UNIWERSYTET JAGIELLOŃSKI
COLLEGIUM MEDICUM
WYDZIAŁ FARMACEUTYCZNY
Z ODDZIAŁEM ANALITYKI MEDYCZNEJ

Rania ABDEL KADER

Wpływ hepatotoksycznych czynników na molekularne zmiany w komórkach wątrobowych oraz mechanizmy naprawy DNA w hepatocytach

Praca doktorska

Promotor: Prof. dr hab. med. Jerzy Jaśkiewicz

Pracę wykonano w Zakładzie Analityki Biochemicznej
Kierownik zakładu: p.o. dr Jadwiga Hartwich

Kraków, 2012

JAGIELLONIAN UNIVERSITY
COLLEGIUM MEDICUM
FACULTY OF PHARMACY

Rania ABDEL KADER

Influence of Hepatotoxic Compounds on the Molecular Changes as well as DNA Repairing Mechanisms in Hepatocytes

PhD Thesis in Pharmaceutical Sciences

Under the supervision of
Prof. Jerzy Jaśkiewicz, MD, PhD

Thesis was performed in Department of Analytical Biochemical,
Director of the Department: Dr. Jadwiga Hartwich

Krakow, 2012

Acknowledgment

I would like to express my deepest thanks and sincere gratitude to Prof. dr hab. med. Jerzy Jaśkiewicz, Professor of Analytical Biochemical, Faculty of Pharmacy, Jagiellonian University – Collegium Medicum, for his kind and continuous supervision, support and great assistance, generous cooperation and valuable guidance throughout the course of this work.

I would like to express my deep gratitude to all staff members of Analytical Biochemical department, Faculty of Pharmacy, Jagiellonian University – Collegium Medicum, my colleagues and faculty staff for their help and support. And, to my family, thank you for your love.

Rania Abdel Kader

LIST OF ABBREVIATIONS

Ac-DEVD-pNA	acetyl-Asp-Glu-Val-Asp-p-nitroanilide
acetyl CoA	acetyl coenzyme A
ALT	alanine aminotransferases
AST	aspartate aminotransferases
ATP	adenosine triphosphate
CCl_4	carbon tetrachloride
CO2	carbon dioxide
CTL	cytotoxic T lymphocytes
CYP450	cytochrome P450
DDB	dimethyl diphenyl bicarboxylate
DDT	dichlorodiphenyltrichloroethane
DEPC	diethylpyrocarbonate
DEVD	Asp-Glu-Val-Asp
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme linked immunosorbent assay
F-100	Fluvastatin 100mg/kg/day group
F-25	Fluvastatin 25mg/kg/day group
F-50	Fluvastatin 50mg/kg/day group
F-75	Fluvastatin 75mg/kg/day group
FD-100	Fluvastatin-100mg/kg/day+100mg/kg/day-DDBgroup
FD-25	Fluvastatin 25mg/kg/day +100mg/kg/day DDB group
FD-50	Fluvastatin 50mg/kg/day +100mg/kg/day DDB group
FD-75	Fluvastatin 75mg/kg/day +100mg/kg/day DDB group
FS-100	Fluvastatin 100mg/kg/day+140mg/kg/day silymarin group
FS-25	Fluvastatin-25mg/kg/day+140mg/kg/day-silymarin group
FS-50	Fluvastatin 50mg/kg/day+140mg/kg/day silymarin group
FS-75	Fluvastatin 75mg/kg/day+140mg/kg/day silymarin group
GGT	gamma glutamyltransferase
GLUT2	glucose transporter -2
GSH	reduced glutathione
HBV	hepatitis B virus
HCL	hydrochloric acid
HCV	hepatitis C virus
HDL	high-density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HRP	horseradish peroxidase
IDL	intermediate-density lipoprotein
LDL	low-density lipoprotein
MES	2-(N-morpholino)ethanesulfonic acid
NaCl	sodium chloride
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NC	Normal control group
OD	optical density
PARP	poly ADP ribose polymerase

PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid)
PMSF	phenylmethyl sulfonyl fluoride
p-NA	p-nitroanilide
RBC	red blood cell
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RNase A	ribonuclease A
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
TCA	tricarboxylic acid
TMB	tetramethylbenzidine
UV	ultraviolet
VLDL	very low density lipoproteins

^{o}C	celsius degree
ΔA/min	difference in Absorbance per minute
µg/ml	microgram per milliliter
µl	microliter
g/dl	gram per deciliter
m^2	meter square
ml	milliliter
mg	milligram
mmol/l	millimoler per liter
nm	nanometer
rpm	revolutions per minute
IU/l	International unit per liter
U/l	unit per liter
U/ml	unit per milliliter
ρg/ml	picogram per liter
ρmol	picomoler

1. Introduction

The liver contributes about 2-3% of the total body weight, thus it is considered the largest organ in the body. It also represents a chemically reactant pool of cells that have a high rate of metabolism, sharing substrates and energy from one metabolic system to another, processing and synthesizing multiple substances that are transported to other tissues and perform numerous other metabolic functions (65).

The primary cell type of the liver that forms the liver lobules is the hepatocyte, which is also known as the hepatic parenchymal cells. Hepatocytes accounts for 80% of the liver volume, but are also account for only 60% of the total number of cells in the liver. The other 40% of the cells are the hepatic nonparenchymal cells that consist of the endothelial cells, Kupffer cells, hepatic stellate cells, and intrahepatic lymphocytes that include pit cells (liver-specific natural killer cells) (65). In this thesis, hepatocytes are regarded as liver cells, as they are the central location for the body's intermediary metabolism. As hepatocytes are particularly rich in endoplasmic reticulum, and they carry out the many functions of the liver, moreover their cytoplasm stores granules of insoluble glycogen and triglycerides (94).

1. Liver function

The liver performs many different functions, and many of these functions interrelate with one another. This becomes especially evident in case of abnormalities in the liver, because many of its functions are disturbed simultaneously. Some of liver functions include filtration and storage of blood, uptake of nutrients via the portal vein, biosynthesis of endogenous compounds, their storage, conversion and degradation into excretable molecules (metabolism), supply the body with metabolites and nutrients, biosynthesis and degradation of almost all plasma proteins and coagulation factors, and formation of bile (65, 94).

Steroid hormones, bilirubin, as well as drugs, ethanol, and other xenobiotics are taken up by liver cells, then inactivated and converted into polar metabolites in order to detoxify and/or excrete them into the bile or urine. In addition, liver temporarily stores energy reserves and nutrients for the body. Also in various liver cells (such as hepatocytes and hepatic stellate cells) certain mineral substances, trace elements, iron and vitamins including: retinol, vitamin A, D, K, folic acid, and B_{12} are stored (94).

1.1. Liver buffering function

The liver is uniquely situated to process and distribute dietary nutrients, because the venous drainage of the gut and pancreas passes through the hepatic portal vein before entry into the general circulation. Thus, after a meal, the liver is bathed in blood containing absorbed nutrients and elevated levels of insulin secreted by the pancreas. During this post-absorptive period, the liver takes up carbohydrates, lipids, and most amino acids. These nutrients are then metabolized, stored, or routed to other tissues (25).

All of the body's tissues must have a constant requirement for energy substrates and nutrients. Although, the body receives these metabolites with food, this supply is irregular and in varying amounts. The liver acts here along with other organs, particularly adipose tissue, as a buffer and storage organ. Its functions primarily serve to cushion fluctuations in the concentration of nutrient substances in the blood, in order to ensure a constant supply to the peripheral tissues (homeostasis) (94). Furthermore, hepatic injury is usually associated with marked distortion of these nutritional status and metabolic functions (26).

In the metabolism of nutrients, a distinction is made between the absorptive state (Fed state) immediately after a meal, the post-absorptive state (Early Fasting state and Fasting state) which starts later, and the switch of the organ metabolism between the these phases depends on the concentration of energy-bearing metabolites in the blood (94). A major goal of the many biochemical alterations in these periods is to maintain glucose homeostasis, or in other words a constant blood-glucose level. A normal functioning liver regulates blood glucose levels, in addition it synthesizes and exports of cholesterol and triglycerides, detoxifies ammonia through urea cycle, forms ketone bodies, synthesizes nucleotide, glycoproteins, proteoglycans and blood proteins, and generates reduced nicotinamide adenine dinucleotide phosphate (NADPH) and five-carbon sugars through pentose phosphate pathway (57).

1.1.1. The Fed state

This state continues for 2-4 hours after food intake. Because of food digestion, the plasma levels of glucose, amino acids, and triglycerides temporarily increase (94).

Figure 1 shows the fate of glucose, amino acids, and fat obtained from food. Glucose passes via portal vein to the liver. Amino acids are partially metabolized in the gut before being released into portal blood. Chylomicrons containing triglycerides are

secreted by the intestinal epithelial cells into lymphatics, and then they enter the thoracic duct, which delivers the chylomicrons to subclavian vein and thence to the rest of the body (68).

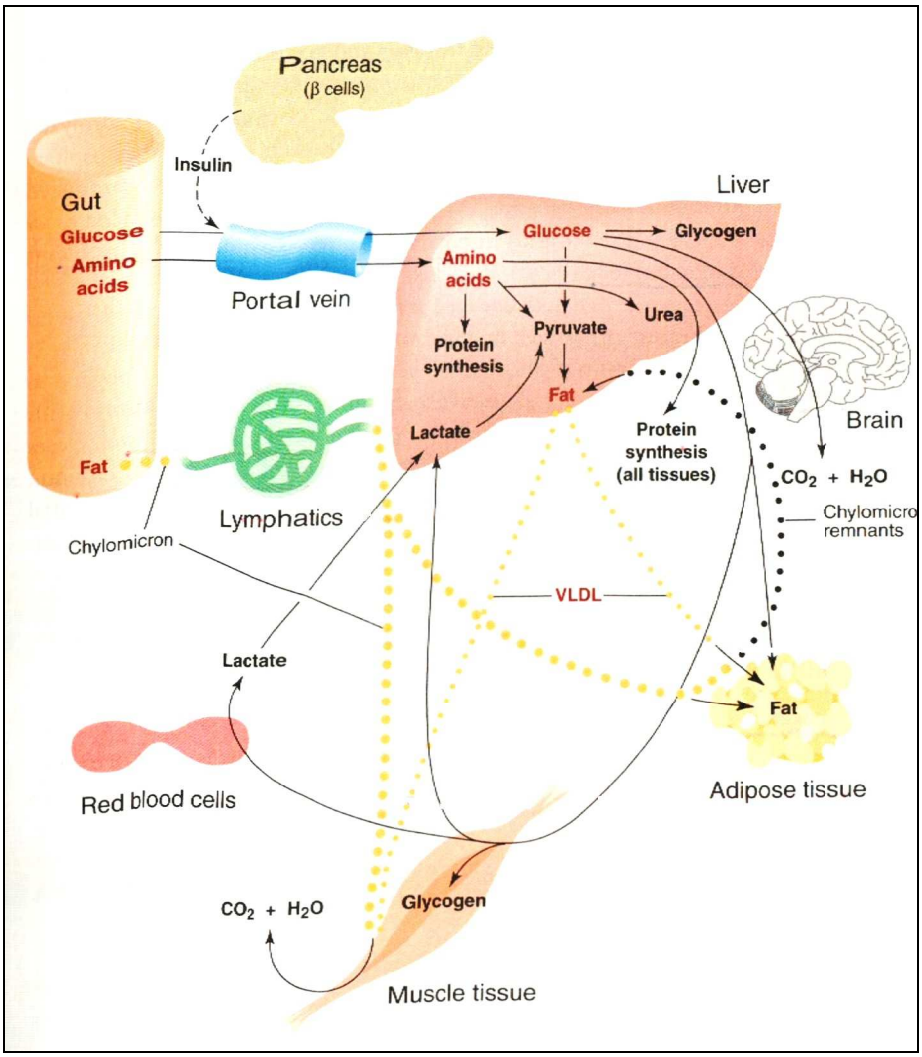


Figure 1: The Fed State (68)

During this post-absorptive period, virtually all tissues use glucose as a fuel (25). Much of the dietary glucose passes through the liver to reach other organs, but the liver can also convert excess dietary glucose into glycogen or into pyruvate and lactate, or it can be used in the pentose phosphate pathway for NADPH generation. Pyruvate can be oxidized into acetyl Coenzyme A (acetyl CoA), which can be converted into triglycerides or oxidized into CO_2 and water (68). In this well-fed state, the liver uses glucose and does not engage in gluconeogenesis, thus the conversion of lactate (produced in peripheral tissue) to glucose is interrupted in this state (68).

Liver removes some absorbed amino acids from portal blood, but most pass through. Moreover, liver metabolizes amino acids only when they are present in high concentration inside the cell. Such excess amino acids can be oxidized completely to carbon dioxide (CO_2), urea, and water, or converted into other intermediates that can be used for lipogenesis. Amino acids that escape the liver are used for protein synthesis or energy in other tissue (68).

Dietary triglyceride reaches the bloodstream as chylomicrons, which hydrolyzed by lipoprotein lipase attached to the surface of endothelial cells in the lumen of the capillaries of various tissues, especially adipose tissue. The liver uptake the remnant chylomicrons, and hydrolyze them by lysosomal lipase. The released fatty acids are re-esterified in hepatocytes and packed into very low-density lipoproteins (VLDL) and secreted into the blood (68).

1.1.2. The Early Fasting state

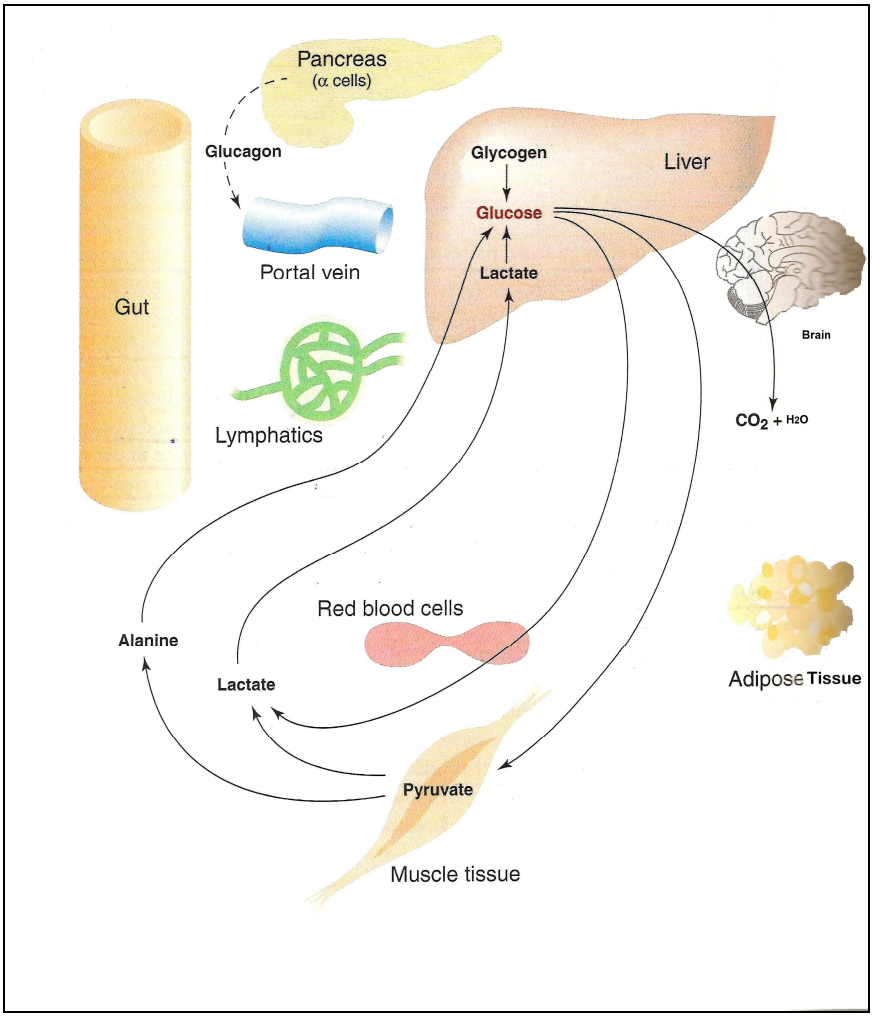


Figure 2: The Early Fasting state (68)

In this state, blood glucose level begins to drop several hours after a meal, leading to a decrease in insulin secretion and a rise in glucagon secretion. As shown in Figure 2, hepatic glycogenolysis maintains blood glucose during early fasting. Lactate and pyruvate, and alanine are diverted from oxidation and fatty acid synthesis into glucose formation, completing Cori cycle. The alanine cycle, in which carbon and nitrogen returns to the liver in the form of alanine, also becomes important (68).

Liver does not use the released glucose, but supplies the other tissues with it. In particular, the brain, adrenal gland medulla, and erythrocytes depend on a constant supply of glucose, as they have no substantial glucose reserves themselves (94).

1.1.3. The Fasting state

Since no dietary fuel enters from the gut and little glycogen is left in the liver after 10-12 hours of fasting state, the body is dependent on hepatic gluconeogenesis, primarily from pyruvate, lactate, glycerol, and Alanine as shown in Figure 3. The brain oxidizes glucose completely to CO_2 and water, hence net glucose synthesis from some other source of carbon is mandatory in fasting. Fatty acids cannot be used for glucose synthesis, but glycerol can be used. However, protein, especially from skeletal muscle, supplies most of the carbon needed for net glucose synthesis (68). As proteins are not stored, and so any breakdown will necessitate a loss of function, the body tends to preserve protein by shifting the fuel being used from glucose to fatty acids and ketone bodies (25).

Because of low blood insulin levels during fasting, lipolysis is greatly activated in adipose tissue, raising the blood level of fatty acids, which are used in preference to glucose by many tissues. In liver, fatty acid oxidation provides most of the adenosine triphosphate (ATP) needed for gluconeogenesis. They are converted to ketone bodies (acetoacetate and β-hydroxybutyrate) by liver mitochondria. Then, they are released into the blood and are a source of energy for many tissues. Once their blood concentration is high enough, ketone bodies enter the brain and serve as an alternative fuel. However, they cannot replace completely the brain’s need for glucose. Ketone bodies suppress proteolysis and branched-chain amino acids oxidation in muscle and decrease alanine release (68).

The working relationship between liver, muscle, and adipose tissue in providing glucose for the brain are shown in Figure 3. Liver synthesizes the glucose, muscle and

gut supply the substrate (alanine), and adipose tissue supplies the ATP (via fatty acid oxidation in the liver) needed for hepatic gluconeogenesis (68).

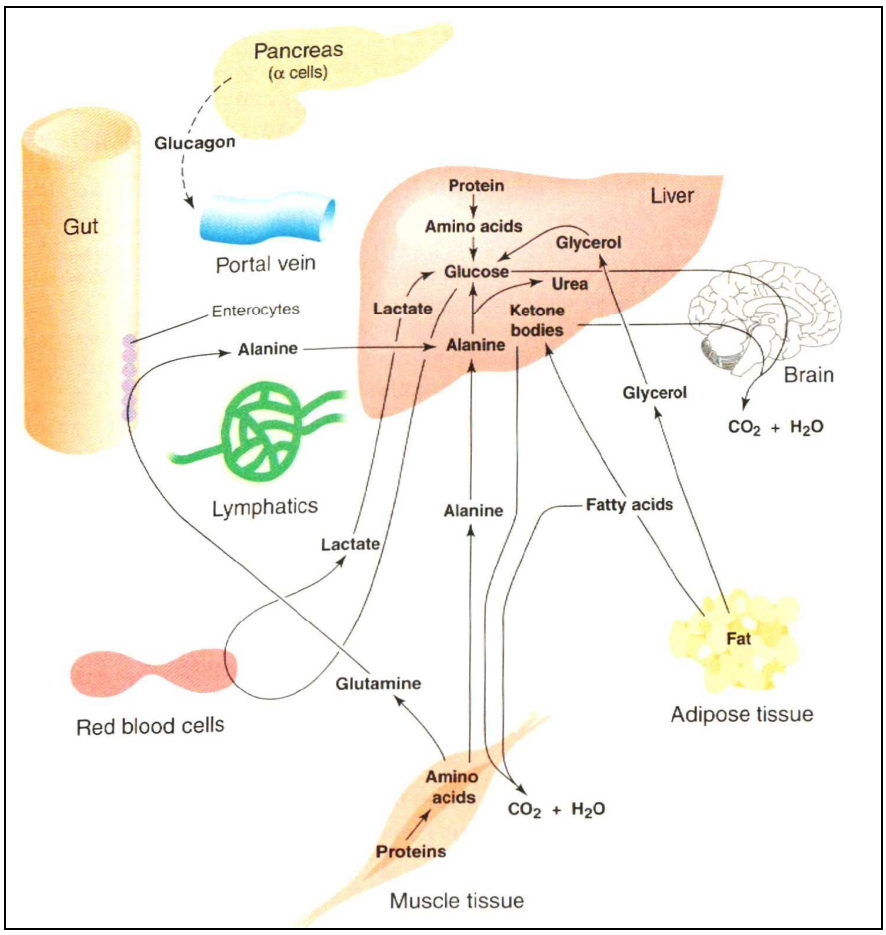


Figure 3: The Fasting state (68)

1.2 Liver effect on some dietary parameters

1.2.1. The body weight

Body weight may be considered as a simple parameter that shows the body physiological function, such as liver. It was noticed that patients suffering from liver dysfunction may manifest complications such as anorexia, weight loss, weakness, and fatigue. As the liver has a central role in nutritional homeostasis, any liver disease leads to abnormalities in nutrient metabolism and subsequent malnutrition. In addition, other reasons for malnutrition may be anorexia, diminished food intake, fat malabsorption, impaired intestinal absorption, and/or abnormal nutrients metabolism and storage (15).

1.2.2. Serum glucose level

One of the primary functions of the liver is to maintain blood glucose concentrations within the normal range. Dietary carbohydrates are digested to monosaccharides, which are absorbed into the blood. The liver removes most of the glucose from the blood and all of the other monosaccharides (galactose and fructose), where they are converted into glucose (65). Liver has the greatest number of ways to utilize glucose. Liver uptake of glucose occurs independently of insulin by means of glucose transporter -2 (GLUT2) (68). Liver is normally a glucose-producing rather than a glucose-consuming tissue, that is why a little of glucose is used to meet liver own energy needs (25).

The absorbed glucose is transported into hepatocytes as glucose 6-phosphate by hexokinase and the liver-specific glucokinase (65, 68). Glucose 6-phosphate can be stored as glycogen, or converted back to glucose, which released back to blood, by glucose 6-phosphatase which found in the lumen of endoplasmic reticulum (68). Alternatively, glucose 6-phosphate is oxidized to pyruvate, then to acetyl CoA, in the pathway of glycolysis. The acetyl group enters the tricarboxylic acid (TCA) cycle, where it is completely oxidized to CO_2 and water. Energy from this oxidative reaction is used to generate ATP (65).

Glucose 6-phospate is also used by pentose phosphate pathway for the production of NADPH, which is needed for reductive synthesis (*de novo* synthesis of fatty acids, and cholesterol), maintenance of reduced glutathione (GSH), a numerous reactions catalyzed by endoplasmic reticulum enzyme system. A vital function of pentose phosphate pathway is the provision of ribose phosphate for the synthesis of the sugar moiety of nucleotides such as ATP and those in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (25, 68).

Glucose 6-phosphate is also used in the glucouronic acid pathway, which is important in drug and bilirubin detoxification. The liver also converts three-carbon precursors (lactate, pyruvate, glycerol, and alanine) into glucose by the process of gluconeogenesis to meet the needs of the other cells and the brain (57, 68, 94).

The blood glucose level is increased pathologically in cases of diabetes, pancreatitis, some hepatic diseases, obesity, stress, and some CNS lesions. In addition, the level rises due to increase in circulatory epinephrine, or due to effect of drugs like alcohol, phenytoin, and steroids. On the other hand, the blood glucose level decreases in

endocrine disorders, insulin overproduction, hypothyroidism, extrapancreatic tumors, hepatic diseases, pediatric abnormalities, enzyme diseases, and malnutrition (117).

1.2.3. Serum protein level

Protein undergo constant turnover; they are constantly being synthesized and degraded, as the body cannot store protein in the same way as glycogen or triglycerides (25). The amino acids released by protein breakdown enter the same pool of free amino acids in the blood as the amino acids from the diet. These free amino acids can be used by all cells for protein synthesis or for biosynthesis of other compounds. As hepatocyte has a well-developed endoplasmic reticulum, Golgi system, and cellular cytoskeleton, all of which function in the synthesis, processing, and secretion of proteins, liver uses the amino acids absorbed from the blood for the synthesis of various proteins (162).

The body cannot dispense with the liver's contribution to protein metabolism for more than a few days without death ensuing. The most important functions of the liver in protein metabolism include deamination of amino acids, removal of ammonia from the body fluids in form of urea. In addition, it forms plasma proteins and the clotting factors, inter-convert of the various amino acids, forms nonessential amino acids, and syntheses other compounds from amino acids such as heme, hormones, neurotransmitters, and purine and pyrimidine bases (65, 162).

Plasma protein depletion causes rapid mitosis of the hepatic cells and growth of the liver to a larger size; these effects are coupled with rapid output of plasma proteins until the plasma concentration returns to normal (65). Such decrease in blood protein levels may be due to poor nutrition, malabsorption, diarrhea, or severe burns, while increase in proteins levels are seen in pathological condition e.g. lupus, hepatitis disease, chronic infections, alcoholism, leukemia, and tuberculosis. In case of liver cirrhosis, protein levels in the blood are also reduced, as liver protein synthesis is reduced (162).

The most abundant plasma protein produced by the liver is albumin, which represents 40 to 60% of the total plasma protein. Albumin serves as a carrier for a large number of hydrophobic compounds, such as fatty acids, steroids, hydrophobic amino acids, vitamins, and pharmacologic agents. It is also an important osmotic regulator in the maintenance of normal plasma osmotic pressure. Albumin is synthesized but it is not stored in the liver, thus it is used as a marker of hepatic synthetic function (94).

Increased albumin level is caused either by dehydration, shock, or by dehydration. Albumin level decreased in cases of sever protein loss (e.g. nephrotic syndrome, burns, protein losing enteropathy), increased albumin turnover (e.g. catabolic states, glucocorticoids administration), decreased protein intake (e.g. malnutrition, very low protein diets), alterations in plasma oncotic pressure (e.g. hypergammaglobulinemia), and liver diseases. Although, plasma albumin is seldom decreased in acute hepatitis, due to its long half-life (20 days), but with the progression of liver disease serum albumin level falls reflecting decreased synthesis, however in the presence of ascites may also reflect albumin dilution (65).

Among other important functions, liver has the ability to synthesize certain amino acids and other important chemical compounds from amino acids. For instance, the nonessential amino acids can all be synthesized in the liver through several stages of aminotransferation. Therefore, aminotransferases enzymes are abundant in the liver, such as alanine aminotransferases (ALT) and aspartate aminotransferases (AST) (65).

1.2.4. Serum lipid profile

The liver plays a central role in the regulation of lipid metabolism. When fuels are abundant, fatty acids derived from the diet or synthesized by the liver are esterified into triglycerides and secreted into the blood in the form of VLDL. However, in the fasting state, the liver converts fatty acids into ketone bodies, and then they are subsequently oxidized for energy or used in biosynthetic pathways (25, 162). The liver also synthesizes cholesterol, which is transported to other tissues as a component of lipoproteins, and excess cholesterol is converted into bile acids in the liver or directly excreted with the bile (94).

