomeruli, interstitial space(s) and T. intima of renal artery. In rabbits of group C and D aorta showed atheromatous lesions. Few atheromas showed ruptured endothelial lining of the T. intima. Foam cells were evident and there was smooth muscle cell proliferation. Liver showed moderate to severe fatty changes. Adrenocortical nodular

hyperplasia, with nodules of the lipid-laden cells loaded within zona fasciculate and zona reticularis and cholesterol crystals in the nodular region were evident. Spleen showed spleenic foam cells and rarefaction of white pulp. In addition, group D rabbits showed calcification in spleenic capsule and trabaculae. Kidneys showed partial to complete loss of glomeruli and pink proteinaecious material accumulation within glomerular spaces. Fibrous tissue proliferation and lymphomononuclear infiltration was also evident.

Calcium demonstration

Von kossa stain was used to demonstrate Ca^{++} within various tissue sections. Calcium was found more in tissues of group B and D rabbits as compared to group A and C rabbits.

Conclusion

Based on various parameters studied, it is concluded that increased intake of cholesterol and subsequent increased intake of vitamin D_3 together aggravated the formation of atheromatous plaques in aorta and heart. It also resulted in significant damage to liver, kidneys, adrenals, spleen and lungs. Thus, high intake of cholesterol along with high intake of vitamin D_3 is more injurious to the cardiovascular and other body systems than cholesterol or vitamin D_3 alone.

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