Although most cells of the body metabolize lipids, certain aspects of there metabolism occur mainly in the liver, such as beta-oxidation of fatty acids and synthesis of phospholipids and most lipoproteins. To derive energy from fatty acids; they are broken-down by beta-oxidation to form acetyl-CoA, which can take place in mitochondria, such reaction occurs in the hepatic cells. The excess of hepatic acetyl-CoA is converted into ketone bodies that are transported throughout the body to be absorbed by other tissues. These tissues reconvert the acetoacetic acid into acetyl-CoA, and then oxidize it in the TCA cycle to liberate tremendous amounts of energy (65).

Ninety percent of the dietary intake and 95% of the fatty acids stored in tissues are triglycerides. Increased triglycerides levels may be present in arthrosclerosis, hypothyroidism, liver diseases, pancreatitis, myocardial infarction, metabolic disorders, toxemia, and nephrotic syndrome. Decreased triglycerides levels may be present in chronic obstructive pulmonary disease, brain infarction, hyperthyroidism, malnutrition, and malabsorption (162).

Cholesterol is a structural component of cell membrane and plasma lipoproteins, and is important in the synthesis of steroid hormones, glucocorticoids, and bile acids. Some of cholesterol is absorbed through the diet, however mostly is synthesized in the liver. The amount of cholesterol from dietary sources and hepatic synthesis is under close hepatic homeostatic control. About 80% of the cholesterol synthesized in the liver is converted into bile salts; the remainder is transported together with phospholipids by the lipoproteins to the tissue cells, as both are used by the cells to form membranes, intracellular structures, and multiple chemical substances that are important to cellular function (65).

Low levels of cholesterol are seen in malnutrition, liver insufficiency, malignancies, anemia, and infection. A high dietary intake and intestinal absorption of cholesterol will reduce the rate of hepatic cholesterol synthesis, in this case the liver acts as a recycling depot for sending excess dietary cholesterol to the peripheral tissue when needed as well as accepting cholesterol from these tissues when required (162).

Lipoproteins, which are essential in lipid blood transportation, are classified into five groups. In order of decreasing size and increasing density, these are chylomicrons, VLDL, IDL (intermediate-density lipoprotein), LDL (low-density lipoprotein), and HDL (high-density lipoprotein). The classes of lipoproteins differ not only in their composition, but also in the ways in which they originate and function. The chylomicrons transport triglycerides from the intestine to the tissues; they are formed in the intestinal mucosa then removed from the blood by the liver. VLDL, IDL, and LDL are closely related to one another. VLDL formed in the liver to transport triglycerides, cholesterol, and phospholipids to other tissues then they are gradually converted into IDL and LDL under the influence of lipoprotein lipase on cells membrane (162).

HDL originates in the liver to transport excess cholesterol obtained from peripheral tissues to the liver, and to exchange proteins and lipids with chylomicrons and VLDL (2). A high level of HDL is an indication of a healthy metabolic system, if

there is no sign of liver disease or intoxication. As HDL metabolism may be abnormal in chronic liver disease. The HDL_2 (more antiatherosclerotic): HDL_3 (less antiatherosclerotic) ratio is usually elevated in cirrhosis, because hepatic lipases enzyme may be deficient or reduced in activity. These changes may result in an overall increase in serum total HDL levels (162).

1.3. Liver impairment

Diseases of the liver can be clinically and biochemically devastating, because no other organ can compensate for the loss of the multitude of functions that liver normally performs. Liver impairment is usually evident by elevated plasma levels of the hepatic enzymes ALT, AST and gamma glutamyltransferase (GGT) (due to hepatocyte injury with a consequent release of these enzymes into the blood), jaundice (an accumulation of bilirubin in the blood caused by inefficient bilirubin conjugation by the hepatocytes), increased blood clotting times (hepatocyte has difficulty producing clotting factors). Also, it may be evident by edema (due to reduced albumin synthesis by hepatocytes leads to a reduction in osmotic pressure of the blood), and hepatic encephalopathy (reduced urea cycle activity in hepatocytes leads to excessive levels of ammonia and other toxic compounds in the central nervous system) (162).

Hepatocytes are well protected against chemical injury by high levels of cytoprotective agents, such as GSH and high levels of antioxidant enzymes, e.g. glutathione peroxidase, superoxide dismutase. In addition, other mechanisms help in protecting and restoring liver function such as the precise regulation of liver growth and functional mass (192).

Hepatic injury or impairment usually involves function and structure, as well as a diminished capacity to respond to toxic stress. Many of these toxic effects result from macromolecular damage, which is irreversible, except for DNA alteration, up to a point, and with sustainable levels of hepatocellular injury, compensatory proliferation occurs (192). Although hepatocytes are normally quiescent cells with low turnover and a long life span, hepatocytes can be stimulated to grow if damage occurs to other cells in the liver. The liver mass has a relatively constant association to the total body mass in adults; deviation from the normal or optimal ratio (due to partial hepatectomy or significant hepatic cell death or injury) is rapidly corrected by hepatic growth. In other words, a proportional rapid increase in hepatocyte replication, as long as the injury is

uncomplicated by viral infection or inflammation (162, 154), and after the original size and volume of the liver are achieved, the hepatocytes revert to their usual quiescent state (65).

In addition, the nature of hepatocytes as intermediate proliferating cells, and their frequency of proliferation are extremely low reflected by a low rate of cell death through apoptosis, enable hepatocytes to avoid sustain DNA damage in the vulnerable S-phase of DNA replication. This renders hepatocytes tolerant to levels of DNA damage that are lethal to other cell types, in addition, hepatocytes are well equipped with DNA repair systems (192).

From previously mentioned data, the liver is usually exposed to many potential harmful agents that in normal situations do not damage the liver cells due to the protective mechanisms and the large repair capacity in these cells. However, during acute and chronic liver diseases, hepatocytes are exposed to increased levels of harmful stress results in disturbed liver function. As a number of diseases can progress into hepatic fibrosis and cirrhosis, liver function becomes inadequate for life when these become greater than cell tolerance (154).

Acute viral hepatitis is characterized by inflammatory cells infiltration, necrosis, hepatocellular degeneration, and regeneration. It can be developed in a short period because of viral hepatitis, drug-induced (e.g., acetaminophen overdose) or toxin-induced (e.g., mushroom derived alpha-Amanitin) toxicity. Chronic hepatic injury occurs in response to a variety of insults, including long-term exposure to viral hepatitis, alcohol abuse, drugs or chemicals, metabolic diseases involving an overload of iron or copper, autoimmune diseases, or congenital abnormalities (95, 163).

Regardless of the insulting agent of the hepatic lesion, the liver will apparently react in five ways: necrosis, degeneration, inflammation, regeneration, and fibrosis. Necrosis may follow practically any lesion where changes are significant. However, before it becomes characteristically necrotic, hepatocytes may become swollen and edematous, with irregularly compact cytoplasm (degenerated). Hepatocytes inflammation is caused by the afflux of inflammatory cells to portal-spaces or parenchyma. Regeneration may take place and is visible by the thickening of hepatocyte cords or their proliferation, and certain disorganization of the parenchyma structure. Fibrosis occurs through increased collagen deposition, should fibrosis persists liver will be divided into regenerating hepatocytes nodules surrounded by scarring tissue

(cirrhosis) (143). However, apoptosis can be added to these signs, as it may also appear together with necrosis during liver injury, as Kupffer cells, leukocytes and lymphocytes are activated, producing pro-inflammatory cytokines and reactive oxygen species, which cause apoptotic and necrotic cell death in hepatocytes (95).

1.4. Liver cell death pathways

Hepatocyte cell death occurs in both acute and chronic liver diseases, moreover these hepatocellular injuries can progress to either apoptosis or necrosis. In addition, the characteristic features of both necrotic and apoptotic cell death can occur in the same tissue and even in the same cell simultaneously (154). Therefore, to understand the hepatocellular injury in any experimental model, both parameters of necrosis and apoptosis should be investigated.

During apoptotic cell death (or programmed cell death), which is ATP dependent and develops more orderly, the following cascade of events occur: DNA condensation, nuclear fragmentation, plasma membrane budding and cell shrinkage. Eventually, the apoptotic cell breaks into small membrane-surrounded fragments called apoptotic bodies (as shown in Figure 4), which are cleared by surrounding neighboring cells and mononuclear cells. In addition, the phagocytic cells were reported to secrete cytokines that inhibit inflammation (95).

On the contrary, necrosis is defined by the loss of plasma membrane permeability barrier, resulting in cell cytolysis (95). It can result from metabolic disruption with energy depletion (loss of ATP), mitochondrial and cellular swelling, and/or activation of degradative enzymes, which cause cell lysis followed by loss of cell constituents into its surroundings (as shown in Figure 4). Thus, necrosis is usually accompanied by inflammation (154). Figure 4 shows the morphological differences between necrosis and apoptosis.

Necrosis and apoptosis pathways may sometimes intercross. When the cell undergo apoptosis may exhaust cellular ATP levels and then progress to secondary necrosis characterized by cell swelling and lysis. On the other hand, if the necrotic cell death mechanism is inhibited, the stress may eventually force the cell into apoptosis. Furthermore, an insult affecting only a few mitochondria would be resolved by autophagy of the damaged cell organelles. If it involves more mitochondria, which release enough cytochrome c to activate the caspase cascade, and if the remaining intact

mitochondria maintain the cellular ATP levels, the cell will undergo apoptosis. However, if the insult is too severe, cellular ATP levels are not maintained and the cell will die by oncotic necrosis (80).

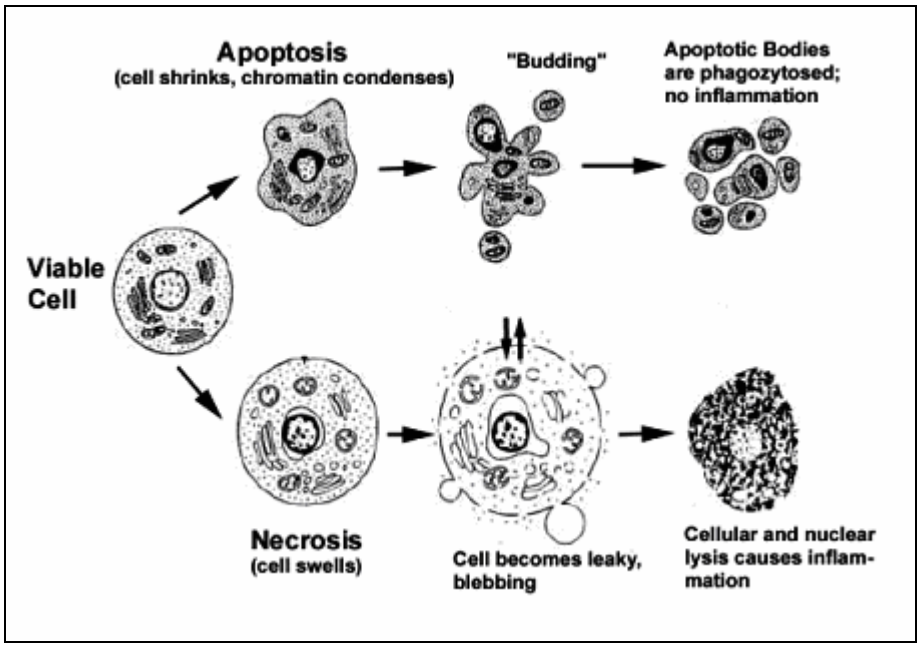


Figure 4: The differences between necrosis and apoptosis (56).

1.4.1. Necrotic hepatocytes death

Necrosis represents an uncontrolled destruction of the cell in response to an external stimulus injury, which result in cellular anoxia and eventual disruption of the plasma membrane with loss of cell contents into the surrounding environment, as shown in Figure 4. This cell necrotic injury is mediated by the interference with the energy supply of the cell, and/or direct damage to cell membranes (41). During hepatocellular damage, varieties of enzymes normally located in the cytoplasm are released into the blood flow, such as aminotransferases, GGT and alkaline phosphatase, as the integrity of the hepatocytes membrane diminishes. Their quantification in plasma is a useful biomarker of the extent and type of hepatocellular damage (136).

In 1989, a panel of 12 European and American experts by consensus defined liver injury by an increase of more than twice the upper limit of the normal range in the levels of serum ALT, or conjugated bilirubin. On the other hand, a combined increase in the levels of AST, alkaline phosphatase, and total bilirubin more than twice the upper limit of the normal range also indicate liver injury (41). Elevations in serum enzyme

levels (ALT and AST) were taken as indicators of hepatocellular, and alkaline phosphatase for cholestatic liver injury (127).

1.4.1.1. Serum aminotransferases levels

Aminotransferases are localized abundantly in hepatocytes, reflecting their role in oxidative phosphorylation and gluconeogenesis. They participate in gluconeogenesis by catalyzing the transfer of amino groups from aspartate or alanine to α-ketoglutaric acid to produce oxaloacetate and pyruvate respectively (as shown in Figure 5), which are important contributors to the TCA cycle (111).

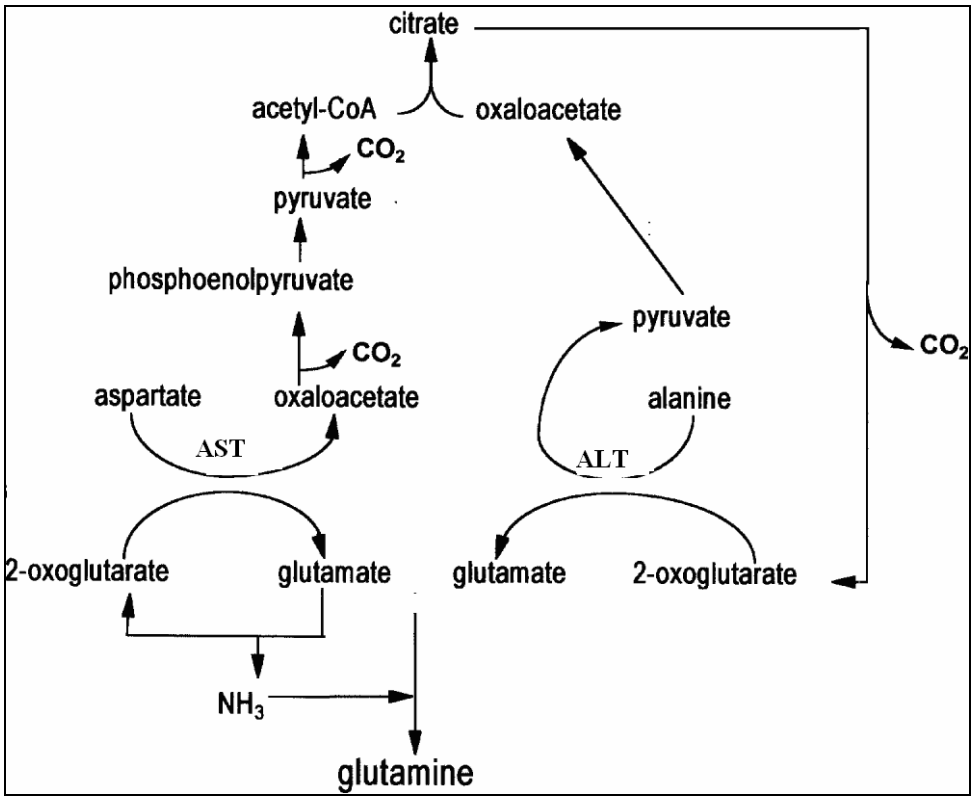


Figure 5: Alanine (ALT) and aspartate (AST) aminotransferases biochemical reactions (47).

Aminotransferases blood serum activities presumably increase because of cellular membrane damage or leakage. The highest increases are observed with acute hepatocellular injuries, caused by xenobiotics or acute viral hepatitis, while cholestasis, alcoholic or chronic liver disease cause slight increases. The degree of increase in ALT activity correlates with the number of hepatocytes damaged, but ALT may also increase following release from hepatocytes during liver repair (13). ALT is highly specific for the liver cell damage, whereas AST is located in liver cells and in the heart, brain,

kidney and skeletal muscle, making this enzyme less specific for liver injury. AST and ALT activity in liver are about 7000 and 3000 times serum activities, respectively. As ALT is exclusively cytoplasmic enzyme, while AST found in both mitochondrial and cytoplasmic forms (13).

ALT was reported to be the more sensitive test in acute and obstructive liver diseases, whereas AST was more sensitive in chronic and infiltrative lesions. The ratio AST/ALT helps in identifying certain etiologic factors causing hepatitis. In most types of liver disease, AST/ALT ratio is usually less than 1.0 in adult patients with acute and chronic (non-alcoholic) hepatitis, whereas, AST/ALT ratio of two is characteristic of severe ischemic hepatic injury, severe hepatotoxic drug injury, or alcoholic hepatitis. In alcoholic patients, pyridoxine deficiency is common that cause a decrease in ALT activity, and alcohol induces release of mitochondrial AST from cells without visible cell damage (73).

Aminotransferases are parameters of hepatocyte damage and turnover. It can be anticipated that the release of aminotransferases correlates quantitatively with the extent and type of cell death and/or its turnover. Moreover, as the release of aminotransferases was lower from apoptotic cells than from necrotic cells, and patients with normal aminotransferases levels had a significantly reduced hepatocellular proliferation rate compared with patients with elevated aminotransferases. It could explain the progression of liver diseases in asymptomatic patients with hepatitis C (HCV) infection and normal values of aminotransferases follow apoptotic cell death (135). In addition, in patients with elevated aminotransferases, necrosis over come apoptotic cell death.

1.4.1.2. Serum gamma-glutamyltransferase level

Although GGT, a membrane bound enzyme, is present in liver, proximal renal tubule, pancreas, and intestine, however GGT activity in serum comes primarily from the liver. Most of GGT is located in cell membranes, where its function is to transport amino acids and peptides into the cell across cell membranes. GGT catalyzes both the transfer of gamma-glutamyl groups from peptides to amino acids and catalyzes the first step in the degradation of extracellular GSH. Thus, it provides cells with a means for the recovery of cysteine, whose adequate supply is critical for protein synthesis and *de novo* synthesis of intracellular GSH. In addition, the metabolism of extracellular GSH can affect other cellular functions by modulating the availability of glutamic acid, which is critical for the growth of normal as well as neoplastic cells (141).

Usually, the increase in GGT levels in patients with chronic liver disease is associated with bile duct damage and fibrosis (57). In addition, GGT is increased in 80-95% of patients with any form of acute hepatitis. Moreover, the enzyme is inducible by chronic alcohol use and by drugs such as phenytoin. Previously, the primary utility of monitoring serum GGT is in the exclusion of bone disease as a cause of increased serum alkaline phosphatase, a condition that does not affect GGT concentrations. Nowadays, measuring GGT is most useful in patients at risk of liver disease, especially due to alcoholism. The half-life of GGT in humans is about 10 days; but in alcohol-associated liver injury the half-life increases to as much as 28 days, suggesting impaired clearance (111).

1.4.2. Apoptosis hepatocytes death

Apoptosis is a controlled active process that may be initiated from within the cell (intrinsic) or in response to a variety of extracellular stimuli (extrinsic). Sometimes, these two apoptotic pathways are closely interrelated at mitochondrial pathway, as extrinsic pathway occasionally require mitochondrial pathway to amplify the relatively weak death receptor-induced apoptotic signal (152). An additional pathway has been presented that involves cytotoxic T lymphocytes (CTL) cell mediated cytotoxicity with perforin-granzyme-dependent killing mechanism (41). Figure 6 shows the three apoptotic pathways: intrinsic, extrinsic and perforin/granzyme mediated pathways.

The extrinsic, or death receptor-mediated pathway, apoptotic pathway is initiated by the activation of membrane-bound death receptors by specific ligands such as tumor necrosis factor-α receptors and Fas ligand. In T-cell mediated cytotoxicity, CTLs are able to kill target cells via the Fas ligand/ Fas receptor interaction (41, 46). The intrinsic mediated signaling, pathway involves a diverse array of non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell. This intrinsic signaling can be stimulated by the absence of certain growth factors, hormones and cytokines, DNA damage by radiation, toxins, hypoxia, hyperthermia, viral infections and free radicals. The resulting stress leads to mitochondrial inner membrane damage, or cellular ATP depletion that can cause mitochondria dysfunction, increasing in mitochondrial permeability transition, cytochrome c release and activation of caspases cascade (41, 46, 152).

The extrinsic, intrinsic, and CTLs pathways join on the same execution pathway. This execution pathway, which is initiated by the cleavage of caspase-3, results in DNA

fragmentation, cytoskeleton degradation, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and at the end uptake by phagocytic cells (41, Figure 6).

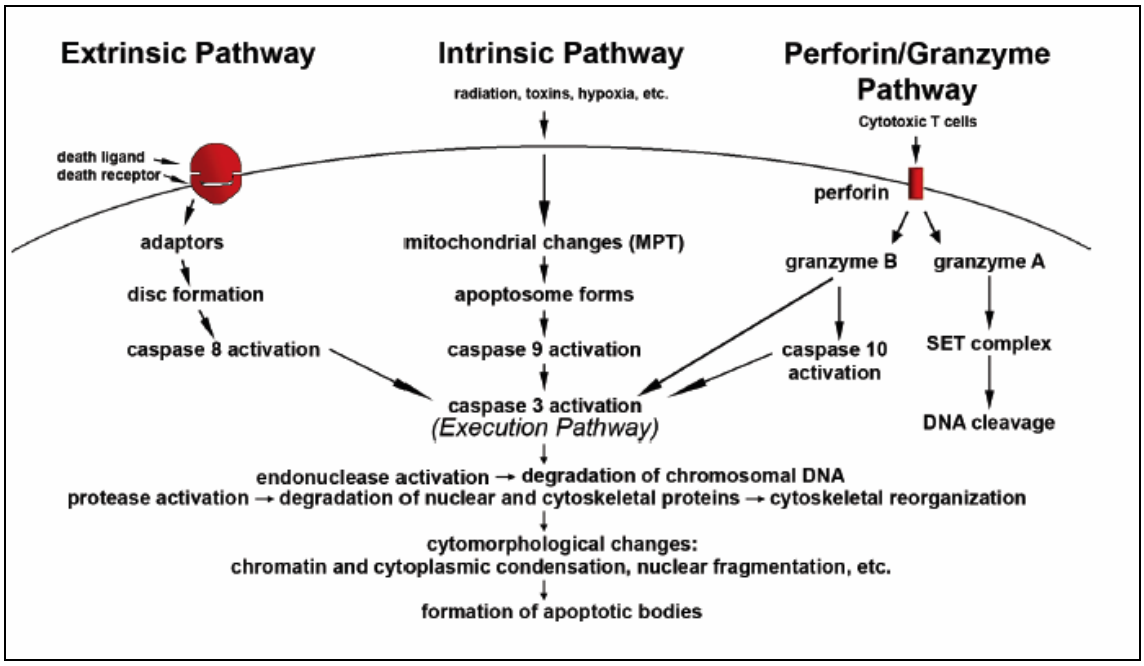


Figure 6: The three apoptotic pathways: intrinsic, extrinsic and perforin/granzyme pathways (41).

1.4.2.1. the tumor suppressor protein - p53

p53 is a key feature of apoptosis that triggered by intracellular stimuli, where the cell recognize itself as damaged and may start to suicide deliberately. The p53 nucleoprotein is a product of the *p53* gene, which has been called the 'guardian of the genome or gatekeeper for growth and division', because of its role in controlling critical checkpoints in response to DNA damage. p53 inactivation or mutation or both is considered a qualification for tumor formation, as failure of normal p53 function may lead to replication of cells with abnormal DNA and hence to malignant transformation. At least half of all malignant tumors were found to have abnormalities of the *p53* gene resulting in abnormal 'mutant' p53 (197).

In response to DNA damage, p53 may induce the expression of a large number of genes lead to cell cycle arrest to allow damaged DNA to be repaired, and if repair is not possible, p53 may initiate apoptosis to prevent the replication of abnormal genetic material (185, 197). When DNA repair is activated early in the p53-induced apoptotic process, the cell death pathway in some circumstance may be reversed (41). p53 can

promote apoptosis by several mechanisms. p53 can directly stimulate death receptor signaling, or stimulate mitochondrial perturbations, as p53 plays an important role in mitochondrial membrane stability. In addition, p53 may have a direct apoptotic genetic role in the mitochondria, or p53 can mediate apoptosis via transcriptional activation of pro-apoptotic genes such as Bcl-2 family members (70, 197).

In its normal state, the tumor suppressor action of wild type p53 is present in the nucleus and mediated by specific DNA binding and protein-protein interactions within the nucleus, although sometimes a secondary event occurs in which wild type p53 is lost. Abnormal cytosolic wild type p53 localization has been observed in a number of cancer cell lines. The cytosolic wild type p53 is stable and inactive, but when it is localized in the mitochondria, it can contribute toward apoptosis. Mitochondrial p53 induces apoptosis by regulating mitochondrial Bcl-2 family proteins, or cytosolic p53 binds to pro-apoptotic Bcl-2-family proteins, leading to permeabilization of mitochondria (70, 197).

1.4.2.2. Cytochrome c

The release of cytochrome c is a critical, early event in the development of apoptosis, especially through intrinsic pathway. Cytochrome c is involved in mitochondrial oxidative phosphorylation (139). Inside mitochondria, cytochrome c operates as part of the respiratory chain by shuttling electrons through its heme group between Complex III (cytochrome c reductase) and Complex IV (cytochrome c oxidase) (194). However, outside mitochondria, cytochrome c triggers the activation of the caspase-9 cascade with Apaf-1, leading to the morphological changes typical for apoptosis such as DNA fragmentation, chromatin condensation, and formation of apoptotic bodies (93).

Cytochrome c is encoded by a nuclear gene and translated by cytosolic ribosomes as apocytochrome c, which is subsequently translocated into the mitochondria where a heme group is attached covalently to form holocytochrome c. Heme binding induces a conformational change of the holocytochrome c which keeps it inside the intermembrane space within the cristae. The apocytochrome c is not able to activate Apaf-1, so signifying that the heme group is essential for its apoptotic function. In this way, the cell is protected from cytosolic cytochrome c during cytochrome c synthesis and translocation into mitochondria (131, 194).

After the release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm, cytosolic cytochrome c forms a complex with Apaf-1 and procaspase-9 binding to ATP, and activates caspase-9 (Figure 7), which in turn activates procaspase-3 and -7 (92, 133, 197).

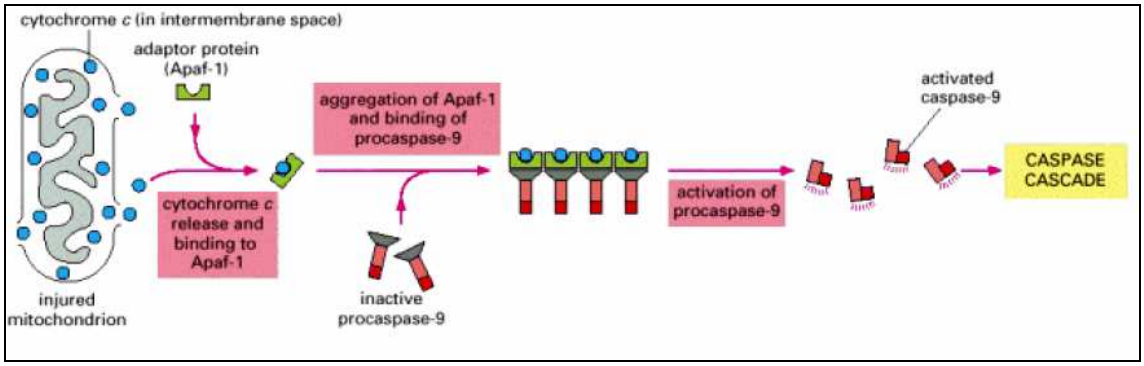


Figure 7: Cytochrome c triggers apoptotic pathway (7).

1.4.2.3. Caspase -3

Molecularly, apoptosis is carried out by caspases, which are represented by a family of cysteine proteases, produced in the cell as zymogens, which are inactive in the cytoplasm until they are cleaved at specific aspartate residues (as shown in Figure 8). Caspases have proteolytic activity and are able to cleave proteins at aspartic acid residues, and these activated caspases cleave many cellular substrates, ultimately leading to cell death (27, 187).

Caspases operating in the apoptotic process can be divided in three groups, initiator, executioner, and inflammatory caspases. Initiators caspases are activated by proapoptotic stimuli (caspase-2,-8,-9,-10) and are involved in the activation of the executioner caspases, which execute the fragmentation of DNA and dissemble cellular structures (caspase-3,-6,-7), and inflammatory caspases (caspase-1,-4,-5) (41, 144). Initiator caspases respond to apoptotic stimuli following particular molecular pro-death cues. Executioner caspases are activated by upstream initiator caspases (Figure 8) and are responsible for proteolytic cleavage of ‘death substrates’, which gives rise to the physical and morphological features of apoptosis. Once executioner caspases are activated, they execute apoptosis through proteolytic cleavage of a number of structural and regulatory proteins within the cell. These proteins include structural proteins, such as actin and nuclear lamin, regulatory proteins, such as p21 and Rb and proteins involved in DNA metabolism and repair, such as poly ADP ribose polymerase (PARP), among many others (187).

Caspase-3 is a key executioner of all apoptotic pathways, which is activated by an initiator caspase, such as caspase-9. Caspase-3 is a cytosolic protein that normally exists as a 32-kD inactive precursor. Moreover, it is cleaved proteolytically into a 17-kD and a 12-kD active heterodimer in cells undergoing apoptosis (27, Figure 8). Caspase-3 preferentially cleaves on the carboxyl side of the tetrapeptide sequence Asp-Glu-Val-Asp (DEVD), a sequence present in PARP, also cleaves sterol regulatory element–binding proteins and several other cellular proteins (125).

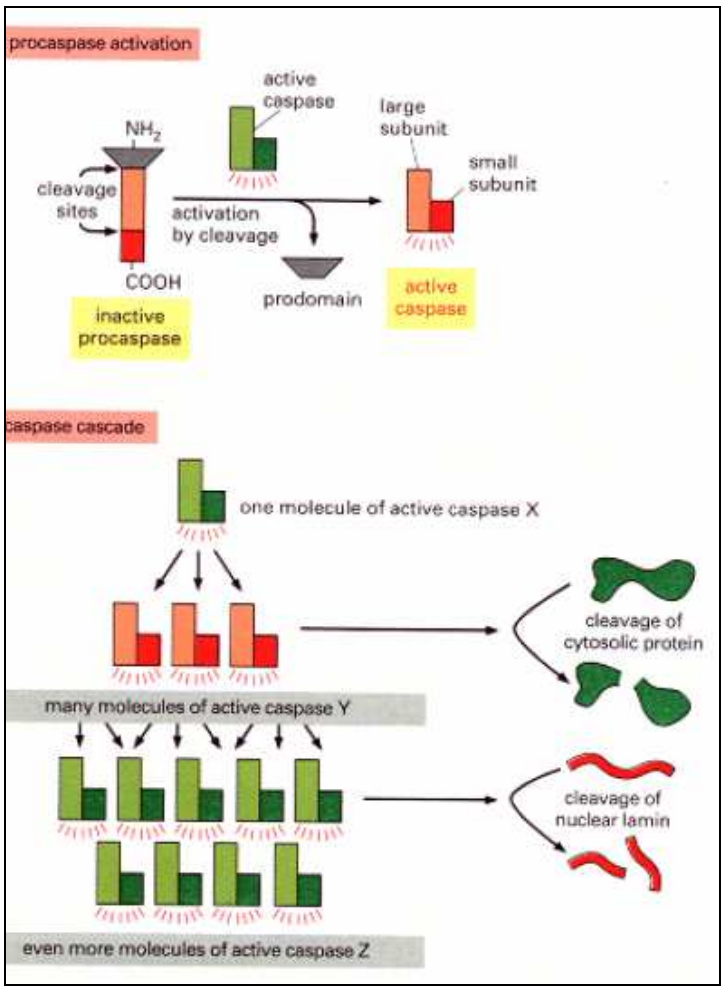


Figure 8: The caspase cascade involved in apoptosis pathway (7)

1.4.2.4. DNA laddering

Different endonucleases, which activated by caspases in cell apoptosis process, mediate two types of DNA cleavage. DNA is initially cleaved into fragments of 300,000 and/or 50,000 base pairs. This large-order DNA cleavage by Ca^{2+}-and Mg^{2+}-dependent endonucleases is often followed by internucleosomal DNA cleavage into fragments of 180–200 base pairs (the so-called ‘‘ladder’’ pattern of DNA cleavage). Thus, a

characteristic “DNA ladder” can be visualized by agarose gel electrophoresis with an ethidium bromide stain and ultraviolet illumination in apoptotic cells (41) as shown in figure 9). However, since DNA fragmentation occurs in the later phase of apoptosis, the absence of a DNA ladder does not eliminate the potential that cells are undergoing early apoptosis. Additionally, necrotic cells can also generate DNA fragments (41).

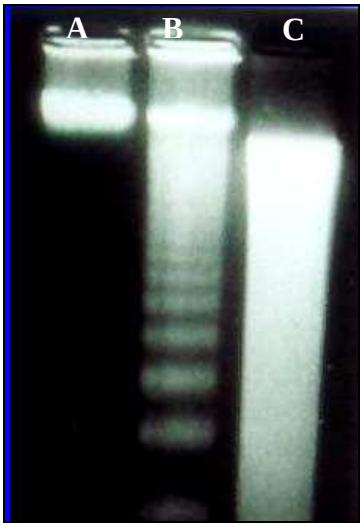


Figure 9: Photo of agarose gel electrophoresis of DNA extracted from different cell cultures (86)

Lane A: DNA of normal viable cells. **Lane B:** DNA of cells exposed to apoptosis. **Lane C:** DNA of necrotic cells.

The apoptotic process appears to be a host defense mechanism against viral infections and hepatocarcinogenesis. That is why; apoptosis of hepatocytes is accepted as a prominent feature of viral hepatitis B (HBV) and HCV (80, 95). However, many viral genomes encode proteins that inhibit host cells apoptosis, especially to escape from host immune attack. Therefore, virus-host interactions may determine viral persistence, extent, and severity of liver inflammation and possibly viral hepatocarcinogenesis (95). As cell death in viral hepatitis may not be caused exclusively through lymphocytes in order to remove damaged cells, but could be influenced by the virus itself (80). Thu, the effect of various hepatoprotective drugs used by hepatitis patients on liver function, and liver cell death pattern must be investigated, and a model of impaired liver is needed to be established on experimental animals first to facilitate such investigation.

1.5. Experimental model

A reproducible animal model of liver disease is highly desirable for appropriate metabolic and therapeutic studies. The criteria for an ideal experimental model should include the development of morphological features similar to that observed in human disease, features such as; gradual progression of pathological changes, high reproducibility and low mortality, reversible and irreversible fibrotic changes with pathophysiological outcome. Many agents have been used as hepatotoxin to induce an experimental hepatic damage model (20). Theses hepatotoxins may react with the basic cellular constituent: proteins, lipids, DNA and ribonucleic acid (RNA), and may induce almost all types of lesions of the liver (20).

To investigate the hepatoprotective effect of new synthetic or natural compound, scientists are used to examine on a model such as mice, rat, rabbit or dog intoxicated. The most common method of producing experimental cirrhosis is by administering variety of chemical compounds such as carbon tetrachloride (CCl_4), ethyl alcohol, dimethyethanolamine and D-galactosamine**,** or physically by ischemia-reperfusion, radiation or partial hepatectomy (142).

Hepatotoxicity can be identified as an injury to the liver that is associated with impaired liver function caused by exposure to a drug or another noninfectious agent. In addition, when liver function is impaired, symptoms and clinically significant disease follow. Drug induced injury to the liver can mimic any form of acute or chronic liver disease, with no risk of spreading a disease. Usually, the acute hepatocellular necrosis caused by drug toxicity clinically resembles viral or ischemic disease; as it is associated with elevated serum aminotransferases levels reflect the release of enzymes from the cytoplasm of dying cells (127).

The drug-induced hepatic disease is either a result of the drug itself or a result of reactive metabolites of the drug that covalently bind to hepatocytes and result in either idiosyncratic (unpredictable), intrinsic (predictable), or immunoallergic hepatitis (87, 105). Intrinsic predictable injury, resulting in hepatocellular necrosis, is dose-related, usually reproducible in animal models, and examples of predictable drug, acetaminophen, and CCl_4. Idiosyncratic unpredictable injury is seen more commonly than predictable toxicity. With unpredictable toxicity, there seems to be no dose relation, and usually there is no animal model, and often seems to be hypersensitivity mediated (e.g., alpha-methyldopa hepatitis) (87).

Many different mechanisms lead to hepatotoxicity. One is disruption of the cell membrane, or covalent binding of the drug to cell proteins that creates new adducts that serve as immune targets, thus inciting an immunologic reaction. Another mechanism is the inhibition of cellular pathways of drug metabolism, or causing an abnormal bile flow leading to cholestasis and jaundice. In addition, programmed cell death (apoptosis) is considered as probable hepatotoxicity mechanisms occurring through tumor necrosis-factor and Fas pathways; and inhibition of mitochondrial function, which lead to accumulation of reactive oxygen species and lipid peroxidation, then cell death (127).

As, Cokca et al. (31) reported that high-dose therapy with statin lead to toxicological situation that mimics viral hepatitis, a new hepatitis model can be induced by over doses of statin in experimental animals. Fluvastatin was investigated as a hepatotoxin for new model of experimental hepatitis, whether it cause injury in intrinsic predictable or immunological pattern.

Fluvastatin

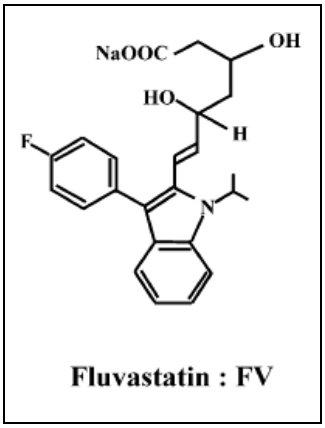


Figure 10: Fluvastatin chemical structure (115)

Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. They effectively reduce cholesterol level (115, 132, 174). Statins are composed of nine unique compounds; they are naturally derived from fungi fermentation or chemically synthesized. Fluvastatin sodium, (T)-(3R*,5S*,6E)-7-(3-p-fluorophenyl)-1-isopropylindol-2-yl)-3,5-dihydroxy-6-heptenoic acid (as shown in Figure 10), is the first fully synthetic HMG-CoA reductase inhibitor (24).

Fluvastatin has a distinct biopharmaceutical profile among statins; it has high rate of absorption, rapid liver uptake, short systemic exposure time (half-life of 1.2 hours), and virtually no active circulating metabolites. Fluvastatin is targeted to the liver, where it is subjected to rapid extensive first-pass metabolism, and 98% of fluvastatin is protein bound (110).

Statins effectively block HMG-CoA reductase from converting HMG-CoA to mevalonic acid, reducing the endogenous *de novo* synthesis of cholesterol in the liver (132). This leads to a decrease in intracellular hepatic cholesterol level, which then induces expression of cell surface LDL receptors, enabling cholesterol to be removed of circulation (196). The safety of statins has been documented extensively; they are remarkably well tolerated, with minor side-effects include constipation, flatulence, dyspepsia, nausea, and gastrointestinal pain (196). The most serious adverse effect is myotoxicity e.g. rhabdomyolysis, and hepatotoxicity that evident by elevated serum aminotransferases levels (132).

Statin mechanism action

Statins as a HMG CoA reductase inhibitor results in depletion of mevalonate, and thus leads to a reduction in cholesterol synthesis, together with reduction in isopentenyl -, geranyl - and farnesyl pyrophosphate, and dolichol and ubiquinone (72, 174). All of these compounds are involved in various essential cell functions (72). Figure 11 outlines the fluvastatin mechanism of action.

Cholesterol is involved in membrane integrity and steroids production, while ubiquinone is involved in electron transport and cell respiration. Isopentenyl pyrophosphate reduction may affect cell biosynthetic activity, while a reduction of geranyl and farnesyl pyrophosphate may potentially affect signal transduction, as they are involved in covalent binding of G proteins, such as the Ras family, to membranes. Dolichol is required for glycoprotein synthesis, and isopentenyladenine, essential for certain transfer RNA function and protein synthesis. The effects of such compounds on cell biosynthetic activity and signal transduction may in turn affect optimal cellular regulation, function, and repair activities (174).

Statins also has some pleiotropic effects, which are independent effects of statin therapy beyond their cholesterol lowering (72). These pleiotropic effects are involved in endothelial function, antioxidant and anti-inflammatory, anticancer, antifibrotic, antiviral and cytostatic properties, and immuno-modulator activity (43, 99, 183).

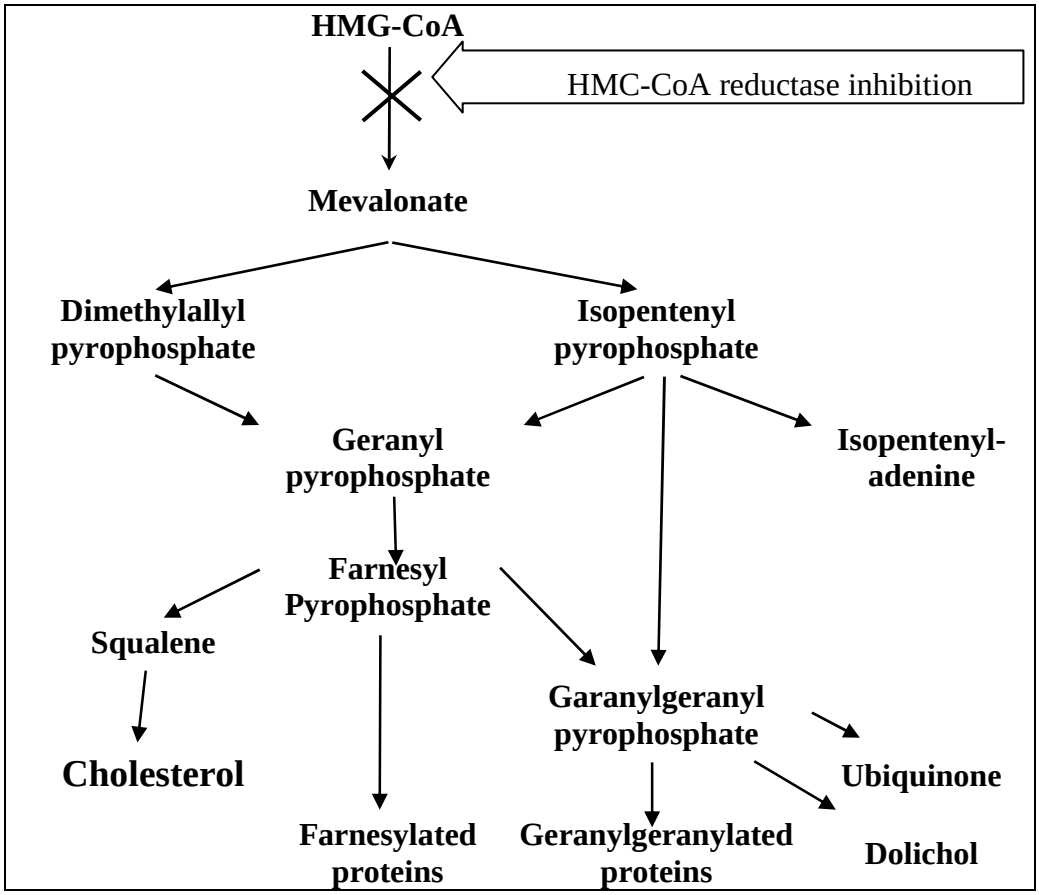


Figure 11 Fluvastatin mechanism of action (196)

Statin side effects

Cholesterol synthesis occurs predominantly in liver, which is the target organ of fluvastatin. Fluvastatin appears in much higher concentrations in the liver than in non-target organs, and the liver is the drug's primary site of both action and side effects, as one of statins most important adverse effects is associated with liver toxicity (167, 174). Moreover, in a subset of patients, who received fluvastatin or another statin, a hepatocellular injury was indicated by elevations in serum aminotransferases levels in dose-dependent manner, and/or by the biochemical abnormalities of liver function (167). These aminotransferases elevations are usually asymptomatic, more prevalent with higher doses or with extended period of drug uptake, and transient as they resolve after discontinuation of the drug (8, 87, 91).

Cokca et al. (31) reported that high-dose therapy with statin lead to toxicological situation that mimics viral hepatitis. In addition, the biochemical tests of hypercholesterolemia patient treated with fluvastatin 80 mg/day for a year showed elevated levels of ALT, AST, lactate dehydrogenase, creatinine, and blood urea

nitrogen. Controlled trials show increases in serum aminotransferases values to more than three times the upper limit of normal in 0.2-2.7% of patients, and rarely, symptomatic liver disease including fatal acute liver failure (87, 115). Because of these reports, experts and manufacturers recommend routine monitoring of liver function test values in patients receiving chronic statin therapy, and advise against statin therapy in patients with active or chronic liver disease (44). In addition, statin therapy should be discontinued in the presence of significant objective evidence of liver dysfunction (46, 120, 157).

Since the early 1990s, there have appeared sporadic reports of statins triggering autoimmune diseases. These diseases include lupus erythematosus, arthralgia, dermatomyositis, polymyositis, hypersensitivity pneumonitis, lichen planus and autoimmune hepatitis that developed a predominantly hepatocellular pattern of injury (8, 91). As drug-induced hepatic toxicity may mimic almost all types of hepatobiliary disease, it even may lead to acute liver failure, and as the prevailing side effect of statins is hepatocellular injury (11). Fluvastatin can be considered as toxic substance causing hepatitis liver damage model in experimental animals, to enable more investigation of various drugs and supplement on damaged liver.

1.6. Hepatic impairment treatment

Chronic hepatitis is a serious global medical problem necessitating effective, inexpensive, and less toxic treatments (186). The rationales for treatment of chronic liver disease are to reduce inflammation, to prevent its progression to fibrosis, cirrhosis, and hepatocellular carcinoma, through the eradication of the insulting agent, e.g. alcohol, HBV and HCV (186).

The effectiveness of antiviral therapy for hepatitis is improving but there is still no effective therapy for evolving alcoholic cirrhosis (109). The only registered drug for viral hepatitis is interferon therapies, however they are already limited by their side effects, expense and route of administration, and they are not efficacious in all cases (172). Recently published reports document that more money is expended annually on alternative therapies than on prescription medicines, suggesting that patients believe that western medical approach is ineffective or that the alternatives are more effective (172).

Herbals have been used for centuries in China and other Far East countries, and recently they have become increasingly popular in western countries (191). The basis

for this increasing interest is the belief that herbals are natural products used for hundreds of years, and thus they must be effective and safe (156, 168). Their use is especially prevalent in persons with chronic diseases, and therefore the fact that those with chronic liver disease seek primary or adjunctive herbal treatment is not surprising (156). The most commonly used herbs in western countries by hepatitis patients were reported to be milk thistle (and its ingredient silymarin), St. John's wort, *Gingko biloba*, ginseng and Echinacea. However, there are some debates whether such herbs actually help patients or not, as Wong and Lee (195) stated that, no alternative or complementary remedy has been shown to affect HCV liver disease.

Herbal medicines for liver disease are also limited by several factors such as most of the herbal drugs lack of standardization, lack of identification of their active ingredients, lack of randomized controlled clinical trials, and lack of full toxicological evaluation. As herbal products are marketed as dietary supplements rather than drugs, they are not subjected to rigorous testing for safety and efficacy (34)

In this thesis, two examples of most popular herbs used by hepatitis patients are investigated in the new challenging experimental model of hepatitis. One is silymarin, the popular drug in western countries; the other is Schisandra derivatives that popular in eastern countries, and both clamed to be safe and useful due to their natural origin.

1.6.1. Silymarin

Silymarin is one of the most known hepatoprotective agent against induced hepatotoxicity due to its plant origin, oral effectiveness, easy availability at an affordable price, and most importantly good safety profile (181). It may prove superior to poly-herbal formulations for its better standardization, quality control and free from contamination from heavy metals and microbial toxins (142).

Silymarin is isolated from milk thistle (*Silybum marianum* L = *Cardus marianum* L*)* (Figure 12). This plant has large bright purple flowers, stout spines, and spiked leaves with white veins, hence the name "milk" thistle (58). The extract of the milk thistle plant has been used to treat chronic liver disease for thousands of years since the time of the ancient Greeks (119, 147). In Roman times, Pliny the Elder [A.D. 77], a noted naturalist, reported that milk thistle was "excellent for carrying off bile". In addition, Nicholas Culpeper [1650], the famous English Herbalist, wrote of silymarin effectiveness in removing obstruction of the liver and spleen (116).

Figure 12: Photo of *Silybum marianum* plant (71)

Milk Thistle is widely used in Europe (as presented in Figure 13), United States, and other countries as a ‘‘liver support’’ (40). In its native Mediterranean region, it has been used for liver ailments since the Greco-Roman era, and today it remains in folk use as a digestive aid, anti-inflammatory, hypotensive, and general tonifier (1). The German Commission currently recommends its use for dyspeptic complaints, toxin-induced liver damage, and hepatic cirrhosis and as a supportive therapy for chronic inflammatory liver conditions (63).

Figure 13: Distribution of milk thistle in European countries (in black) (4)

Silymarin is the most commonly used herbal product to treat a range of liver and gallbladder disorders, including hepatitis, cirrhosis, and jaundice, and to protect the liver against poisoning from toxins, including snakebites, insect stings, mushroom poisoning and alcohol (4, 50, 63, 147, 156).

Silymarin constituents

Silymarin is found in the entire plant, but it is concentrated in the fruit and seeds. The active complex in mile thistle is composed of flavonolignans –silybin (A & B),

isosilybin (A & B), silydianin, silychrstine, isosilychristin and one flavonoid (taxifolin). These chemicals form a complex known as silymarin, comprising up to 80% of milk thistle extract (96). Silybin A and B are the most abundant flavonolignans in silymarin and together comprise the fraction of silymarin known as silybinin (104). Figure 14 represents the most of the chemical constituent found in *Silybum marianum* plant.

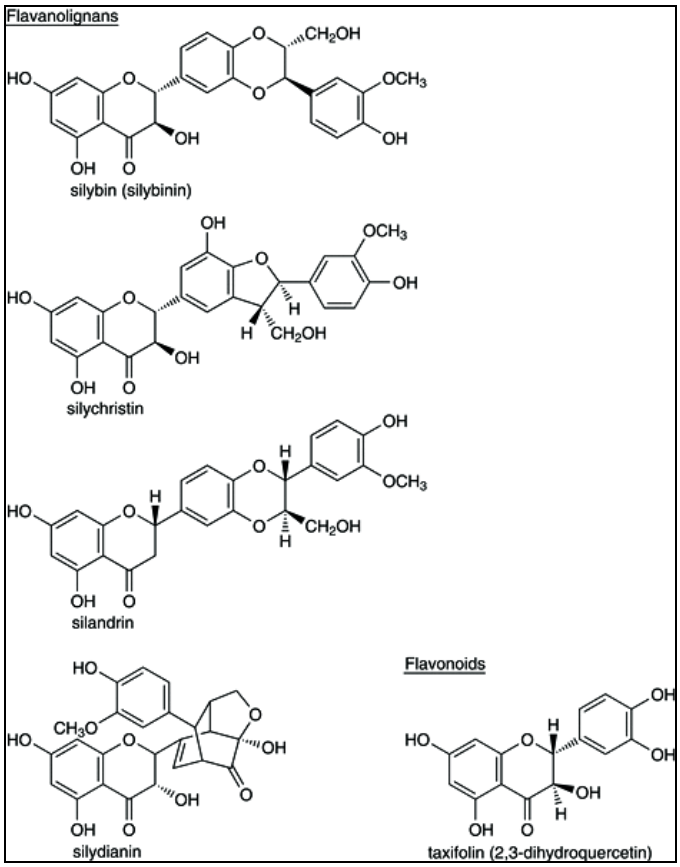


Figure 14: *Silybum marianum* active constituents (104)

Silymarin pharmacokinetic and toxicity

Silymarin has a short half-life, with rapid conjugation in the liver, and primary excretion in bile. It is absorbed in the intestine, concentrates in the bile, and probably has an enterohepatic circulation so that it continues to recycle through the liver. Results from rat and human studies indicate that the highest concentration is found in liver, suggesting a localization of silymarin effects to liver (198).

Silymarin has been described to be very well tolerated, even at high doses, as higher doses and/or more frequent oral dosing of silymarin currently available forms are more prescribed in order to control hepatic inflammation (122). Silymarin has shown to be generally non-toxic, the only contraindications are pregnancy and allergy to the daisy/composite family. Silymarin did not show side effects when administered to

adults in an oral dose range of 240–900 mg/day in two or three divided doses, or when administered in an oral dose 100 mg/kg/day to rats for 22 weeks (58). However, a laxative effect with an increased bile flow and secretion may appear at higher doses of more than 1500 mg/ day silymarin (4).

Silymarin mechanism of action

Hepatoprotective activity of silymarin has been demonstrated by various researchers from all over the world against partial hepatectomy, radiation, cold ischemia models, and toxicated models in experimental animals using acetaminophen, CCl_4, ethanol, D-galactosamine, iron overload, phenylhydrazine, and *Amanita phalloides* toxin (4, 40, 142). The hepatoprotective action of silymarin in fatal fulminant hepatic failure following *Amanita phalloides* (death cap) mushroom poisoning was noted in experimental animals and humans, where it showed higher efficiency when silymarin was given soon after exposure. Silymarin was also found to protect the liver tissues from injury caused by microcystin, halothane and alloxan (61, 97).

Several pharmacological studies have been carried out on the active components of milk thistle, silymarin and silybinin. It has been found that these substances exert hepatoprotective, antioxidant, anti-inflammatory and antifibrotic properties. In addition, they stimulate protein biosynthesis and liver regeneration, increase lactation and possess anti-viral, anti-proliferative and immuno-modulatory activity (5, 6, 45, 140, 178). Silymarin hepatoprotective is thought to work via more than one mechanism (40, 61, 97). One of proposed mechanisms is that silymarin inhibit the hepatotoxin binding to receptor sites on the hepatocyte membrane, regulating the membrane permeability, and increasing membrane stability. It can also interact directly with cell membrane components to prevent any abnormalities in the content of lipid fraction to maintain normal fluidity.

Another mechanism is that silymarin stimulate ribosomal RNA polymerase and subsequent protein synthesis with hepatocyte regeneration. Silymarin has a capacity to regulate the nuclear expression, by means of a steroid-like effect, essential for restoring the structural proteins and damaged enzymes. Silymarin also can inhibit the hepatic cytochrome P450 (CYP450) detoxification system; this effect could explain the hepatoprotective activities against *Amanita phalloides* intoxication, as the toxin becomes lethal for hepatocytes only after being activated by the CYP450 system. The most popular mechanism is that silymarin act as a free-radical scavenger and

antioxidant; and protect against lipid peroxidation. This result in silymarin ability to increase the cellular content of GSH, reduce the oxidation glutathione and induces superoxide dismutase to enhance its level in the liver and intestine. Silymarin also was reported to inhibit stellate cells transformation into myofibroblasts, which leads to cirrhosis formation. Moreover, silymarin has some modulating activity against the immune response (61).

Silymarin was reported to tend to normalize elevated aminotransferases (ALT and AST) levels (142). Silymarin also shown to improve other markers of liver function (GGT and bilirubin) in patients with liver disease of various etiology, including those exposed to toxic levels of toluene or xylene or solvents, paints, and glues that cause acute or chronic liver damage (58, 190).

Silymarin is favored prescribed drug among physicians to hepatitis patients, however there is still a debate concerning its efficiency. Dhiman and Chawla (34) do not support recommending this herbal compound for the treatment of liver disease, as silymarin did not reduce mortality nor improved the biochemistry and histology among patients with chronic liver disease. Moreover, Seeff et al. (156) reported that silymarin has no beneficial effect on serum ALT or HCV RNA levels. They noted that silymarin use was strongly correlated with patients' higher education, and data analysis showed fewer liver-related symptoms and better quality-of-life parameters in users. However, after reanalysis only fatigue, nausea, liver pain, anorexia, muscle and joint pain, and general health remained significantly better in silymarin users.

1.6.2. Dimethyl diphenyl bicarboxylate – DDB

Dimethyl diphenyl bicarboxylate (DDB) has been registered as liver support medication in China, and it is currently used for the treatment of acute and chronic HBV and HCV in far east countries and in other countries (2, 83, 169).

DDB is a synthetic mimic of the natural product "schisandrin C", a component of *Fructus Schizandrae* (103). Figure 15 represents the ripe and dried fruit of *Schizandrae* plant. *Schizandrae,* or Schisandra, is a plant that has been used in traditional Chinese and Japanese medicine, and there are several species, including *Schisandra chinensis*, which is native to Northeastern China and Korea (128).

Schisandra has long been used for a wide variety of conditions including asthma, dyspnea, cough, mouth dryness, spontaneous diaphoresis, nocturnal diaphoresis, nocturnal emission, insomnia, amnesia, dysentery, diarrhea, impotence and kidney

problems (159). In the past two decades, the crude drug prepared from schisandra has been developed as an alternative medicine for the treatment of various liver diseases; as it is claimed to protect the liver from injuries induced by hepatotoxin, improve liver function, and stimulate liver cell re-growth (67). These findings encouraged its use in human trials for the treatment of hepatitis.

Figure 15: Photo of ripe and dried *Fructus Schizandrae* (189)

Schisandra constituents

The fruit of *Schisandra chinensis* contains dibenzocyclooctadiene lignans, which reported to have a variety of pharmacological effects such as antioxidant, anti-inflammatory, hepatoprotective and anti-tumor effects (27, 64, 114, 121, 161, 164). In addition, these lignans are believed to be responsible for the effect of lowering the elevated liver aminotransferases in plasma, suppressing lipid peroxidation, and enhancing GSH mediated anti-oxidation (77, 78).

These dibenzocyclooctadiene lignans include schisandrin A, B and C, deoxyschisandrin, g-schisandrin, pseudo-g-schisandrin, isoschizandrin, gomisin A, gomisin N and wuweizisu C, schisandrol and schisandra esters (137). Although some of these components isolated from schisandra have interesting pharmacological activities such as schisandrin A, B and C, yet their content in the plant is very low and their total synthesis is very difficult. To develop new anti-hepatitis drugs, a number of analogues of schisandrin C, were synthesized and screened; and DDB is one of them (112). DDB chemical name is (dimethyl-4,4’-dimethoxy-5,6,5’,6’-dimethylene-dioxybiphenyl-2,2’-dicarboxylate), its empirical formula is $C_{20}H_{18}O_{10}$, and its molecular weight = 418.36.

Figure 16 shows the similarity between the chemical structure of synthetic drug “DDB” and the chemical structure of natural component “schizandrin c” that found in schisandra fruits.

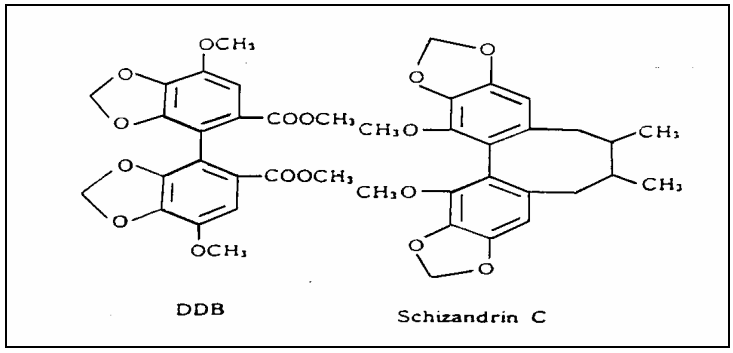


Figure 16: Chemical structure similarity of DDB and schisandrin C (108)

DDB is widely used for the treatment of chronic hepatitis, as in China. DDB has been tested clinically on patients with HBV and the results indicate that it markedly improves impaired liver functions, such as normalization of elevated serum ALT level. This was reported in most of patients after 1-3 months of treatment; moreover, it improved the abnormal bilirubin and serum albumin to globulin ratio, and relieved patients' symptoms (103, 113).

Many studies reported that DDB improve liver functions in various experimental models induced by e.g. CCl_4, D-galactosamine, thioacetamide, aflatoxin β1, erythromycin or prednisolone (2, 42, 113). Moreover, it improved liver functions in ethanol-induced humoral immunosuppression and dimethylnitrosamine-induced fibrogenesis (83, 89).

DDB mechanism of actions

The presence of hepatic enzymes, such as ALT and AST, in blood plasma is commonly used as an indirect index of hepatocellular damage. And numerous studies demonstrated that DDB reduce serum elevated ALT activity in animal models and in humans, thus DDB is considered by some authors a hepatoprotective drug. However, DDB did not improve liver histology, and the ALT normalization sometimes occurs only during the treatment period, and did not show a sustainable reducing affect on AST level (188). This creates a conflict whether it is a true hepatoprotective drug or it just masks the ALT elevated activity.

Although, the exact mechanism involved in the improvement of hepatic aminotransferases after DDB treatment is not clearly known, yet several researchers

suggested its hepatoprotective mechanisms, at same time others debate such proposed mechanisms. Some of the proposed mechanisms are that DDB has antioxidant action, anti-lipid peroxidation action, and anti-cytolysis action (42). However, contrary to these theories, Ip et al. (79) reported that, DDB did not protect from CCl_4 intoxication and did not enhance hepatic glutathione redox status and mitochondrial reduced glutathione activity.

Another mechanism depends on postulating that DDB induce CYP450 enzymes, thus increase the detoxification capability of liver, which may result in increase in the activity of glutathione peroxidase, glutathione reductase, and glutathione-S-transferase (203). Yet, Kim et al. (88) stated that DDB forms a metabolite-P450 complex, which selectively inhibits the CYP450 3A4 activity. Moreover, treatment with DDB was suggested to offer a protection by keeping the structural integrity of liver cell membrane against hepatotoxin challenge as a membrane-stabilizing agent (2, 69, 136).

Another mechanism suggested by Li (108) is that DDB has an anabolic action. After treatment with DDB, the protein metabolic processes of hepatitis patients were improved, with increase in serum albumin levels (2, 3, 38, 203). Another study shows that DDB increases liver protein and glycogen synthesis (203). However, Kang et al. (82) stated that DDB did not restore the lowered plasma proteins and albumin contents in dimethylnitrosamine treated rats. On the other hand, studies done by Fu and Liu (51) showed that, DDB would be beneficial to the repair of the damaged liver cells, because it increases the concentration of liver microsomal proteins, and increases liver free ribosomal proteins and RNA.

Other mechanisms were also suggested such as anti-carcinogenic action, and immuno-modulatory action (89, 169). Moreover, the increased hepatic blood flow rate by DDB might be one of its hepatoprotective mechanisms, as typically patients with chronic liver disease, such as liver cirrhosis and liver fibrosis showed decreased hepatic blood flow rate (83). However, DDB had not shown a significant improvement in most of the parameters expressing synthetic liver function in hepatitis C patients, although patients' claim of health improvement feeling (19).

2. Aim of the study

The aim of this thesis is divided into two parts; establish a new hepatitis experimental model, and investigate the effect of drugs used as hepatoprotective on liver cell function and death in challenging hepatitis experimental model.

In order to explore a new experimental model that mimics gradual progressive hepatitis injury, fluvastatin was used as hepatotoxin, as fluvastatin is usually well-tolerated chemical drug that cause liver toxic symptoms resembling hepatitis upon high doses or prolonged administration.

Fluvastatin was administered in gradual toxic dosages (25, 50, 75 and 100 mg/kg) for seven subsequent days. Such doses were used to obtain gradual hepatic damage, and to avoid complete or uncontrolled liver impairment. In addition, these fluvastatin doses were administered to easily available laboratory animals (Wistar rats), to make this model reproducible and easily maintained in various laboratories.

Since many drugs are used as alternative and complementary therapy for patients with chronic liver diseases, prescribed by the physicians or over counter, based on the claim that they are hepatoprotective, do not harm the liver and improve the general physical condition. In this thesis, two examples of such drugs (silymarin and DDB) were chosen to investigate more closely the hepatoprotective action in challenging hepatitis pattern and assess their protective effect on the hepatocytes damage on cellular and molecular basis.

In order to challenge these two popular hepatoprotective drugs to exert a hepatoprotective effect while liver is under stressful toxic progressive condition, these drugs were tested in healthy rats and rats intoxicated with gradual toxic doses of fluvastatin. Parameters of hepatocytes function such as hepatic enzymes, proteins, lipid profile and glucose levels, and markers of hepatocytes necrosis and apoptosis were observed, measured, and compared to normal group values and respective toxicated groups.

3. Materials and Methods

3.1. Animals

Ninety (90) male Wister rats (6 weeks old, 150–175 g body weight) were obtained from the Animal House, Faculty of Pharmacy, Jagiellonian university-collegium medicum (UJ-CM). The animal room has been maintained at 25-27^{o}C and suitable humidity conditions. Animals were housed in stainless-steel cages, and they were subjected to 12 hours light–dark cycle lights from 6:00 to 18:00. They had a free access to food (rats' standard chow diet) from 18:00 until 9:00, and a free access to water all the time**.**

All animals were handled and treated in compliance with UJ-CM Ethical Research Council guidelines, and the thesis research plane was approved by the Research Animal Care, Ethical Committee of UJ-CM. Animals were acclimatized for two days prior to the experiment, then they were randomly assigned to different groups.

3.2. Drugs and Dosages

The drugs used were obtained from international available drug stores, while reagents were obtained in analytical grade from the commercial analytical and chemical companies. Drugs were suspended in sterile distilled water, and they were given to fasted rats as a gastric gavage by oral needle between 17:00 and 18:00, when the daily wave of hepatic DNA synthesis was at its peak (62).

- Fluvastatin (Lescol 40mg, Novartis Pharmaceuticals, Poland) was administered daily to rats in four gradual oral doses of fluvastatin for 7 days (25, 50, 75 and 100 mg/kg/day).
- Silymarin (Legalon, MADAUS GmbH, Cologne, Germany) was administered daily with and without the previous stated fluvastatin toxic doses to rats in a fixed oral dose 140 mg/kg/day (180) for 7 days.
- Dimethyl diphenyl bicarboxylate (DDB, Beijing Union Pharmaceutical Factory, P.R. China) was administered daily with and without the previous stated fluvastatin toxic doses to rats in a fixed oral dose 100 mg/kg/day (188) for 7 days.

3.3. Study design

Animals were classified into four groups:

Group I	**Normal group**	**(NC)**	6 healthy rats received water for 7 days.
Group II	**Fluvastatin toxicated group**	24 rats divided into four subgroups each contains 6 rats, received for 7days:	
		(F-25)	25 mg/kg/day fluvastatin
		(F-50)	50 mg/kg/day fluvastatin
		(F-75)	75 mg/kg/day fluvastatin
		(F-100)	100 mg/kg/day fluvastatin
Group III-A	**Silymarin group**	6 rats, received for 7 days 140mg silymarin/kg/day	
Group III-B	**Silymarin and fluvastatin group**	24 rats divided into four subgroups each contains 6 rats, received for 7 days	
		(FS-25)	(25mg fluvastatin+140mg silymarin) /kg/day
		(FS-50)	(50mg fluvastatin+140mg silymarin) /kg/day
		(FS-75)	(75mg fluvastatin+140mg silymarin) /kg/day
		(FS-100)	(100mg fluvastatin+140mg silymarin) /kg/day
Group IV-A	**DDB group**	6 rats, received for 7 days 100mg DDB/kg/day	
Group IV	**DDB and fluvastatin group**	24 rats divided into four subgroups each contains 6 rats, received for 7 days	
		(FD-25)	(25mg fluvastatin + 100mg DDB) /kg/day
		(FD-50)	(50mg fluvastatin + 100mg DDB) /kg/day
		(FD-75)	(75mg fluvastatin + 100mg DDB) /kg/day
		(FD-100)	(100mg fluvastatin + 100mg DDB) /kg/day

3.4. Sample collection

After seventh day of experiment, fasted animals were anesthetized by intra-muscular injection of Ketamine hydrochloride salt overdose. Liver were rapidly collected, clamped frozen in liquid nitrogen, and stored at -80°C until analysis. Blood was collected by heart puncture, allowed to clot. Then, the serum was separated, divided in small amounts of samples, and stored at -80°C until analysis. Rats' body weight was recorded daily from the first to the last day of experiment. At the day of animal sacrifice, the weight of the liver and kidney were recorded before freezing the liver.

3.5. Liver biochemical tests:

3.5.1. Serum glucose

Serum glucose was determined colorimetrically according to the method of Trinder (179) using a BioMaxima kit (Cat. No. 1-033-0400, BioMaxima, Lublin, Poland). The quantification of glucose depended on enzymatic oxidation of glucose into gluconic acid. This oxidation was accompanied with hydrogen peroxide production, which combined with phenol and 4-aminoantipyrine forming colored complex. The resulting color is proportional at 500 nm to the amount of glucose present in serum.

Test reagent:

A glucose solution (5.55 mmol/l) is used as standard solution. Test reagent consisted of 250 mmol/l phosphate buffer (pH 7.5), >20 kU/l glucose oxidase, >1.5 kU/l peroxidase, 0.4 mmol/l 4-aminoantipyrine, 5 mmol/l phenol and 2 mmol/l ethylenediaminetetraacetic acid (EDTA).

Test procedure:

In two cuvettes, 10 µl of serum and 10 µl of standard were each mixed with 1 ml of test reagent. The optical density (OD) of the sample and standard were measured after 10 minutes incubation, at room temperature against a blank reagent at 500 nm.

Calculation:

The concentration of serum glucose was calculated according to the equation: Glucose (mmol/l) = (OD sample / OD standard) × standard concentration

3.5.2. Serum total protein:

Total protein concentration was determined according to the method of Gornall (60) using a Biolabo kit (Cat. No. 80016, Biolabo reagents, Maizy, France). The colorimetric quantification of serum total protein is based on the principle of Biuret reaction. The peptide bond of amino acids in protein react with copper II in an alkaline solution to yield cupric ions, those ions complex with sodium potassium tartarate forming blue color. This blue color intensity is proportional to total protein concentration, and measured at 550 nm.

Test reagents:

A bovine albumin solution (6 g/dl) is used as total protein standard solution. Biuret reagent is composed of 10 mmol/l sodium potassium tartarate, 3 mmol/l potassium iodide, 370 mmol/l sodium hydroxide and 3 mmol/l copper II sulphate.

Procedure:

In two cuvettes, 20 µl of serum was mixed with 1 ml of Biuret reagent in one cuvette, and with 1 ml saline in the other. In another cuvette, 20 µl of standard solution was mixed with 1 ml of Biuret reagent. The OD of the sample and standard were measured after 10 minutes incubation at room temperature against a blank reagent at 550 nm.

Calculation:

The concentration of serum total protein was calculated according to the equation:

Total protein (g/dl) = [(OD sample assay – OD sample blank) / (OD standard)] × standard concentration

3.5.3. Serum albumin quantity:

Albumin was determined according to the method of Doumas et al. (37) using a BioMaxima kit (Cat. No. 1-003-0200, BioMaxima, Lublin, Poland). The colorimetric quantification of serum albumin depends on the reaction between albumin (in a buffered solution) with bromocresol green. The resulting product has a blue green color that is proportional at 630 nm to the albumin concentration.

Test reagents:

A bovine albumin solution (4 g/dl) is used as albumin standard solution. Color reagent is composed of 0.15 mmol/l bromocresol green, and 75 mmol/l succinic acid.

Procedure:

In two cuvettes, 10 µl of serum and 10 µl of standard solution were each mixed with 1 ml of Color Reagent. The OD of the sample and standard were measured after 2 minutes incubation at room temperature against a blank reagent at 630 nm.

Calculation:

The concentration of serum albumin was calculated according to the equation:

Albumin (g/dl) = (OD sample assay / OD standard) × standard concentration

3.5.4. Serum total cholesterol:

Total cholesterol was determined according to the method of Allain et al. (9) using a BioMaxima kit (Cat. No. 1-023-0200, BioMaxima, Lublin, Poland). The colorimetric quantification of serum total cholesterol depends on the enzymatic hydrolysis of cholesterol esters by cholesterol esterase to cholesterol and fatty acid. Free

cholesterol is then oxidized by cholesterol oxidase into cholesterol-4-en-3-on, and hydrogen peroxide that combined with phenol and 4-aminoantipyrine forming colored complex. The resulting color is proportional at 550 nm to the amount of total cholesterol present in serum.

Test reagent:

A cholesterol solution (5.2 mmol/l) is used as standard solution. The reagent is composed of 50 mmol/l 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.4), >0.4 kU/l cholesterol esterase, >0.1 kU/l cholesterol oxidase, >1.5 kU/l peroxidase, 4 mmol/l phenol, 0.3 mmol/l 4-aminoantipyrine, 25 mmol/l magnesium sulphate, and 7 mmol/l sodium cholate.

Procedure:

In two cuvettes, 10 µl of serum and 10 µl of standard were each mixed with 1 ml of test reagent. The OD of the sample and standard were measured after 10 minutes incubation at room temperature against a blank reagent at 550 nm.

Calculation:

The concentration of serum cholesterol was calculated according to the equation: Total cholesterol (mmol/l) = (OD sample / OD standard) × standard concentration

3.5.5. Serum triglycerides:

Triglyceride was determined according to the method of Fossati and Prencipe (49) using a BioMaxima kit (Cat. No. 1-053-0200, BioMaxima, Lublin, Poland). The colorimetric quantification of serum triglyceride depends on the enzymatic hydrolysis of triglyceride by lipoprotein lipase, producing glycerol and fatty acid. Glycerol was phosphorelated with ATP and glycerol kinase. The produced glycerol-3-phosphatase is then oxidized by glycerol kinase to produce dihydroxyacetone phosphate and hydrogen peroxide. Hydrogen peroxide is combined with 4-chlorophenol and 4-aminoantipyrine forming colored complex. The resulting color is proportional at 550 nm to the amount of triglyceride present in serum.

Test reagent:

A triglyceride solution (2.28 mmol/l) is used as standard solution. Reagent is composed of 50 mmol/l piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.0), >5.6 kU/l lipoprotein lipase, >0.64 kU/l glycerol kinase, >1.6 kU/l peroxidase, >3.2 kU/l glycerol phosphate oxidaze, 2 mmol/l ATP, 2.7 mmol/l 4-chlorophenol, and 0.4 mmol/l 4-aminoantipyrine.

Procedure:

In two cuvettes, 10 µl of serum and 10 µl of standard were each mixed with 1 ml of test reagent. The OD of the sample and standard were measured after 10 minutes incubation at room temperature against a blank reagent at 550 nm.

Calculation:

The concentration of serum triglyceride was calculated according to the equation: Triglyceride (mmol/l) = (OD sample / OD standard) × standard concentration

3.5.6. Serum High Density Lipoprotein (HDL) fraction:

HDL fraction was determined, according to the method of Burstein et al. (21), by separating HDL cholesterol fraction from other cholesterol fractions and then measure cholesterol concentration using a BioMaxima kit (Cat. No. 1-029-0200, BioMaxima, Lublin, Poland). The mixture (phosphotungstic acid and magnesium chloride) precipitate chylomicrons, LDL and VLDL fractions, and leave HDL cholesterol fraction soluble in supernatant. Then, the colorimetric quantification of serum HDL cholesterol fraction proceeded as total cholesterol measurement until a colored complex is formed. The resulting color is proportional at 550 nm to the amount of HDL cholesterol fraction present in serum.

Test reagents:

A HDL solution (1.3 mmol/l) is used as standard solution. Precipitating reagent is composed 1.0 mmol/l phosphotungstic acid, 50 mmol/l magnesium chloride, and 6.94 mmol/l sodium benzoate. Cholesterol reagent is composed of 50 mmol/l MES buffer (pH 6.4), >0.4 kU/l cholesterol esterase, >0.1 kU/l cholesterol oxidase, >1.5 kU/l peroxidase, 4 mmol/l phenol, 0.3 mmol/l 4-aminoantipyrine, 25 mmol/l magnesium sulphate, and 7 mmol/l sodium cholate.

Procedure:

In two vials, 500 µl of serum and 500 µl of standard were each mixed with 500 µl of Precipitating reagent. After they were let to stand for 20 minutes in room temperature, mixtures were centrifuged at 4000 ×g. The carefully collected supernatant was then subjected to the previous method of total cholesterol measurement, where 50 µl of sample and standard supernatant were mixed in two cuvettes with 1 ml of cholesterol reagent. After 10 minutes incubation at room temperature, the OD of the sample and standard were measured against a blank at 550 nm.

Calculation:

The concentration of serum HDL was calculated according to the equation: HDL (mmol/l) = (OD sample / OD standard) × standard concentration.

3.5.6. Serum aminotransferases activities

Both aminotransferases (ALT and AST) were determined colorimetrically according to the method described by Reitman and Frankel (148).

3.5.6.1. Serum alanine aminotransferase (ALT)

ALT [E.C. 2.6.1.2.] activity was determined using Biolabo kit (Cat. No. 92027, Biolabo reagents, Maizy, France) according to the reaction equation:

The ALT catalyses the transfer of an amino group from alanine to 2-oxoglutarate, producing a pyruvate molecule. The produced pyruvate reacts with 2,4-dinitrophenyl hydrazine in an alkaline medium to produce 2,4-dinitrophenyl hydrazone, which has brown color. The color intensity has absorbance at 505 nm, which is proportional to the ALT activity in the reaction mixture.

Test reagents:

Substrate solution contains 2 mmol/l 2-oxoglutarate, 200 mmol/l L-alanine and 100 mmol/l phosphate buffer (pH 7.5). Color reagent contains 1 mmol/l 2,4-dinitrophenyl hydrazine and 1 mol/l hydrochloric acid (HCL). Alkaline solution consists of 0.4 mol/l sodium hydroxide (NaOH).

Procedure:

After reagents and serum were let to warm in room temperature, 1 ml of substrate solution was incubated for 5 minutes at 37^{o}C in waterbath. Then, 200 µl of serum was added, mixed and incubated at 37^{o}C in waterbath. After 30 minutes, 1 ml of the color reagent was added, and the color was allowed to develop at room temperature for 20 minutes at room temperature before adding 5 ml of alkaline solution. The OD of the resultant color was measured at 505 nm against reagent blank after 5 minutes.

Calculation:

The ALT activity of each sample was calculated as International Units (IU)/l from the standard curve. This standard curve was plotted by the use of standards provided within the kit (standard units against their absorbance at 505nm). Figure 17 represent the ALT standard curve used in this study.

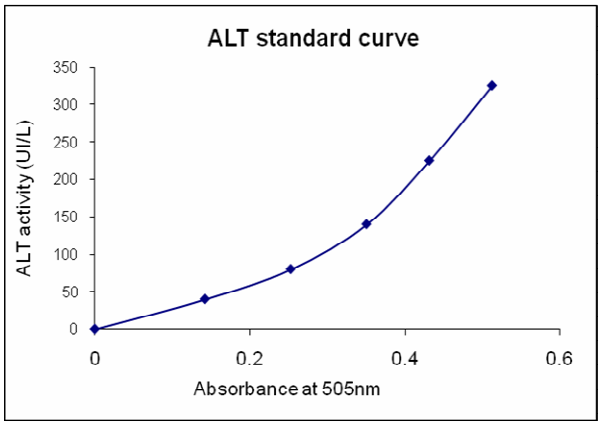


Figure 17: ALT standard curve

3.5.6.2. Serum aspartate aminotransferase (AST)

AST [E.C. 2.6.1.1.] activity was determined using Biolabo kit (Cat. No. 92025, Biolabo reagents, Maizy, France). The colorimetric determination of serum AST activity was done according to the reaction equation:

2-oxoglutarate + L-aspartate $\xrightleftharpoons{\text{AST}}$ L-glutamate + oxaloacetates

The AST catalyses the transfer of an amino group from aspartate to 2-oxoglutarate, producing an oxaloacetate molecule. The produced oxaloacetate reacts with 2,4-dinitrophenyl hydrazine in an alkaline medium to produce 2,4-dinitrophenyl hydrazone, which has brown color. This color intensity has absorbance at 505 nm, which is proportional to the AST activity in the reaction mixture.

Test reagents:

Substrate solution contains 2 mmol/l 2-oxoglutarate, 200 mmol/l L-aspartate and 85 mmol/l phosphate buffer (pH 7.5). Color reagent contains 1 mmol/l 2,4-dinitrophenyl hydrazine and 1 mol/l HCL. Alkaline solution consist of 0.4 mol/l NaOH.

Procedure:

After reagents and serum were let to stand in room temperature, 1 ml of substrate solution was incubated for 5 minutes at 37^{o}C in waterbath. Then, 200 µl of serum was added, mixed and incubated at 37^{o}C in waterbath. After 60 minutes, 1 ml of the color reagent was added, and the color was allowed to develop at room temperature for

20 minutes at room temperature before adding of 5 ml of NaOH solution. The intensity of the resultant color was determined at 505 nm against reagent blank after 5 minutes.

Calculation:

The AST activity for each sample was calculated as IU/l from the standard curve. This standard curve was plotted by the use of standards provided within the kit. (standard units against their absorbance at 505nm). Figure 18 represent the AST standard curve used in this study.

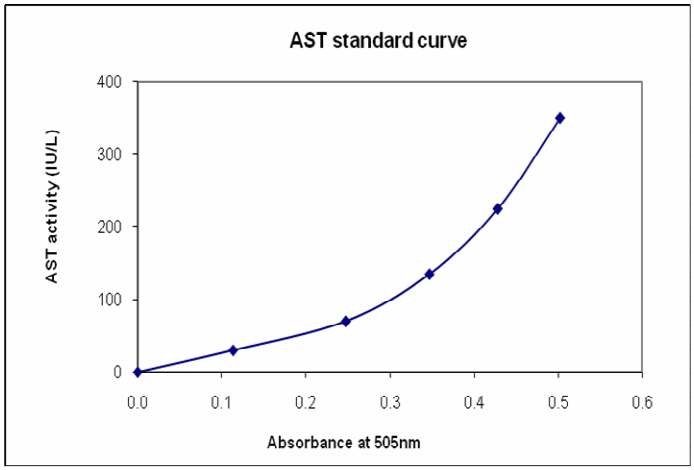


Figure 18: AST standard curve

3.5.7. Serum gamma-glutamyltransferase (GGT)

GGT [E.C. 2.3.2.2.] activity was determined kinetically according to the method described by Szasz (171) using BioMaxima kit (Cat. No. 1-228-0060, BioMaxima, Lublin, Poland). The kinetic determination of GGT activity was done according to the reaction equation:

L-γ-glutamyl-3-carboxy-4-nitroanilid + glycylglycine $\xrightarrow{\text{GGT}}$ L-γ-glutamyl-glycylglycine + 5-amino-2-nitrobenzoate

The GGT catalyses the transfer of γ-glutamyl group from γ-glutamyl-3-carboxy-4-nitroanilid to glycylglycin. The increase of absorbance at 405 nm due to yellow color of 5-amino-2-nitrobenzoate is proportional to the GGT activity in the reaction.

Test reagents:

Test Solution reagent contains 138 mmol/l glycylglycine, and 138 mmol/l TRIS buffer (pH 8.25), 22 mmol/l L-γ-glutamyl-3-carboxy-4-nitroanilide, and 20 mmol/l MES buffer (pH 6).

Procedure:

To 1 ml of Test Solution, 100 µl of serum was added, mixed in cuvette. The cuvette was incubated in 30^{o}C in thermostat, and after 1 minute, the initial absorbance at 405nm against air was recorded. The absorbance readings were repeated at 60 seconds intervals for 3 minutes. Then, the average absorbance change per minute (ΔA/min) was calculated.

Calculation:

The GGT activity was calculated based on the following formula:

GGT activity (U/l) = $(Vt \times 10^5) / (\varepsilon \times l \times Vs) \times \Delta A/min$

Vt – Total reaction volume = 1.10 ml

l – Path-length of the cuvette = 1 cm

ε – Molar absorbance of 5-amino-2-nitrobenzoate at 405 nm = 950 m^2/mol

Vs – Sample volume = 0.10 ml

GGT activity (U/l) = $(1.10 \times 10^5) / (950 \times 1 \times 0.10) \times \Delta A/min$

= 1158 × ΔA/min

3.6. Hepatic apoptotic tests

3.6.1. Hepatic samples preparation:

A whole cell protein extraction buffer was prepared according to Le Minh et al. (102) for caspase 3 and cytochrome c extraction. Liver tissue was homogenized by a Dounce homogenizer, in 1 ml lysis buffer containing 10 mmol/l Tris (pH 7.5), 10 mmol/l NaCl, 1 mmol/l EDTA, 0.5% Triton X-100, and 0.2 mmol/l phenylmethyl sulfonyl fluoride (PMSF). Then, extract was incubated for 30 minutes on ice. Before homogenization, a freshly prepared protease inhibitor cocktail containing 0.5 mg leupeptin, 1 mmol/l dichlorodiphenyltrichloroethane (DDT), 1 mmol/l ethylene glycol tetraacetic acid (EGTA) and 0.5 mg trypsin inhibitor I, was added to the buffer. The homogenate was centrifuged at 10000 ×g for 15 minutes at 4^{o}C. The supernatant was saved as whole protein fraction of hepatic tissue.

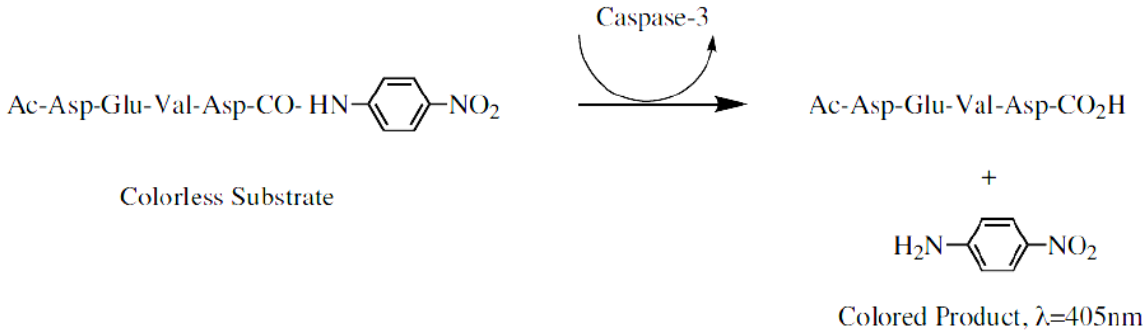
Low salt radioimmunoprecipitation assay (RIPA) buffer was prepared for p53 protein preparation according to p53 pan enzyme linked immunosorbent assay (ELISA) kit (Roche Diagnostic) with some modification. Liver extracts were prepared by detergent lysis containing 20 mmol/l Tris, 0.5 mmol/l EDTA, 0.1% Triton X-100, 0.5% sodium deoxycholate, and 0.05% sodium dodecyl sulfate (SDS). Before

homogenization, a freshly prepared 2 µg/ml Leupeptin and 1 mmol/l PMSF was added to the buffer. While, samples were placed on ice, 1 volume of RIPA buffer was added to liver tissue. Using a Dounce homogenizer, liver tissue was homogenized. Then, extracts were centrifuged at 10000 ×g for 10 minutes at 4°C. The supernatant was used to estimate hepatic p53 concentration.

Protein concentrations of liver extracted samples were determined using the Biuret protein assay (Biolabo kit, Cat. No. 80016, Biolabo reagents, Maizy, France) with bovine serum albumin (6 g/dl) as standard.

3.6.2. Hepatic caspase-3 activity:

Caspase-3 was determined colorimetrically using microplate reader according to the method described in Assay designs/Stressgen kit (Cat. No. 907-013, Assay designs, USA). The colorimetric determination of liver caspase-3 activity was conducted according to the following reaction.



Active Caspase-3 enzyme which is present in liver homogenate, cleaves the colorless substrate of acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-p-NA), to release yellow p-nitroanilide (p-NA) that can be measured by absorbance at 405 nm. One unit of caspase-3 activity was defined as the amount of enzyme needed to convert 1ρmol of colorimetric substrate per minute at 30°C.

Test reagents:

Standard reagents were consisting of serial concentration of desiccated caspase-3 enzyme. Calibrator reagent contains a solution of 50 µmol/l p-NA in Caspase-3 Reaction Buffer. Active Caspase-3 Reaction Buffer contains 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer freshly mixed with desiccated dithiothreitol (DTT). Caspase-3 Colorimetric substrate solution was mixed freshly in active Reaction Buffer. Stop Solution consists of 1 mol/l HCl.

Assay Procedure

Standards, blank, calibrator and samples were assayed in duplicate in clean half-area Microtiter Plate. Blank wells were filled with 50 µl of Active Caspase-3 Reaction Buffer, while calibrator wells were filled with 125 µl of p-NA Calibrator. Fifty µl of Standards (1000, 500, 250, 125 and 62.5 U/ml), and 50 µl of liver homogenate samples, equivalent to 100 µg protein, were pipetted into empty wells.

Into each well, except the p-NA Calibrator wells, 75 µl of Caspase Colorimetric Substrate was added. The plate was mixed gently, covered with a sealer, and then incubated for 3 hours at 37°C. At the end of the 3 hours, a 10 µl of Stop Solution was added into each well, including the p-NA Calibrator wells. The OD of microplate content was read at 405 nm, against the blank.

Calculation:

The concentration of caspase-3 enzyme in standard solutions was determined by the amount of p-NA released, through correlation with p-NA calibrator OD. The actual concentration of active caspase-3 standard was quantified by comparison of the OD of standards with OD of p-NA calibrator. To calculate the concentration of active caspase-3 in the samples, the average OD of each standard and sample was subtracted from blank. Then, the activity of caspase-3 in samples was obtained from standard curve.

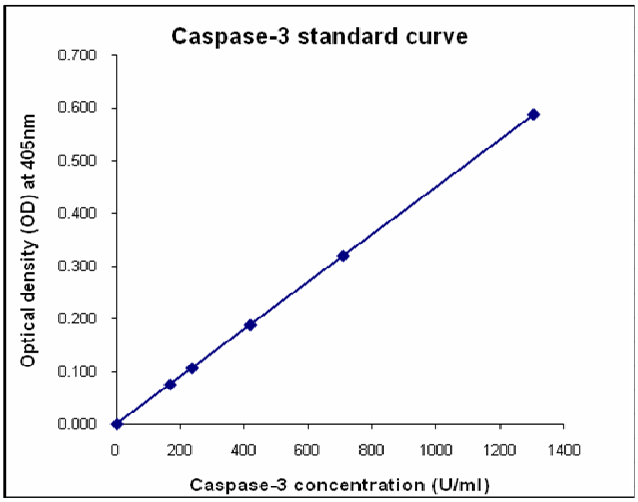


Figure 19: Caspase-3 standard curve

The standard curve plotted the average OD for each standard versus actual concentration of active Caspase-3 for the standards. Figure 19 represents the caspase 3 standard curve used in this study. The concentration of active Caspase-3 in the samples can be determined by interpolation.

3.6.3. Hepatic cytochrome c:

For the quantification of cytochrome c concentration in whole liver cells extract, a photometric two-steps-enzyme immunoassay was used. The results were read by microplate reader according to the method of Cytochrome c Protein Quantity Microplate Assay MitoSciences kit (Cat. No. MSA41, MitoSciences, Oregon, USA).

The assay is based on a quantitative sandwich ELISA principle, where cytochrome c is immune-captured within the microplate wells, and its concentration is determined by adding a cytochrome c specific antibody conjugated with horseradish peroxidase (HRP), where peroxidase changes the coloring substrate from colorless to blue.

Test reagents:

The kit contains a 96-well microplate with a monoclonal antibody pre-bound to the wells of the microplate. It also contains the required reagents; sample buffer, detergent, blocking solution, wash buffer, detector antibody, development solution, and HRP label solution.

Sample Preparation

Liver whole cell protein extracts were re-suspended in sample buffer to the same concentration (1 mg/ml). One tenth volume of detergent was added and mixed with samples, and then 9 volumes of blocking solution were added to each sample. Samples were centrifuged at 20000 ×g for 20 minutes. The supernatant was then carefully collected, and used as sample for the test.

Assay Procedure

Buffer control (200 µl of blocking solution) was used as a background reference, and 200 µl of each diluted sample was added to each well. The microplate was then covered and incubated for 3 hours at room temperature. The bound monoclonal antibody has immobilized the cytochrome c in the wells. Emptying the wells, by quickly turning the plate upside down and shaking out any remaining liquid, then each well was washed by 300 µl of washing solution. Detection solution (200 µl) was added to each well, and the plate was covered once again and incubated for 1 hour at room temperature. Again, the wells of the plate were emptied, and washed each with 300 µl of washing solution. The wells again were emptied, and then 200 µl of HRP Label solution were added to each well. Then, the plate was covered and incubated for 1 hour at room temperature. The wells were emptied again, and washed 5 times by adding 300 µl of washing solution to each well. The wells were then emptied and 200 µl of development solution was added to each well. The reaction was stopped after 10 minutes by the

addition of 100 µl of 1.5 mol/l HCl to each well and then measure the endpoint OD at 450 nm. The quantity of cytochrome c was expressed as µg/ml.

3.6.4. Hepatic p53:

For the quantification of p53 in liver cell homogenates, a photometric one-step-enzyme- immunoassay was used. The results were read by microplate reader according to the method of p53 pan ELISA kit (Cat. No. 11 828 789 001, Roche Diagnostics, Warsaw, Poland).

The assay is based on a quantitative sandwich ELISA principle (as shown in Figure 20). The biotin-labeled capture antibody is pre-bound to the streptavidin-coated Microtiter plate. In one single incubation step, the p53-containing suspension (sample or standard) reacts with capture antibody and peroxidase-labeled detection antibody to form a stable immuno-complex. The peroxidase bound in the complex react with tetramethylbenzidine (TMB) as color substrate developing a color, which can be measured at 450 nm. This photometrically determined color is proportional to the concentration of p53.

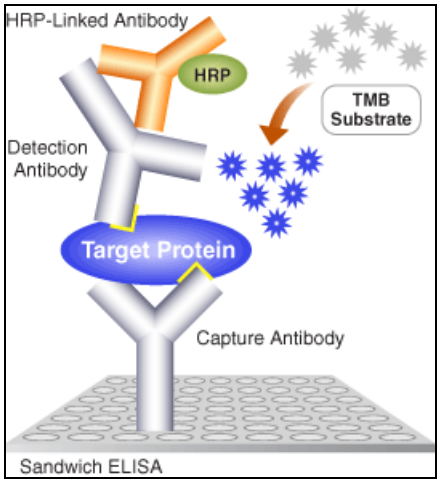


Figure 20: Sandwich ELISA (one-step-enzyme- immunoassay) (39)

Test reagents:

Streptavidin coated Microtiter plate was pre-coated with monoclonal from mouse anti-p53-biotin. The kit contains polyclonal anti-human-p53 antibody from sheep labeled with pan-peroxidase, human p53 standards (0.883 pg/ml), incubation buffer, washing buffer, TMB color substrate solution, and Stop solution.

Assay Procedure

In duplicate, 100 µl of standards and samples diluted in incubation buffer were pipetted very carefully into the wells coated with anti-p53-biotin (capture antibody).

Then, 100 µl of anti-p53-pan-peroxidase (detection antibody linked with HRP enzyme) were added to all wells. The plate was then covered, and incubated for 2 hours at 15-25°C on a 300 rpm shaker. After 2 hours, well content was removed thoroughly by suction. Wells were rinsed five times with 300 µl washing buffer, and then washing solution was carefully removed. In order to develop a color, 200 µl of the photometric TMB substrate solution were added into the wells. Then again, the plate was covered tightly and incubated 20 minutes at room temperature on a shaker at 300 rpm, protected from light, thus the developed color is sufficient for photometric detection at 450 nm within 5 minutes. Before reading the OD of developed color, a 50 µl of stop solution was added to each well. Then, the plate was incubated for 1 minute at room temperature on the shaker at 300rpm.

Calculation:

The average of the double OD readings from standards/samples was calculated. Then, a standard curve was constructed by plotting the average OD values of standards versus the p53 standard concentrations.

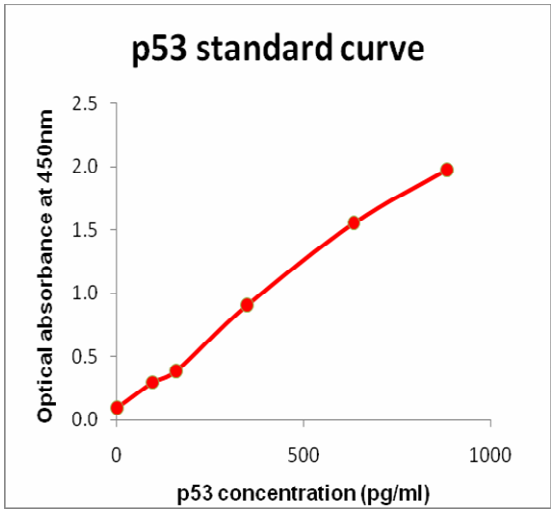


Figure 21: p53 standard curve

Sample concentrations then was determined from the p53 standard curve. Figure 21 represents the p53 standard curve that was used in this study. Then, sample p53 concentration was divided on sample protein concentration, and results were expressed as p53 pg/mg protein.

3.6.5. Hepatic DNA electrophoresis:

DNA fragmentation assay

Hepatic DNA was isolated and purified by AxyPrep Multisource Genomic DNA Miniprep Kit (Cat No. AP-MN-MS-GDNA-50, Axygen Biosciences, Union City, USA). The isolation employed a special kit's lysis buffer and proteinase K to release genomic DNA efficiently from other biologic materials. Contaminating proteins, pigments, carbohydrates and lipids were separated from the genomic DNA by unique two–phase partition. Then the free genomic DNA was bound to a special AxyPrep column, where residual impurities and salt were removed. After washing and desalting, the purified DNA was eluted in a Tris buffer.

Frozen liver tissue (20 mg) was homogenized by a mortar and pestle in liquid nitrogen and 650 µl lysis buffer and 0.9 µl of ribonuclease A (RNase A) was added during homogenization. Then, the homogenate was collected and incubated at 65°C for 5 minutes. Samples were then centrifuged at 12000 ×g for 2 minutes to clarify the cell homogenate. To the supernatant, 400 µl phase-partition buffer (pre-chilled to 4^{o}C) was added twice, mixed by vortex at top speed for 30 seconds, then centrifuged at 12000 ×g for 2 minutes.

The blue upper phase was then aspirated off as much as possible, while the colorless clear lower phase (containing DNA) was transferred to a Spin-filter in a 2 ml Microfuge tube and centrifuged for 1 minute at 12000 ×g. Then, the filter discarded and 400 µl of DNA binding buffer was added to the flow-through, mixed by repeated inversion. The whole binding mixture was then transferred to Miniprep column, and then centrifuged at 12000 ×g for 1 minute. By this step, DNA was entrapped inside Miniprep column.

The filtrate was discarded, and then the columns were washed by 500 µl of Wash buffer and centrifuged at 12000 ×g for 1 minute. Then, columns were washed again twice by 700 µl alcoholic desalting buffer and centrifuged at 12000 ×g for 1 minute. In order to elute purified samples' DNA, the Miniprep columns were transferred into a clean tube, and 100 µl of pre-warmed 2.5 mmol/l Tris-HCl (pH 8.5) buffer was added. After 1 minute stand at room temperature, sample columns were centrifuged for 1 minute at 12000 ×g to collect DNA content.

Measurement of DNA concentration

The DNA concentration was measured by spectrophotometric estimation, as nitrogenous bases absorb UV light, the more concentrated the DNA solution, the more UV light it will absorb. The samples were diluted (1:50) in diethylpyrocarbonate (DEPC) water in specific cuvettes, then the optical absorbance at 260 nm was measured against blank (DEPC water).

The concentration of pure double-stranded DNA with an optical absorbance at 260 nm of 1.0 is 50 mg/ml. In addition, the ratio of readings at 260 nm and 280 nm provides an estimate of purity of the nucleic acid to avoid contamination with protein or phenol, where DNA absorbance at 260 / absorbance at 280 = 1.8 ± 0.15. The following formula is used to determine the DNA concentration of a solution:

DNA concentration (mg/ml) = 50 mg/ml × OD at 260 nm × dilution factor

3.6.6. DNA electrophoresis and DNA ladder imaging

The purified DNA was loaded onto a 2% agarose gel in 0.5% TBE buffer (89 mM Tris, 80 mM boric acid, and 0.2 mM EDTA, pH 8.0). The gel with DNA fragments was stained with ethidium bromide after electrophoresis (180 V / 90 mA, 45minutes) with a 100-1000 bp standard DNA ladder maker (Invitrogen, CA, USA). A picture of gel was taken under UV light to image the DNA migration and ladder formation.

3.7. Statistical analysis

Collected and calculated data were coded and verified, prior to computer data entry. Grubb's test was used to identify the outliers (14). Descriptive statistics were applied; mean, standard deviation (SD) and standard error of the mean (SEM). Test of homogeneity was done using F test for equal variances. Tests of significance were applied to test null hypothesis, student t test for homogenous data, while Mann-Whitney test for non-homogenous data.

Groups were compared to normal group values. The *p* value of each group difference to normal group was listed in tables together with mean ± SEM values. Groups were presented as box and whiskers: min to max, which gives indication of data mean, median and distribution. Correlations were analyzed by Spearman rank correlation test, only significant correlation was presented in table, in form of Spearman r value and *p* value. A *p* value of less than 0.05 was considered statistically significant.

Statistical analysis and Graphic presentation were performed using the statistical program GraphPad Prism 5.

3.8. Instruments

Instruments used were compact shaker (Lab Dancer, IKA, Germany), benchtop pH meter (SevenEasy pH, Mettler Toledo, Switzerland), UV-VIS spectrophotometer (Varian CARY-100 BIO, Maryland, United States), thermostat (TS-92, EMCO, Poland), waterbath (MLL147, AJL electronic, Poland), analytical balance (RADWAG WPA 60/k, Radom, Poland), microplate reader (HumaReader HS Microtiter Plate Reader, Human GmbH, Germany), microplate washer (Humawash, human GmbH, Germany), high speed brushless centrifuge (MPW-350R, MPW Med Instruments, Poland), waterbath shaker (type 357, ELPIN+, Poland), homogenizer (Ultra-Turrax T18 basic IKA, Germany), and -80^{o}C deep freezer (HERAfreeze, Heraeus, Germany).

In addition, for DNA electrophoresis: UV-photometer (Biometra Gene Ray, Göttingen, Germany), gel casting and electrophoresis apparatus (Bio-Rad, Richmond, CA), benchtop UV transilluminator (Herola UVT - 14 M), photocamera with dark chamber, and Zoom Browser EX program were used.

4. Results

4.1. Body and organs weight

This study was done on 90 male Waster rats. Six rats were administered filtered tap water for seven subsequent days, and they were considered a normal control group (NC). The remaining 84 rats (6 rats per group) were subjected to fluvastatin toxic doses ranging from 25 to 100 mg/kg/day for 7 days, with and without simultaneous treatment by silymarin (140 mg/kg/day) or DDB (100 mg/kg/day). Animals' body weight was recorded from the first day of the experiment to the day they were sacrificed (the 7^{th} day). Liver and kidneys weight were also recorded at time of scarification, and their weight ratio to body weight was calculated.

Animals' survival rate was 100% in all groups, except in highly toxicated group with 100 mg/kg/day fluvastatin without treatment and with silymarin was 6/8 (75%).

The results of groups III-A (rats received only silymarin) and IV-A (rats received only DDB) did not show any significant difference from normal control group, thus they were not presented in the results.

4.1.1 Body weight difference

Although animals were freely admitted to standard chew diet for 7 days, rats receiving fluvastatin toxic doses had showed a negative effect on rats' body weight starting from the smallest toxic dose. In F-25 rats, the body weight gain had decreased insignificantly by 37%, when compared to normal group. Rats started progressively to loss body weight significantly instead of gaining, as the fluvastatin dose increased to 50, 75 and 100 mg/kg/day. (Table 1a and Figure 21 A)

Table 1-a: Rats' body weight difference in normal group, and groups toxicated with gradual doses of fluvastatin for 7 days.

Animal Groups		**Fluvastatin dose for 7 days**	**The body weight difference (g) Mean ± SEM**	**Significance *(p value)* versus NC**
Normal group	**NC**	0 mg/kg/day	**+27.67 ± 2.28**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**+17.50 ± 3.82**	ns (0.0649)
	F-50	50 mg/kg/day	**-3.67 ± 5.49**	***(0.0064)**
	F-75	75 mg/kg/day	**-14.00 ± 6.80**	*** (0.0022)**
	F-100	100 mg/kg/day	**-21.83 ± 2.96**	*** (0.0022)**

***: Significant difference from normal control. Ns: No significant difference from normal control**

When silymarin was given simultaneously with fluvastatin toxic doses, the weight gain in FS-25 rats was 49% of normal, and it was less than the weight gain recorded in

F-25 rats by 14%. In FS-50 rats, the weight gain was only 9% of normal, but it was still weight gain rather than weight loss as recorded in F-50 group. Notably, the weight loss in highly toxicated groups FS-75 and FS-100 was more pronounced than in animals receiving the same fluvastatin toxic doses alone. (Table 1 a,b and Figure 21 B-E)

Table 1-b: Rats' body weight difference in normal group, and groups received 140mg/kg/day silymarin with gradual doses of fluvastatin for 7 days.

Animal Groups		Fluvastatin dose for 7 days	The body weight difference (g) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**+27.67 ± 2.28**	
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**+13.67± 4.40**	***(0.0247)**
	FS-50	50 mg/kg/day	**+2.67± 5.94**	***(0.0022)**
	FS-75	75 mg/kg/day	**-20.83 ± 2.36**	***(0.0022)**
	FS-100	100 mg/kg/day	**-23.00 ± 6.82**	***(0.0022)**

*** Significant difference from normal control. ns No significant difference from normal control**

The Chinese drug, DDB, had showed effect that is more protective in low fluvastatin toxic doses than silymarin. The weight gain, in FD-25 rats was 61% of normal group, nearly similar to the weight gain in F-25 rats. In FD-50 rats, the weight gain was 19% of normal, but it still was weight gain rather than weight loss as recorded in F-50 group and was twice the weight gain in FS-50. The weight loss in highly toxicated groups (FD-75 and FD-100) was less pronounced, when compared to animals receiving fluvastatin toxic doses alone or even animals receiving silymarin with fluvastatin toxic doses. (Table 1 a,b,c and Figure 21 B-E)

Table 1-c: Rats' body weight difference in normal group, and groups received 100mg/kg/day DDB with gradual doses of fluvastatin for 7 days.

Animal Groups		Fluvastatin dose for 7 days	The body weight difference (g) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**+27.67 ± 2.28**	
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**+16.83 ± 3.56**	***(0.0370)**
	FD-50	50 mg/kg/day	**+5.17 ± 3.86**	***(0.0022)**
	FD-75	75 mg/kg/day	**-6.00 ± 7.22**	***(0.0022)**
	FD-100	100 mg/kg/day	**-9.67 ± 6.31**	***(0.0022)**

*** Significant difference from normal control. ns No significant difference from normal control**

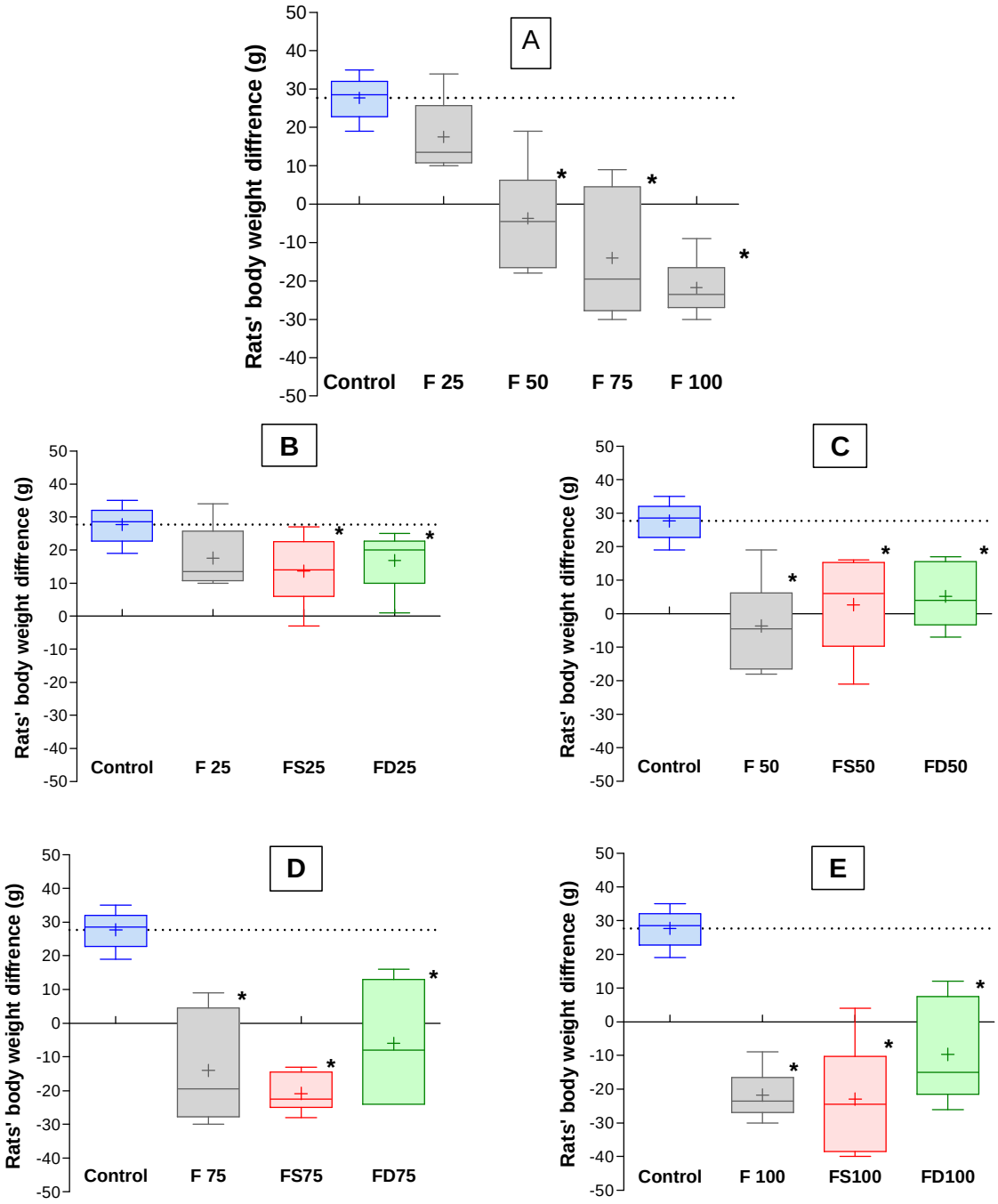


Figure 22: Rats' body weight diffrence
A) in control group vs. fluvastatin toxicated with gradual doses groups, and in control group vs. groups toxicated for 7 days with B) 25, C) 50, D) 75, E) 100 mg/kg/day fluvastatin, without and with treatment (silymarin and DDB).

F X = rats received X mg/kg/day fluvastatin for 7 days
FS X = rats received for 7 days X mg/kg/day fluvastatin +140 mg/kg silymarin,
FD X = rats received for 7 days X mg/kg/day fluvastatin + 100mg/kg DDB
*Significant difference when compared to normal group

4.1.2 Liver weight ratio

Relative liver weight decreased in F-25 rats when compared to normal group, however relative liver weight showed a gradual increase as fluvastatin dose increased. In groups treated with silymarin, the relative liver weight tended to decrease in highly toxicated groups (FS-75 and FS-100). While, in DDB treated groups, the relative liver weight was normal in all groups. (Table 2)

Table 2: Rats' relative liver weight in normal group, and groups toxicated with gradual doses of fluvastatin (without and with 140 mg/kg/day silymarin or 100 mg/kg/day DDB treatment) for 7 days.

Animal groups		Fluvastatin dose for 7days	Relative liver weight (%) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**4.22 ± 0.12**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**3.27 ± 0.19**	***(0.0081)**
	F-50	50 mg/kg/day	**3.32 ± 0.37**	ns (0.1481)
	F-75	75 mg/kg/day	**3.60 ± 0.38**	ns (0.1081)
	F-100	100 mg/kg/day	**4.02 ± 0.20**	ns (0.5745)
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**4.67 ± 0.14**	ns (0.0627)
	FS-50	50 mg/kg/day	**3.87 ± 0.28**	ns (0.1727)
	FS-75	75 mg/kg/day	**3.43 ± 0.17**	***(0.0129)**
	FS-100	100 mg/kg/day	**3.25 ± 0.24**	***(0.0101)**
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**4.40 ± 0.13**	ns (0.3350)
	FD-50	50 mg/kg/day	**4.03 ± 0.39**	ns (1.0000)
	FD-75	75 mg/kg/day	**3.95 ± 0.18**	ns (0.1727)
	FD-100	100 mg/kg/day	**4.18 ± 0.22**	ns (0.7479)

*** Significant difference from normal control.** **ns No significant difference from normal control**

4.1.3 Kidneys weight ratio

Relative kidneys weight was normal in nearly all groups, especially in animals treated with DDB. However, in F-100 group and FS-100, a significant increase in relative kidneys weight was recorded. (Table 3)

Table 3: Rats' relative kidneys weight in normal group, and groups toxicated with gradual doses of fluvastatin (without and with 140 mg/kg/day silymarin or 100 mg/kg/day DDB treatment) for 7 days.

Animal groups		Fluvastatin dose for 7days	Relative kidneys weight (%) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**0.92 ± 0.04**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**0.88 ± 0.03**	ns (0.5536)
	F-50	50 mg/kg/day	**1.02 ± 0.07**	ns (0.3682)
	F-75	75 mg/kg/day	**0.95 ± 0.05**	ns (0.6775)
	F-100	100 mg/kg/day	**1.18 ± 0.04**	***(0.0047)**
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**0.85 ± 0.02**	ns (0.2324)
	FS-50	50 mg/kg/day	**0.90 ± 0.04**	ns (0.7980)
	FS-75	75 mg/kg/day	**0.95 ± 0.05**	ns (0.6775)
	FS-100	100 mg/kg/day	**1.06 ± 0.02**	*** (0.0273)**
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**0.92 ± 0.06**	ns (0.8015)
	FD-50	50 mg/kg/day	**0.88 ± 0.05**	ns (0.6313)
	FD-75	75 mg/kg/day	**0.95 ± 0.04**	ns (0.6734)
	FD-100	100 mg/kg/day	**0.98 ± 0.06**	ns (0.5082)

*** Significant difference from normal control.** **ns No significant difference from normal control**

4.2. Biochemical results

4.2.1. Blood glucose

Fluvastatin liver toxicity caused a significant decrease in serum glucose level to 60-70% glucose level recorded in normal group. Treatment with silymarin helped to maintain glucose level within normal range only at fluvastatin lowest toxic doses (FS-25 and FS-50). At FS-75 and FS-100, silymarin could not stop the glucose decrease, as it significantly decreased nearly to 50% normal level.

DDB treatment significantly attenuated the glucose decrease only in FD-25 group. While, in FD-50 and FD-75 groups, serum glucose level decreased significantly to 73 and 61% normal level. Notably, serum glucose level in FD-100 was increased to normal values. (Table 4 and Figure 22)

Table 4: Rats' serum glucose concentration in normal group, and groups toxicated with gradual doses of fluvastatin (without and with 140 mg/kg/day silymarin or 100 mg/kg/day DDB) for 7 days.

Animal groups		Fluvastatin dose for 7days	Glucose (mmol/l) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**8.82 ±0.65**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**5.97 ±0.45**	*** (0.0049)**
	F-50	50 mg/kg/day	**6.24 ±0.49**	*** (0.0103)**
	F-75	75 mg/kg/day	**5.58 ±0.73**	*** (0.0080)**
	F-100	100 mg/kg/day	**5.31 ±0.87**	*** (0.0090)**
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**8.19 ±0.91**	ns (0.5892)
	FS-50	50 mg/kg/day	**7.30 ±0.22**	ns (0.0712)
	FS-75	75 mg/kg/day	**4.41 ±0.17**	*** (0.0013)**
	FS-100	100 mg/kg/day	**4.42 ±0.54**	*** (0.0007)**
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**8.48 ±0.38**	ns (0.6685)
	FD-50	50 mg/kg/day	**6.44 ±0.48**	*** (0.0152)**
	FD-75	75 mg/kg/day	**5.39 ±0.74**	*** (0.0061)**
	FD-100	100 mg/kg/day	**7.12 ±0.81**	ns (0.1332)

*** Significant difference from normal control.** **ns No significant difference from normal control**

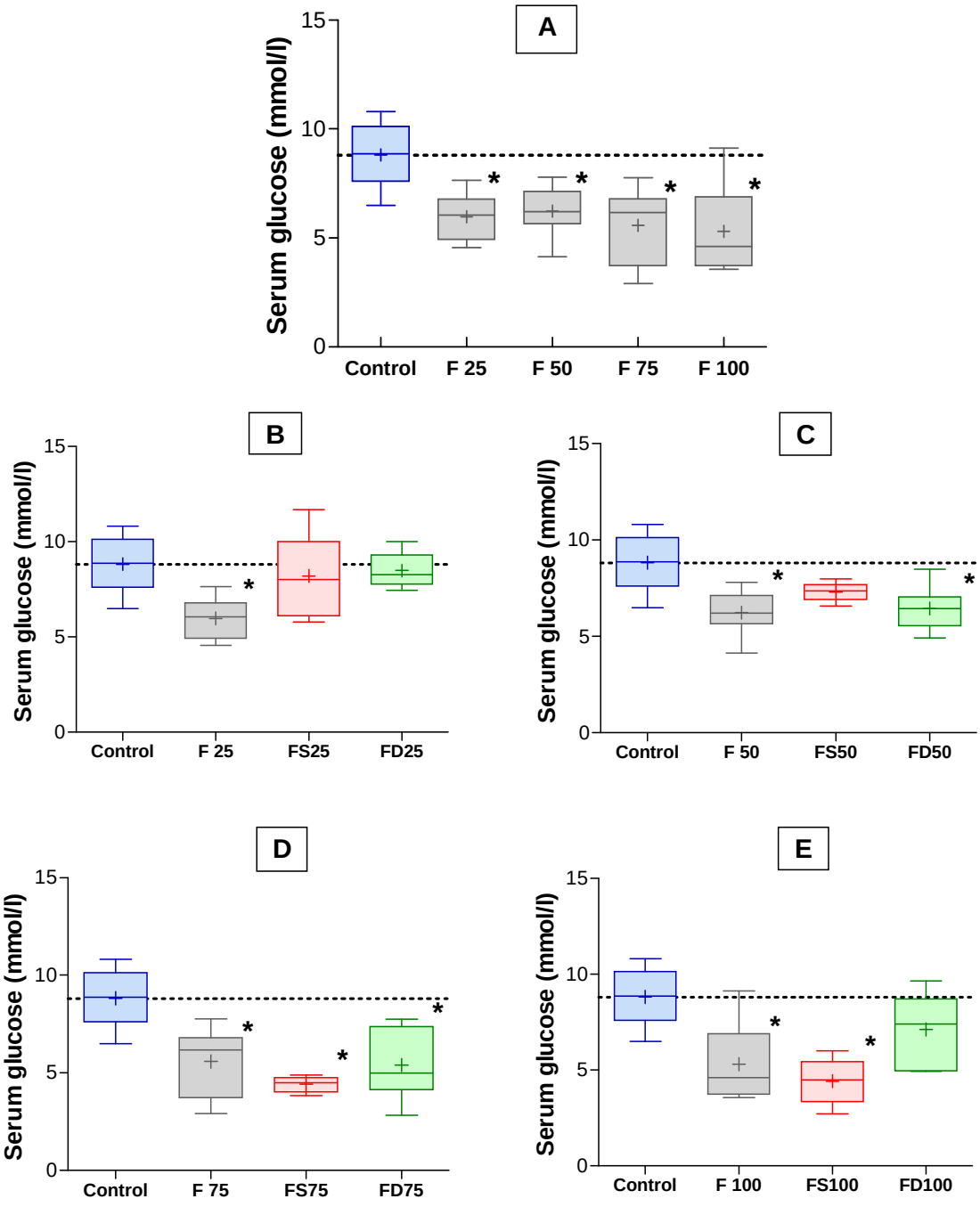


Figure 23: Rats' serum glucose concentration
A) in control group vs. fluvastatin toxicated with gradual doses groups, and in control group vs. groups toxicated for 7 days with B) 25, C) 50, D) 75, E) 100 mg/kg/day fluvastatin, without and with treatment (silymarin and DDB).

F X = rats received X mg/kg/day fluvastatin for 7 days
FS X = rats received for 7 days X mg/kg/day fluvastatin +140 mg/kg silymarin,
FD X = rats received for 7 days X mg/kg/day fluvastatin + 100mg/kg DDB
*Significant difference when compared to normal group

4.2.2. Serum protein profile:

4.2.2.1. Total protein

Total Protein concentrations were in all groups normalized, except in one group F-25 increased by 30% of the normal value. In rats treated simultaneously with silymarin (FS-25) and DDB (FD-25) with 25 mg/kg/day fluvastatin for 7 days, total protein concentration significantly decreased by 30% than F-25. (Table 5)

Table 5: Rats' serum total protein concentration in normal group, and groups toxicated with gradual doses of fluvastatin (without and with 140mg/kg/day silymarin or 100 mg/kg/day DDB) for 7days.

Animal groups		Fluvastatin dose for 7days	Total protein (g/dl) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**4.63 ±0.23**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**6.01 ±0.40**	*** (0.0136)**
	F-50	50 mg/kg/day	**4.83 ±0.16**	ns (0.4802)
	F-75	75 mg/kg/day	**4.63 ±0.26**	ns (0.9963)
	F-100	100 mg/kg/day	**4.78 ±0.32**	ns (0.7139)
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**4.50 ±0.08**	ns (0.6188)
	FS-50	50 mg/kg/day	**4.94 ±0.16**	ns (0.2908)
	FS-75	75 mg/kg/day	**4.47 ±0.16**	ns (0.5817)
	FS-100	100 mg/kg/day	**4.87 ±0.11**	ns (0.4075)
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**4.58 ±0.22**	ns (0.8760)
	FD-50	50 mg/kg/day	**4.99 ±0.28**	ns (0.3361)
	FD-75	75 mg/kg/day	**4.88 ±0.07**	ns (0.3550)
	FD-100	100 mg/kg/day	**4.84 ±0.12**	ns (0.4259)

*** Significant difference from normal group.** **ns No significant difference from normal group.**

4.2.2.2. Albumin

Albumin concentration in groups toxicated with low doses of fluvastatin (F-25, FD-25 and F-50, FS-50, FD-50) showed a tendency to increase, then albumin returned to normal values at higher toxic doses (F-75, FS-75, FD-75 and F-100, FS-100, FD-100), weather rats received treatment or not. (Table 6 and Figure 23)

Table 6: Rats' serum albumin concentration in normal group, and groups toxicated with gradual doses of fluvastatin (without and with 140mg/kg/day silymarin or 100 mg/kg/day DDB) for 7days.

Animal groups		Fluvastatin dose for 7days	Albumin (g/dl) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**2.49 ±0.07**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**2.86 ±0.13**	*** (0.0313)**
	F-50	50 mg/kg/day	**3.02 ±0.06**	*** (0.0002)**
	F-75	75 mg/kg/day	**2.58 ±0.04**	ns (0.2940)
	F-100	100 mg/kg/day	**2.32 ±0.11**	ns (0.2064)
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**2.66 ±0.09**	ns (0.1559)
	FS-50	50 mg/kg/day	**2.86 ±0.10**	*** (0.0108)**
	FS-75	75 mg/kg/day	**2.47 ±0.04**	ns (0.7811)
	FS-100	100 mg/kg/day	**2.50 ±0.16**	ns (0.9470)
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**3.10 ±0.09**	*** (0.0005)**
	FD-50	50 mg/kg/day	**2.88 ±0.08**	*** (0.0042)**
	FD-75	75 mg/kg/day	**2.65 ±0.04**	ns (0.0748)
	FD-100	100 mg/kg/day	**2.49 ±0.02**	ns (0.9965)

*** Significant difference from normal group.** **ns No significant difference from normal group.**

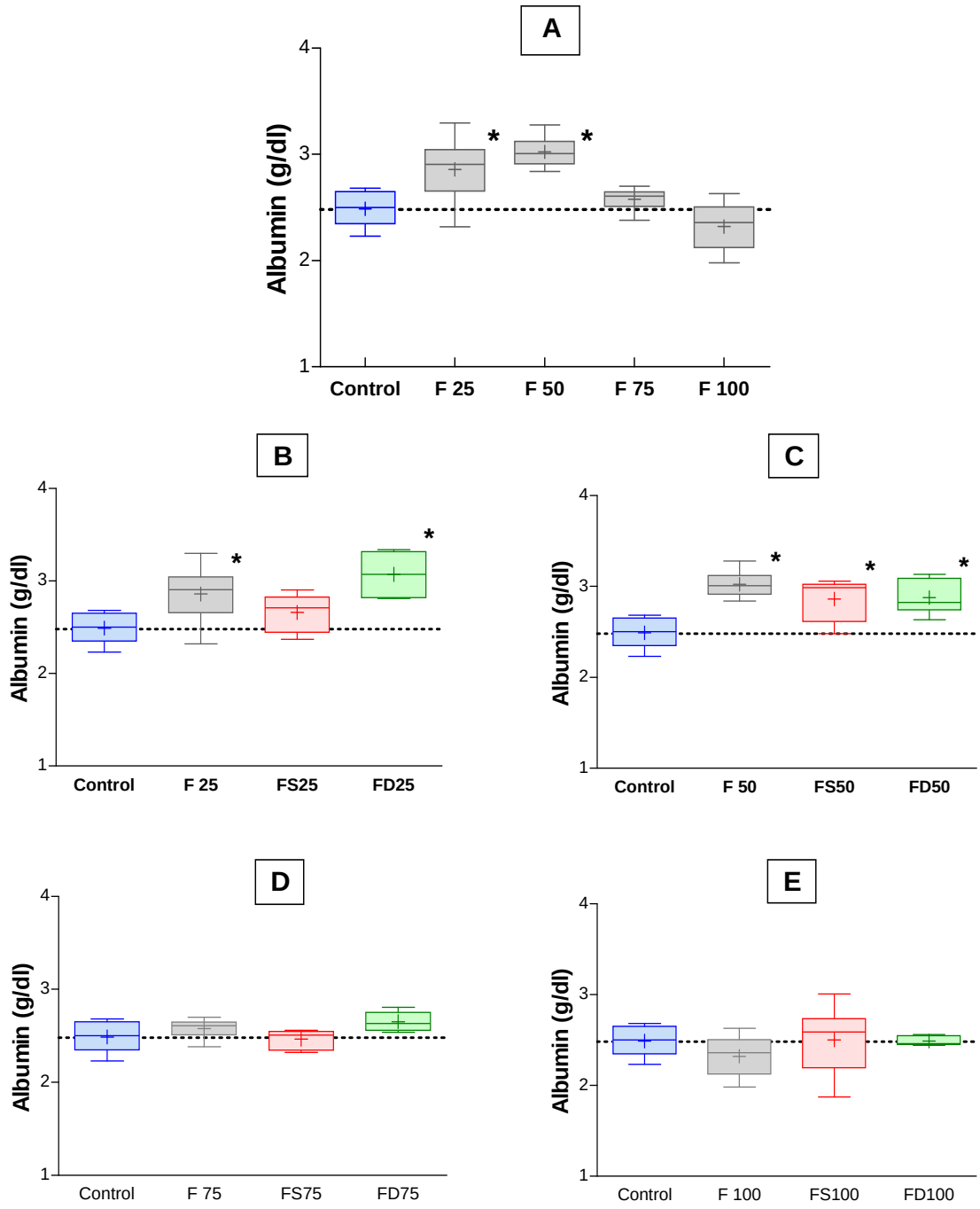


Figure 24: Rats' serum albumin concentration
A) in control group vs. fluvastatin toxicated with gradual doses groups, and in control group vs. groups toxicated for 7 days with B) 25, C) 50, D) 75, E) 100 mg/kg/day fluvastatin, without and with treatment (silymarin and DDB).

F X = rats received X mg/kg/day fluvastatin for 7 days
FS X = rats received for 7 days X mg/kg/day fluvastatin +140 mg/kg silymarin,
FD X = rats received for 7 days X mg/kg/day fluvastatin + 100mg/kg DDB
*Significant difference when compared to normal group

4.2.3. Serum lipid profile:

4.2.3.1. Cholesterol

Although fluvastatin inhibits *denovo* cholesterol synthesis, its toxic doses showed no action on serum cholesterol profile in toxicated rats. Serum cholesterol was normal in all groups, except in one group FD-25 as cholesterol concentration significantly increased by 26% when compared to normal group. However, as the fluvastatin dose increased in groups treated with DDB, cholesterol concentration returned to normal level. (Table 7)

Table 7: Rats' serum cholesterol concentration in normal group, and groups toxicated with gradual doses of fluvastatin (without and with 140mg/kg/day silymarin or 100 mg/kg/day DDB) for 7days.

Animal groups		Fluvastatin dose for 7days	Cholesterol (mmol/l) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**1.84 ±0.06**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**1.77 ±0.18**	ns (0.7131)
	F-50	50 mg/kg/day	**2.09 ±0.31**	ns (0.4678)
	F-75	75 mg/kg/day	**2.05 ±0.15**	ns (0.2298)
	F-100	100 mg/kg/day	**2.09 ±0.33**	ns (0.4848)
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**2.04 ±0.24**	ns (0.4634)
	FS-50	50 mg/kg/day	**1.78 ±0.08**	ns (0.5313)
	FS-75	75 mg/kg/day	**1.95 ±0.08**	ns (0.3051)
	FS-100	100 mg/kg/day	**1.89 ±0.32**	ns (0.8878)
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**2.32 ±0.16**	*** (0.0186)**
	FD-50	50 mg/kg/day	**1.67 ±0.07**	ns (0.0949)
	FD-75	75 mg/kg/day	**1.95 ±0.17**	ns (0.5708)
	FD-100	100 mg/kg/day	**1.77 ±0.18**	ns (0.7280)

*** Significant difference from normal group.** **ns No significant difference from normal group.**

4.2.3.2. Triglycerides

Serum triglyceride showed a tend to increase as the toxic fluvastatin dose increased, however all values were significantly less than normal by 60, 35, 40, 30% in F-25, F-50, F-75 and F-100 groups, respectively. Treatment with silymarin and DDB normalized triglyceride concentration in groups toxicated with 25mg/kg/day fluvastatin (FS-25 and FD-25), where triglyceride value was significantly higher than F-25. Serum triglyceride was recorded significantly lower than normal by nearly 50% in FS-100 group. On the other hand, in similar group treated with DDB (FD-100) triglyceride level was nearly normal. (Table 8 and Figure 25)

Table 8: Rats' serum triglycerides concentration in normal group, and groups toxicated with gradual doses of fluvastatin (without and with 140mg/kg/day silymarin or 100 mg/kg/day DDB) for 7days.

Animal groups		Fluvastatin dose for 7days	Triglycerides (mmol/l) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**0.88 ±0.10**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**0.34 ±0.04**	*** (0.0009)**
	F-50	50 mg/kg/day	**0.57 ±0.08**	*** (0.0332)**
	F-75	75 mg/kg/day	**0.53 ±0.05**	*** (0.0086)**
	F-100	100 mg/kg/day	**0.62 ±0.08**	ns (0.0668)
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**0.62 ±0.08**	ns (0.0679)
	FS-50	50 mg/kg/day	**0.54 ±0.05**	*** (0.0110)**
	FS-75	75 mg/kg/day	**0.53 ±0.04**	*** (0.0069)**
	FS-100	100 mg/kg/day	**0.40 ±0.09**	*** (0.0051)**
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**0.70 ±0.07**	ns (0.1526)
	FD-50	50 mg/kg/day	**0.56 ±0.08**	*** (0.0253)**
	FD-75	75 mg/kg/day	**0.55 ±0.06**	*** (0.0139)**
	FD-100	100 mg/kg/day	**0.61 ±0.10**	ns (0.0667)

*** Significant difference from normal control.** **ns No significant difference from normal control**

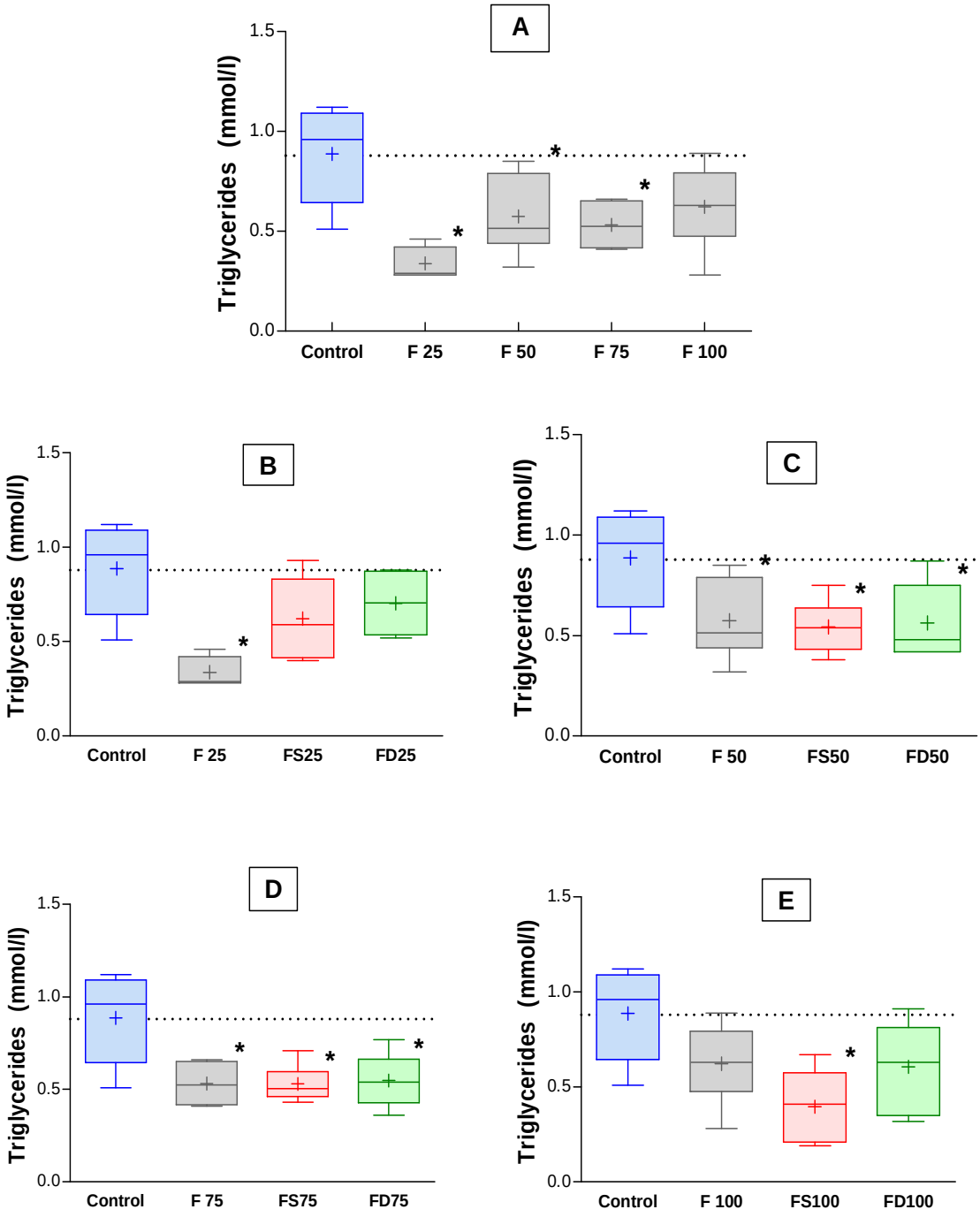


Figure 25: Rat's serum triglycerides concentration
A) in control group vs. fluvastatin toxicated with gradual doses groups, and in control group vs. groups toxicated for 7 days with B) 25, C) 50, D) 75, E) 100 mg/kg/day fluvastatin, without and with treatment (silymarin and DDB).

F X = rats received X mg/kg/day fluvastatin for 7 days
FS X = rats received for 7 days X mg/kg/day fluvastatin +140 mg/kg silymarin,
FD X = rats received for 7 days X mg/kg/day fluvastatin + 100mg/kg DDB
* significant difference when compared to notmal goup

4.2.3.3. High density lipoprotein fraction (HDL)

HDL concentration showed significant increased but starting from F-50. Treating toxicated rats with silymarin and DDB, caused an increase in HDL level in FS-25 and FD-25 when compared to similar group receiving fluvastatin only (F-25). Moreover, treatment with silymarin and DDB succeeded to lower HDL increase but only at 50 mg/kg/day fluvastatin in FS-50 and FD-50. (Table 9 and Figure 26)

Table 9: Rats' serum HDL concentration in normal group, and groups toxicated with gradual doses of fluvastatin (without and with 140 mg/kg/day silymarin or 100 mg/kg/day DDB) for 7days.

Animal groups		Fluvastatin dose for 7days	HDL (mmol/l) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**1.26±0.06**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**1.05 ±0.10**	ns (0.1697)
	F-50	50 mg/kg/day	**1.81 ±0.17**	*** (0.0214)**
	F-75	75 mg/kg/day	**1.79 ±0.05**	*** (0.0049)**
	F-100	100 mg/kg/day	**1.75 ±0.30**	ns (0.3768)
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**1.84 ±0.27**	ns (0.0913)
	FS-50	50 mg/kg/day	**0.76 ±0.11**	*** (0.0025)**
	FS-75	75 mg/kg/day	**1.60 ±0.21**	ns (0.3768)
	FS-100	100 mg/kg/day	**1.80 ±0.31**	ns (0.1601)
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**1.47 ±0.10**	ns (0.1096)
	FD-50	50 mg/kg/day	**0.65 ±0.06**	*** (<0.0001)**
	FD-75	75 mg/kg/day	**1.90 ±0.25**	ns (0.0559)
	FD-100	100 mg/kg/day	**1.37 ±0.13**	ns (0.4800)

*** Significant difference from normal control.** **ns No significant difference from normal control**

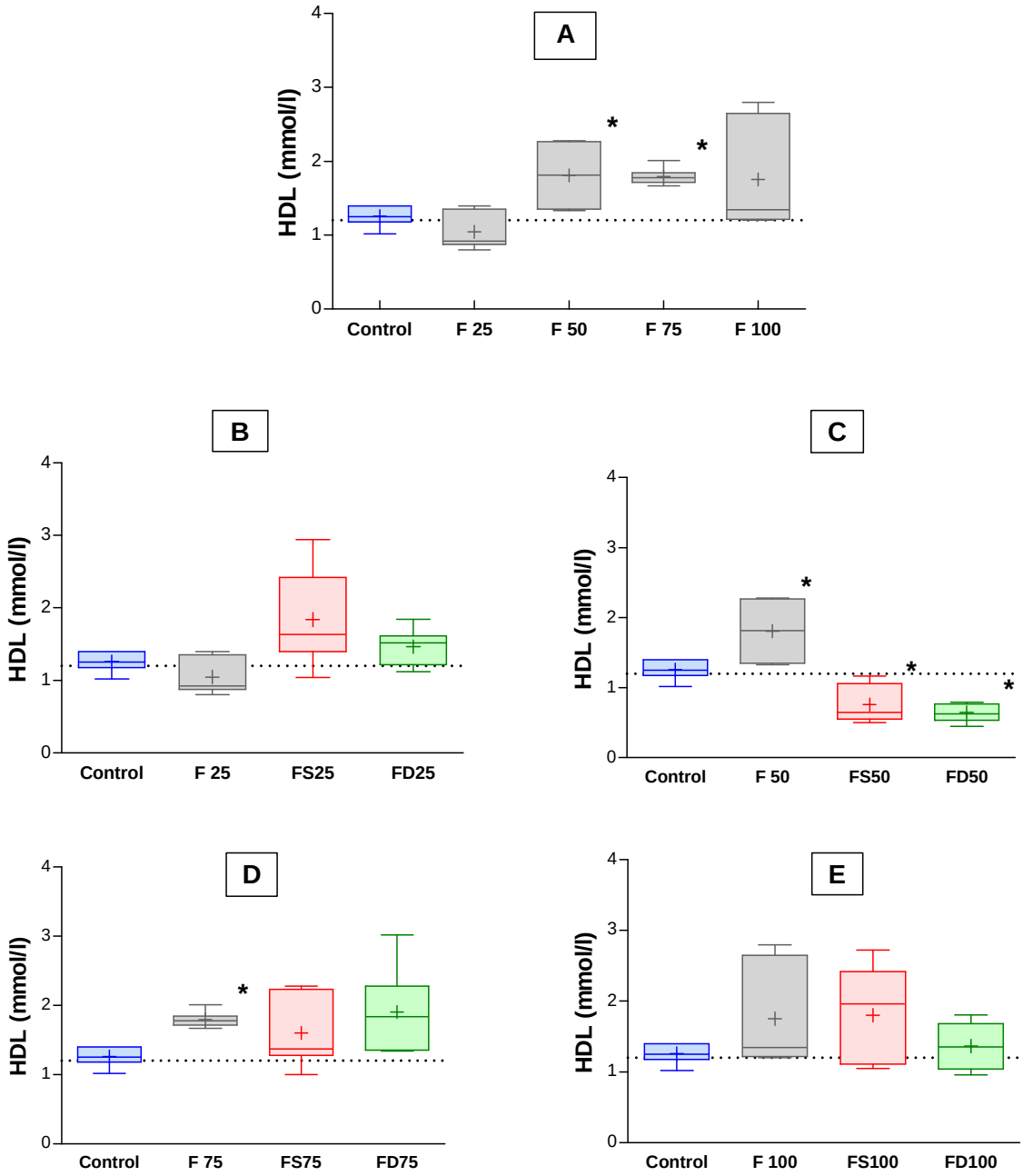


Figure 26: Rat's serum HDL fraction concentration A) in control group vs. fluvastatin toxicated with gradual doses groups, and in control group vs. groups toxicated for 7 days with B) 25, C) 50, D) 75, E) 100 mg/kg/day fluvastatin, without and with treatment (silymarin and DDB).

F X = rats received X mg/kg/day fluvastatin for 7 days
FS X = rats received for 7 days X mg/kg/day fluvastatin +140 mg/kg silymarin,
FD X = rats received for 7 days X mg/kg/day fluvastatin + 100mg/kg DDB
*Significant difference when compared to normal group

4.2.4. Serum liver enzymes profile:

4.2.4.1. Alanine aminotransferase (ALT):

When fluvastatin toxic dose increased, the level of ALT significantly increased in rats toxicated with gradual doses of fluvastatin for 7 days. In F-25 and F-50 groups, ALT level increased to nearly twice the normal group level (225 and 229%, respectively). As the dose increased to 75 mg/kg/day fluvastatin (F-75), ALT level increased 4 times (430%), and at 100 mg/kg/day fluvastatin (F-100) ALT level doubled to 8 times (786%) the normal level. (Table 10-a and Figure 27A)

Table 10-a: Rats' serum alanine aminotransferase (ALT) activity in normal group, and groups toxicated with gradual doses of fluvastatin for 7 days.

Animal groups		Fluvastatin dose for 7days	ALT (IU/l) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**30.39 ±5.65**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**68.36 ±6.65**	*** (0.0043)**
	F-50	50 mg/kg/day	**69.49 ±22.54**	ns (0.3095)
	F-75	75 mg/kg/day	**130.80 ±43.23**	*** (0.0411)**
	F-100	100 mg/kg/day	**238.87 ±42.82**	*** (0.0022)**

*** Significant difference from normal.** **ns No significant difference from normal**

In rats receiving silymarin simultaneously with fluvastatin, the increase in ALT level was suppressed to 1.5 times (151%) the normal level in FS-25 group, thus ALT level is 75% significantly less than F-25. As the fluvastatin dose was doubled to 50 mg/kg/day (FS-50), the ALT level reached to nearly twice (212%) normal value. As the fluvastatin dose reached 75 mg/kg/day (FS-75), the ALT level drastically increased to 11 times (1114%) normal level. This increase was significantly higher (by seven times) than ALT recorded in similar group receiving only fluvastatin. ALT level decreased a bit in FS-100, compared to FS-75, but it was still significantly higher than normal level by 7 times (670%). (Table 10-b and Figure 27A)

Table 10-b: Rats' serum alanine aminotransferase (ALT) activity in normal group, and groups received 140 mg/kg/day silymarin with gradual doses of fluvastatin for 7 days.

Animal groups		Fluvastatin dose for 7days	ALT (IU/l) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**30.39 ±5.65**	
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**45.99 ±3.53**	*** (0.0411)**
	FS-50	50 mg/kg/day	**64.35 ±12.84**	*** (0.0260)**
	FS-75	75 mg/kg/day	**338.60 ±26.99**	*** (0.0022)**
	FS-100	100 mg/kg/day	**203.50 ±35.01**	*** (0.0043)**

*** Significant difference from normal control.** **ns No significant difference from normal control**

The ALT level, in DDB treated rats, had a different behavior than silymarin treated groups. In rats receiving DDB while toxicated with fluvastatin, the ALT level increased to nearly twice the normal value, and was nearly steady in spite of fluvastatin increasing dose. Notably, FD-75 showed a normal ALT level. (Table 10-c and Figure 27B-E)

Table 10-c: Rats' serum alanine aminotransferase (ALT) activity in normal group, and groups received 100 mg/kg/day DDB with gradual doses of fluvastatin for 7 days.

Animal groups		Fluvastatin dose for 7days	ALT (IU/l) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**30.39 ±5.65**	
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**66.36 ±7.73**	*** (0.0087)**
	FD-50	50 mg/kg/day	**67.28 ±15.13**	*** (0.0411)**
	FD-75	75 mg/kg/day	**48.90 ±9.17**	ns (0.1320)
	FD-100	100 mg/kg/day	**76.30 ±15.43**	*** (0.0411)**

*** Significant difference from normal control.** **ns No significant difference from normal control**

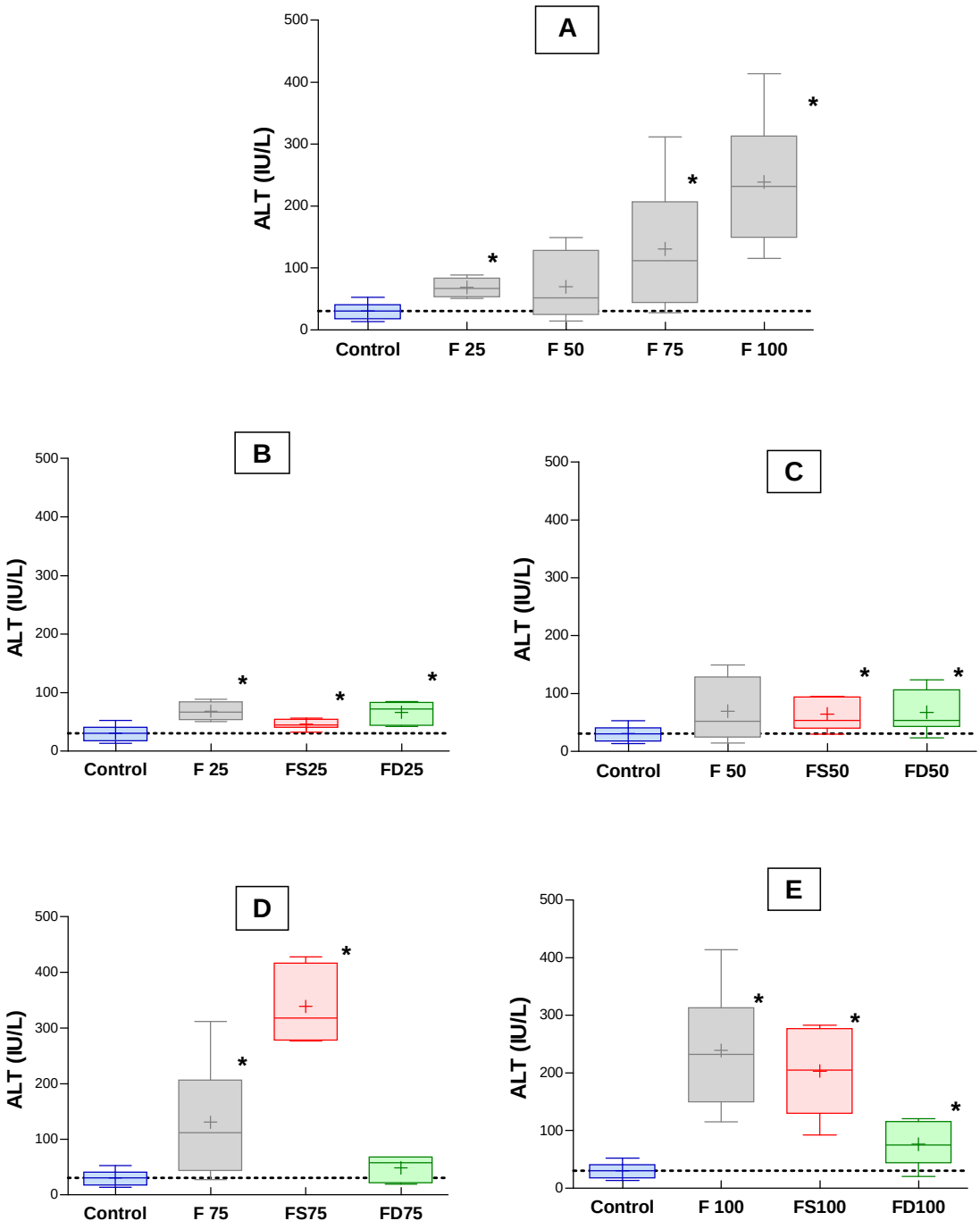


Figure 27: Rats' serum alanine aminotransferase (ALT) activity A) in control group vs. fluvastatin toxicated with gradual doses groups, and in control group vs. groups toxicated for 7 days with B) 25, C) 50, D) 75, E) 100 mg/kg/day fluvastatin, without and with treatment (silymarin and DDB).

F X = rats received X mg/kg/day fluvastatin for 7 days
FS X = rats received for 7 days X mg/kg/day fluvastatin +140 mg/kg silymarin,
FD X = rats received for 7 days X mg/kg/day fluvastatin + 100mg/kg DDB
*Significant difference when compared to normal group

4.2.4.2. Aspartate aminotransferase (AST):

AST level significantly increased more slowly than ALT as fluvastatin doses increased. In F-25 group, AST level increased to 173% the normal group level. As the dose doubled to 50 mg/kg/day fluvastatin (F-50), AST reached twice (229%) the normal level, and as dose increased to 75 mg/kg/day (F-75), AST level increased 4 times (373%). At 100 mg/kg/day fluvastatin (F-100), AST level increased to only 473% the normal level. (Table 11-a and Figure 28A).

Table 11-a: Rats' serum aspartate aminotransferase (AST) activity in normal group, and groups toxicated with gradual doses of fluvastatin for 7 days.

Animal groups		Fluvastatin dose for 7days	AST (IU/l) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**112.30 ±12.17**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**194.07 ±13.20**	*** (0.0043)**
	F-50	50 mg/kg/day	**257.31 ±52.52**	*** (0.0152)**
	F-75	75 mg/kg/day	**419.20 ±68.15**	*** (0.0022)**
	F-100	100 mg/kg/day	**531.27 ±46.88**	*** (0.0022)**

*** Significant difference from normal.** **ns No significant difference from normal**

In rats receiving silymarin simultaneously fluvastatin, AST level increased twice (191%) the normal level in FS-25 group, so it increased 18% when compared to F-25. As the fluvastatin dose was doubled to 50 mg/kg/day (FS-50), the AST level reached to nearly thrice (270%) normal value. Then, AST level raised to 5 times (506%) the normal value at 75 mg/kg/day fluvastatin (FS-75), and at FS-100 AST was 4 times (433%) the normal. (Table 11-b and Figure 28 B-E)

Table 11-b: Rats' serum aspartate aminotransferase (AST) activity in normal group, and groups received 140 mg/kg/day silymarin with gradual doses of fluvastatin for 7 days.

Animal groups		Fluvastatin dose for 7days	AST (IU/l) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**112.30 ±12.17**	
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**214.60 ±12.11**	*** (0.0022)**
	FS-50	50 mg/kg/day	**303.35 ±54.42**	*** (0.0043)**
	FS-75	75 mg/kg/day	**568.00 ±44.92**	*** (0.0022)**
	FS-100	100 mg/kg/day	**485.94 ±61.35**	*** (0.0022)**

*** Significant difference from normal control.** **ns No significant difference from normal control**

In DDB treated rats while toxicated with fluvastatin, AST level increased to 246, 206 and 282% the normal value in FD-25, FD-75 and FD-100, respectively. Strangely, the AST level recorded in FD-50 was higher than rest groups, as it reached 4 times (409%) the normal value. This indicate that AST at this group was 139% more than FS-50, and significantly higher by 180% when compared to F-50. (Table 10-c and Figure 27 B-E)

Table 11-c: Rats' serum aspartate aminotransferase (AST) activity in normal group, and groups received 100 mg/kg/day DDB with gradual doses of fluvastatin for 7 days.

Animal groups		Fluvastatin dose for 7days	AST (IU/l) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**112.30 ±12.17**	
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**276.78 ±48.08**	*** (0.0043)**
	FD-50	50 mg/kg/day	**458.93 ±71.28**	*** (0.0022)**
	FD-75	75 mg/kg/day	**231.60 ±16.82**	*** (0.0043)**
	FD-100	100 mg/kg/day	**316.84 ±53.61**	*** (0.0043)**

*** Significant difference from normal control.** **ns No significant difference from normal control**

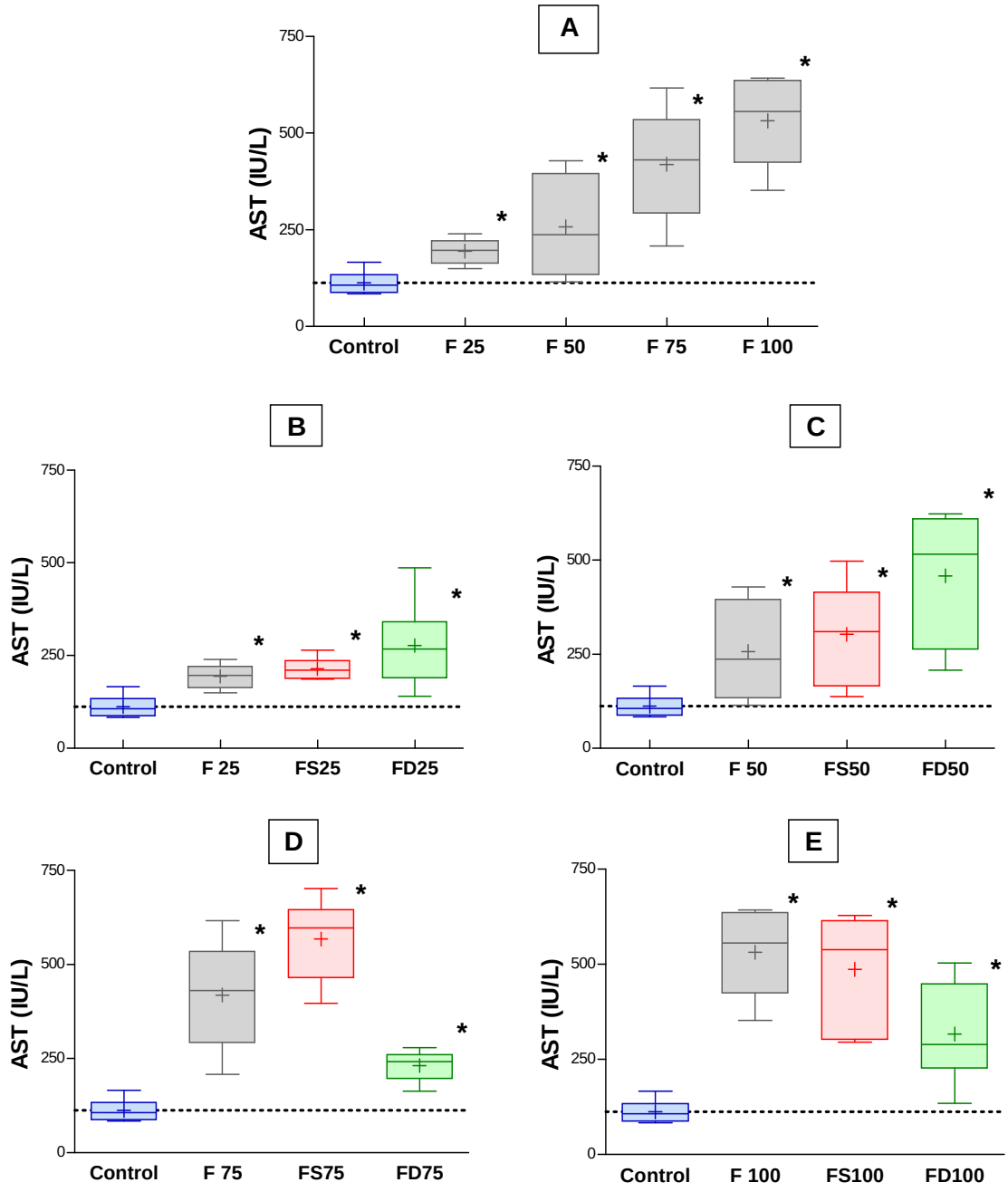


Figure 28: Rats' serum aspartate aminotransferase (AST) activity A) in control group vs. fluvastatin toxicated with gradual doses groups, and in control group vs. groups toxicated for 7 days with B) 25, C) 50, D) 75, E) 100 mg/kg/day fluvastatin, without and with treatment (silymarin and DDB).

F X = rats received X mg/kg/day fluvastatin for 7 days
FS X = rats received for 7 days X mg/kg/day fluvastatin +140 mg/kg silymarin,
FD X = rats received for 7 days X mg/kg/day fluvastatin + 100 mg/kg DDB
*Significant difference when compared to normal group

4.2.4.3. gamma glutamyltransferase (GGT):

As fluvastatin dose increased, GGT level increased slowly. However, all GGT values were significantly less than normal value by 88, 80, 85, and 58% in F-25, F-50, F-75, and F-100 groups. Treating the toxicated rats with silymarin and DDB, showed similar increase in GGT, but starting from 50 mg/kg/days Fluvastatin (FS-50 and FD-50). (Table 12 and Figure 29 A)

The lowest dose of Fluvastatin accompanied with silymarin (FS-25) showed high GGT level even higher than normal value by 25%. Treatment with DDB (FD-25) showed nearly normal GGT level (90%). Interestingly, in heavily toxicated rats FS-100, the GGT increased until it nearly reached the normal value. (Table 12 and Figure 29 B-E)

Table 12: Rats' serum gamma glutamyltransferase (GGT) activity in normal group, and groups toxicated with gradual doses of fluvastatin (without and with 140 mg/kg/day silymarin or 100 mg/kg/day DDB) for 7days.

Animal groups		**Fluvastatin dose for 7days**	**GGT (IU/l) Mean ± SEM**	**Significance *(p value)* versus NC**
Normal group	**NC**	0 mg/kg/day	**18.11 ±3.09**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**2.18 ±0.39**	*** (0.0037)**
	F-50	50 mg/kg/day	**3.54 ±0.70**	*** (0.0059)**
	F-75	75 mg/kg/day	**2.65 ±0.64**	*** (0.0045)**
	F-100	100 mg/kg/day	**7.57 ±2.22**	*** (0.0380)**
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**22.80 ±1.99**	ns (0.2305)
	FS-50	50 mg/kg/day	**2.05 ±0.32**	*** (0.0036)**
	FS-75	75 mg/kg/day	**2.16 ±0.32**	*** (0.0037)**
	FS-100	100 mg/kg/day	**16.16 ±0.96**	ns (0.6021)
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**15.81 ±0.96**	ns (0.5106)
	FD-50	50 mg/kg/day	**2.41 ±0.55**	*** (0.0041)**
	FD-75	75 mg/kg/day	**2.99 ±0.51**	*** (0.0048)**
	FD-100	100 mg/kg/day	**4.38 ±0.57**	*** (0.0072)**

*** Significant difference from normal group.** **ns No significant difference from normal group.**

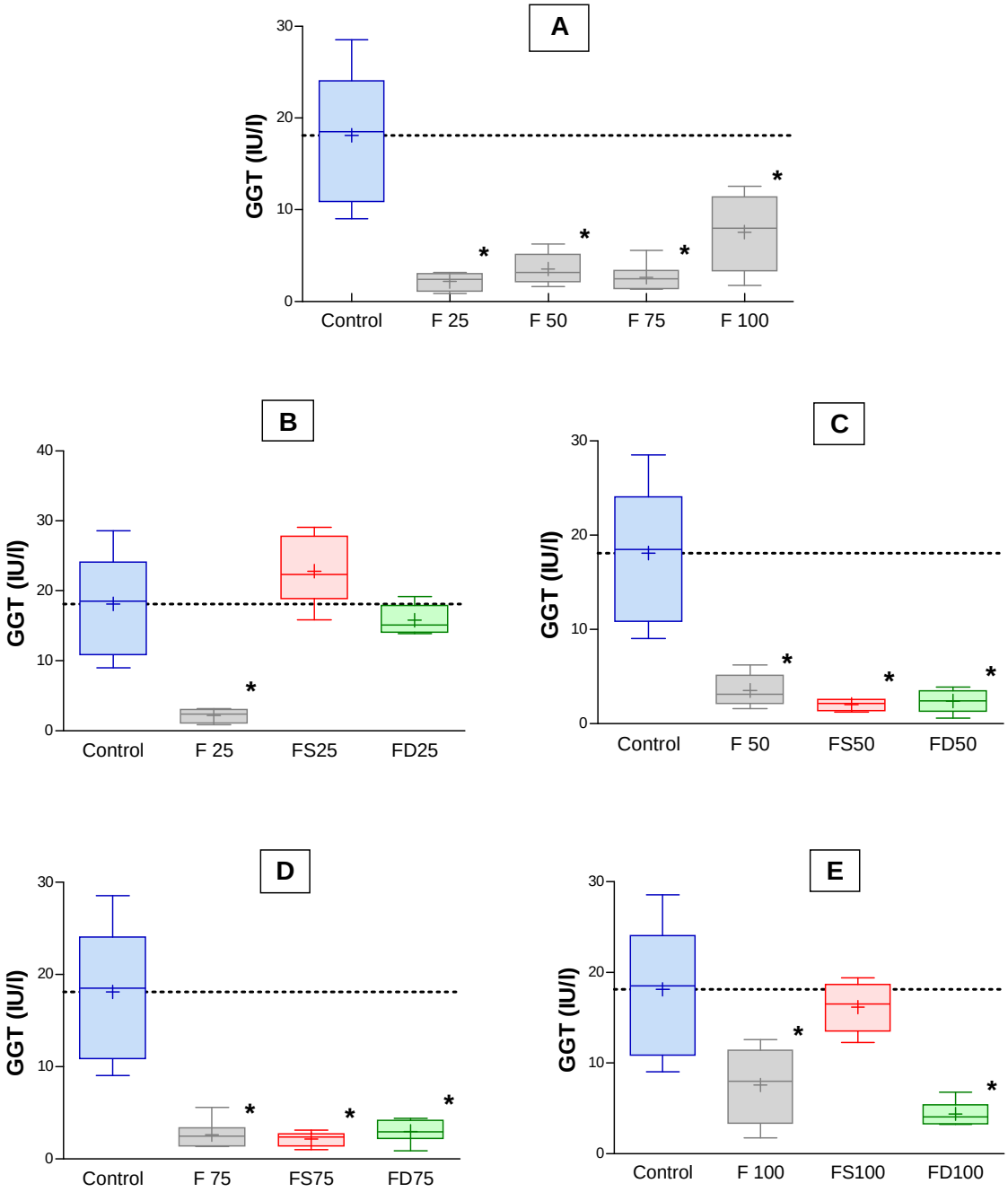


Figure 29: Rats' serum gamma glutamyltransferase (GGT) activity A) in control group vs. fluvastatin toxicated with gradual doses groups, and in control group vs. groups toxicated for 7 days with B) 25, C) 50, D) 75, E) 100 mg/kg/day fluvastatin, without and with treatment (silymarin and DDB).

F X = rats received X mg/kg/day fluvastatin for 7 days
FS X = rats received for 7 days X mg/kg/day fluvastatin +140 mg/kg silymarin,
FD X = rats received for 7 days X mg/kg/day fluvastatin + 100 mg/kg DDB
*Significant difference when compared to noral group

4.3. Hepatic apoptosis parameters

4.3.1. Hepatic Caspase-3

Fluvastatin lowest toxic dose 25 mg/kg/day (F-25) did not affect hepatic caspase-3 activity. However, as fluvastatin dose increased from 50 to 100 mg/kg/day (F-50, F-75, F-100), caspase-3 activity showed a significant increased by nearly 50-70%. (Table 13 and Figure 30 A)

When rats were treated with silymarin and DDB while toxicated with fluvastatin, caspase-3 activity significantly increased as fluvastatin dose increased, except in FS-25 and FD-25. In groups FS-50, FS-75 and FS-100, caspase-3 increased significantly than normal level by 70, 100 and 90%, respectively. On the other hand, in FD-50, FD-75 and FD-100 groups, caspase-3 significantly increased to 50-60% the normal level. (Table 13 and Figure 30)

Table 13: Rats' hepatic caspase-3 activity in normal group, and groups toxicated with gradual doses of fluvastatin (without and with 140 mg/kg/day silymarin or 100 mg/kg/day DDB) for 7days.

Animal groups		Fluvastatin dose for 7days	Hepatic caspase-3 (Unit/0.1mg) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**889.04 ±134.31**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**887.77 ±151.70**	ns (0.9951)
	F-50	50 mg/kg/day	**1311.84 ±82.43**	*** (0.0260)**
	F-75	75 mg/kg/day	**1488.69 ±193.70**	*** (0.0245)**
	F-100	100 mg/kg/day	**1382.58 ±136.15**	*** (0.0262)**
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**1303.51 ±147.68**	ns (0.0618)
	FS-50	50 mg/kg/day	**1481.65 ±110.09**	*** (0.0066)**
	FS-75	75 mg/kg/day	**1756.87 ±101.27**	*** (0.0007)**
	FS-100	100 mg/kg/day	**1684.80 ±113.18**	*** (0.0010)**
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**1297.58 ±125.26**	ns (0.0503)
	FD-50	50 mg/kg/day	**1314.43 ±37.78**	*** (0.0225)**
	FD-75	75 mg/kg/day	**1461.76 ±83.78**	*** (0.0085)**
	FD-100	100 mg/kg/day	**1325.17 ±144.90**	*** (0.0495)**

*** Significant difference from normal group.** **ns No significant difference from normal group.**

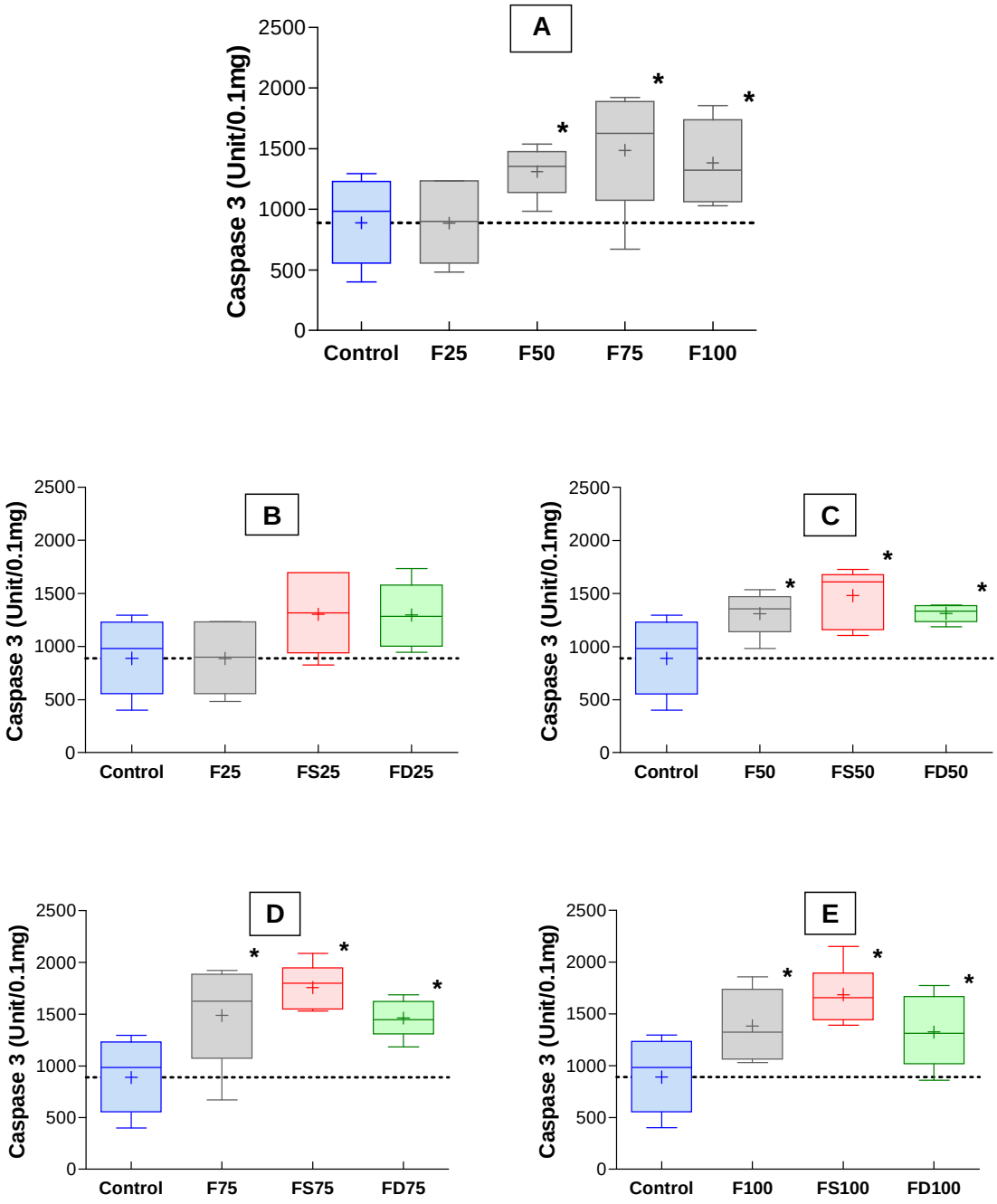


Figure 30: Rats' hepatic caspase-3 activity
A) in control group vs. fluvastatin toxicated with gradual doses groups, and in control group vs. groups toxicated for 7 days with B) 25, C) 50, D) 75, E) 100 mg/kg/day fluvastatin, without and with treatment (silymarin and DDB).

F X = rats received X mg/kg/day fluvastatin for 7 days
FS X = rats received for 7 days X mg/kg/day fluvastatin +140 mg/kg silymarin,
FD X = rats received for 7 days X mg/kg/day fluvastatin + 100mg/kg DDB
*Significant difference when compared to normal group

4.3.2. Hepatic cytochrome C

Hepatic cytochrome C concentration showed no significant difference from normal values, and all values were varying from 94-109% the normal value. (Table 14 and Figure 31)

Table 14: Rats' hepatic cytochrome c concentration in normal group, and groups toxicated with gradual doses of fluvastatin (without and with 140 mg/kg/day silymarin or 100 mg/kg/day DDB) for 7days.

Animal groups		Fluvastatin dose for 7days	Cytochrome c (ng/mg) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**2.94 ±0.09**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**3.03 ±0.13**	ns (0.8867)
	F-50	50 mg/kg/day	**2.91 ±0.09**	ns (0.4719)
	F-75	75 mg/kg/day	**3.16 ±0.10**	ns (0.9835)
	F-100	100 mg/kg/day	**3.19 ±0.10**	ns (0.2307)
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**2.77 ±0.08**	ns (0.0907)
	FS-50	50 mg/kg/day	**2.83 ±0.07**	ns (0.1797)
	FS-75	75 mg/kg/day	**3.03 ±0.12**	ns (0.8925)
	FS-100	100 mg/kg/day	**2.94 ±0.10**	ns (0.6449)
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**2.88 ±0.12**	ns (0.4108)
	FD-50	50 mg/kg/day	**2.89 ±0.10**	ns (0.4263)
	FD-75	75 mg/kg/day	**2.79 ±0.09**	ns (0.1271)
	FD-100	100 mg/kg/day	**3.02 ±0.17**	ns (0.9402)

*** Significant difference from normal control.** **ns No significant difference from normal control**

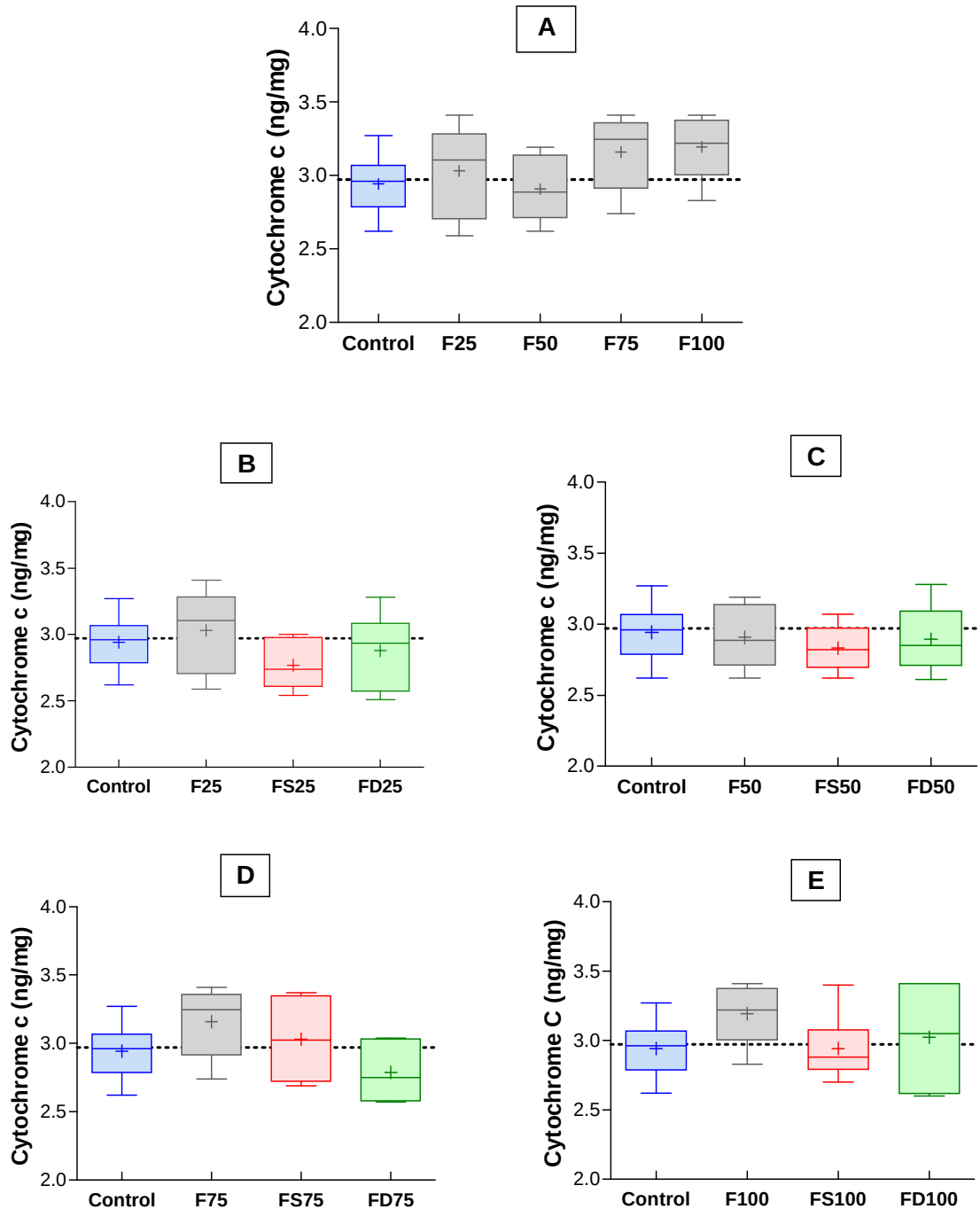


Figure 31: Rats' hepatic cytochrome c concentration A) in control group vs. fluvastatin toxicated with gradual doses groups, and in control group vs. groups toxicated for 7 days with B) 25, C) 50, D) 75, E) 100 mg/kg/day fluvastatin, without and with treatment (silymarin and DDB).

F X = rats received X mg/kg/day fluvastatin for 7 days
FS X = rats received for 7 days X mg/kg/day fluvastatin +140 mg/kg silymarin,
FD X = rats received for 7 days X mg/kg/day fluvastatin + 100mg/kg DDB
*Significant difference when compared to normal group

4.3.3. Hepatic p53

No significant difference in p53 concentration was recorded, although nearly in all groups p53 decreased by 40-60% the normal mean value. However, FS-25 group showed increase nearly by 50% the normal p53 value, and in similar group treated with DDB (FD-25) p53 was just 70% the normal value. (Table 15 and Figure 32)

Table 15: Rats' hepatic p53 concentration in normal group, and groups toxicated with gradual doses of fluvastatin (without and with 140 mg/kg/day silymarin or 100 mg/kg/day DDB) for 7days.

Animal groups		Fluvastatin dose for 7days	p53 (pg/mg) Mean ± SEM	Significance *(p value)* versus NC
Normal group	**NC**	0 mg/kg/day	**7.98 ±2.22**	
Fluvastatin toxicated groups	**F-25**	25 mg/kg/day	**3.84 ±0.95**	ns (0.1460)
	F-50	50 mg/kg/day	**4.41 ±0.83**	ns (0.1632)
	F-75	75 mg/kg/day	**3.06 ±0.15**	ns (0.0783)
	F-100	100 mg/kg/day	**3.79 ±0.67**	ns (0.1310)
Silymarin and fluvastatin groups	**FS-25**	25 mg/kg/day	**11.26 ±3.02**	ns (0.4031)
	FS-50	50 mg/kg/day	**4.92 ±1.40**	ns (0.2712)
	FS-75	75 mg/kg/day	**3.65 ±0.15**	ns (0.1096)
	FS-100	100 mg/kg/day	**4.74 ±0.93**	ns (0.2084)
DDB and fluvastatin groups	**FD-25**	25 mg/kg/day	**5.66 ±1.35**	ns (0.3944)
	FD-50	50 mg/kg/day	**4.16 ±0.95**	ns (0.1453)
	FD-75	75 mg/kg/day	**3.03 ±0.41**	ns (0.0800)
	FD-100	100 mg/kg/day	**4.56 ±0.57**	ns (0.1964)

*** Significant difference from normal control.** **ns No significant difference from normal control**

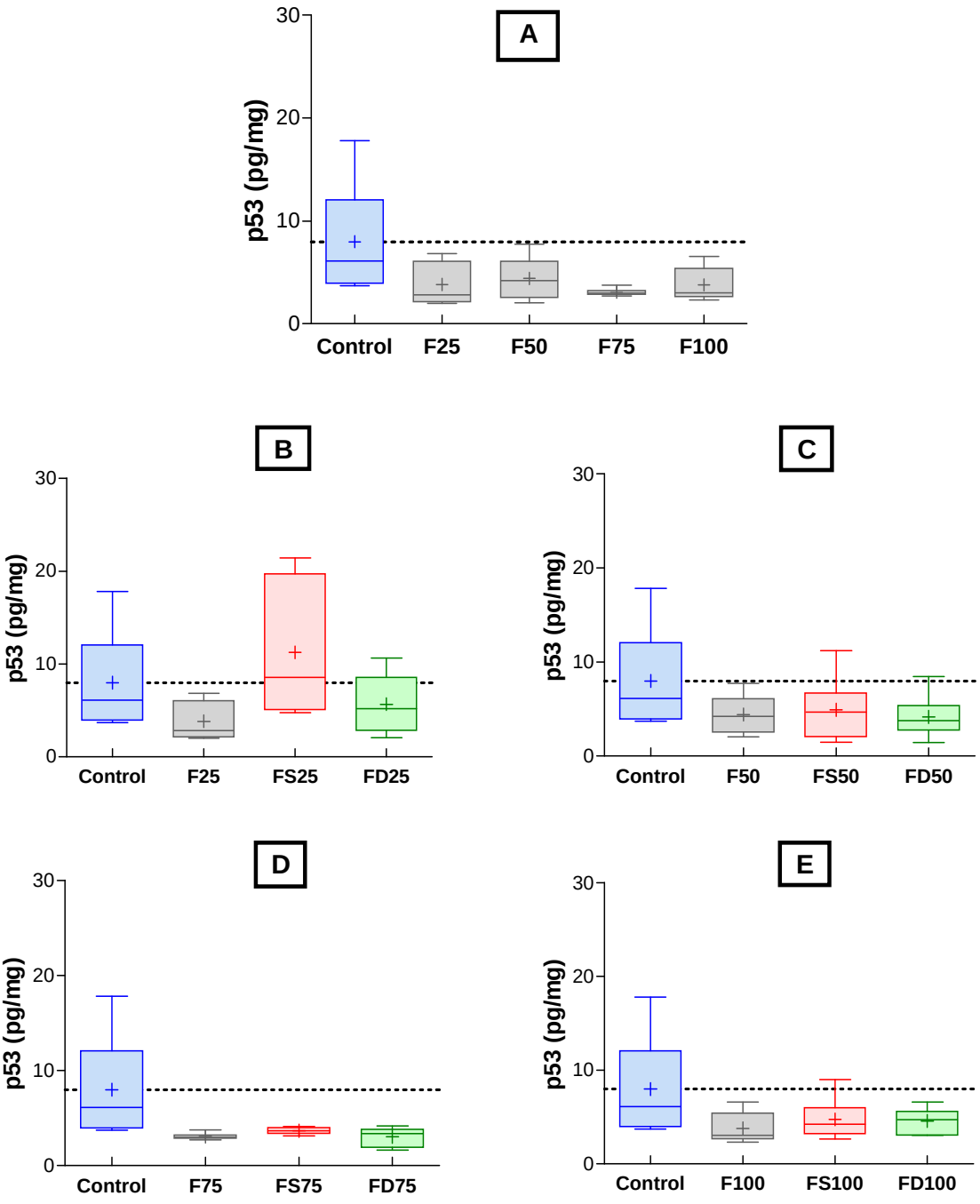


Figure 32: Rats' hepatic p53 concentration
A) in control group vs. fluvastatin toxicated with gradual doses groups, and in control group vs. groups toxicated for 7 days with B) 25, C) 50, D) 75, E) 100 mg/kg/day fluvastatin, without and with treatment (silymarin and DDB).

F X = rats received X mg/kg/day fluvastatin for 7 days
FS X = rats received for 7 days X mg/kg/day fluvastatin +140 mg/kg silymarin,
FD X = rats received for 7 days X mg/kg/day fluvastatin + 100mg/kg DDB
*Significant difference when compared to noramzl group

4.3.4. DNA electrophoresis

DNA electrophoresis showed no DNA ladder pattern in fluvastatin toxicated groups just fragmentation, with distinctive 700bp band shown.

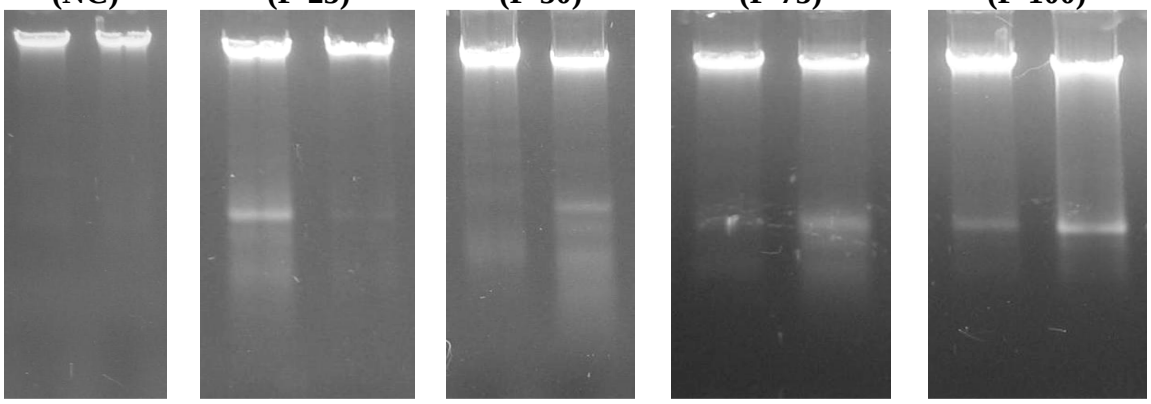


Figure 33: Hepatic DNA electrophoresis in normal group, and groups toxicated with gradual doses of fluvastatin for 7 days

In groups treated with silymarin, fragmentation and DNA ladder pattern appeared at low toxic doses (FS-25 and FS-50), however as fluvastatin dose increase to 75 (FS-75) and 100mg/kg/day (FS-100) a more distinctive DNA ladder pattern start to appear.

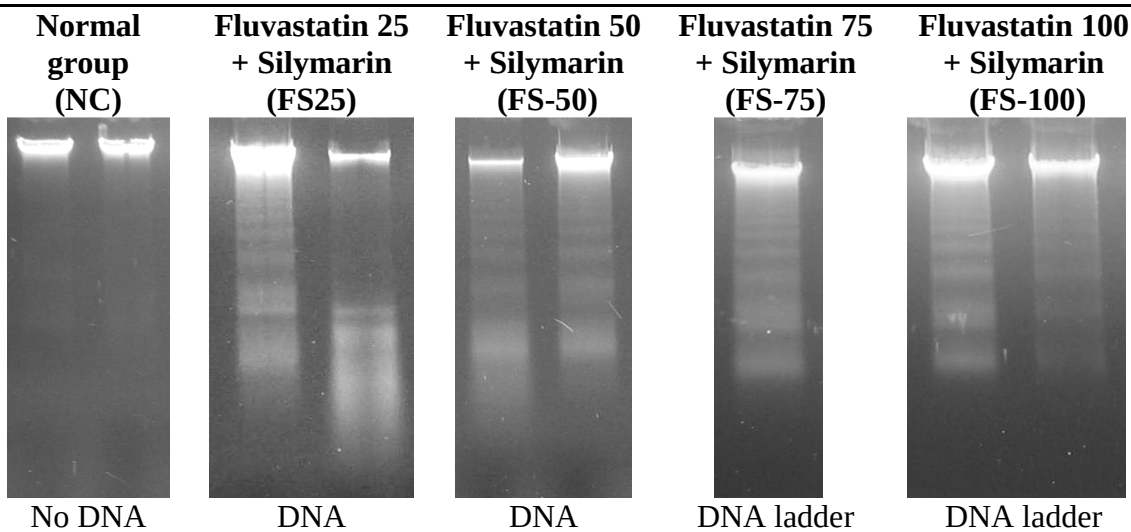


Figure 34: Hepatic DNA electrophoresis in normal group, and groups received 140mg/kg/day silymarin with gradual doses of fluvastatin for 7 days.

At DDB treatment, mixed DNA pattern are shown. First at FD-25 was similar to FS-25 showing DNA fragmentation and to F-25 showing distinctive band at 700bp, at FD-50 and FD-75 a more necrotic pattern, last at FD-100 DNA ladder start to appear.

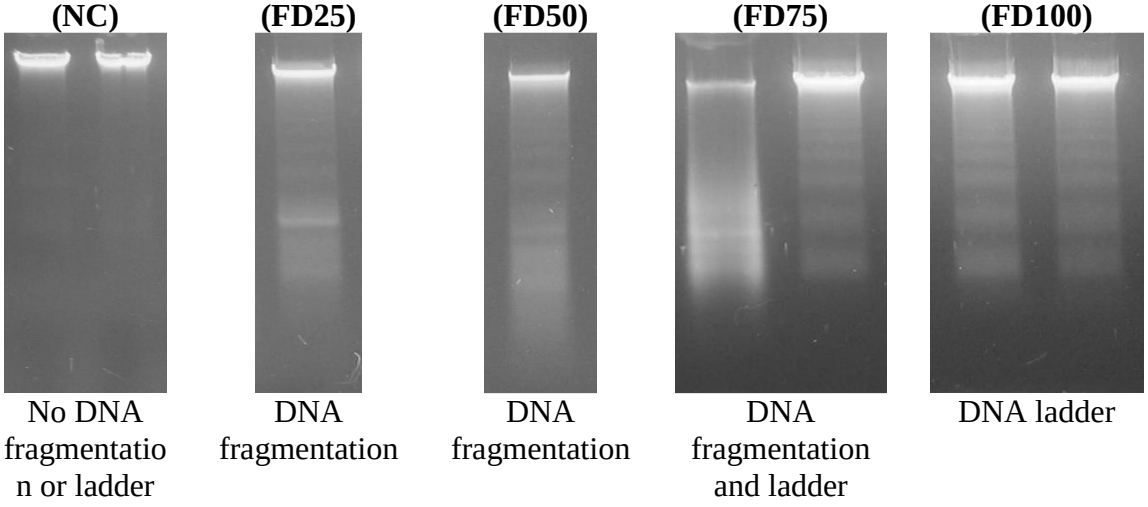


Figure 35: Hepatic DNA electrophoresis in normal group, and groups received 100 mg/kg/day DDB with gradual doses of fluvastatin for 7 days

4.4. Correlation between various parameters:

The increase in fluvastatin dose was accompanied with an increase in relative kidneys weight, ALT, AST, and HDL levels. At same time, the increase in dose was accompanied with a decrease in body weight gain, glucose, and albumin levels. As for apoptotic parameters, both caspase-3 ($r = +0.42$) and p53 ($r = -0.28$) had a significant weak correlation with fluvastatin dose.

Rats' serum glucose level has been positively correlated with body weight gain, relative liver weight, GGT, and triglyceride. At same time, the increase in relative kidney weight, ALT, AST and caspase-3 levels was accompanied with decrease in glucose level.

Hepatic inflammatory markers (ALT, AST, and GGT) were correlated among them selves and with body and organs weight. Moreover, ALT correlated negatively with triglyceride, and AST correlated negatively with albumin. From serum protein parameters, only albumin correlated with fluvastatin dose, body weight gain, relative kidneys weight, AST and of course with total protein level.

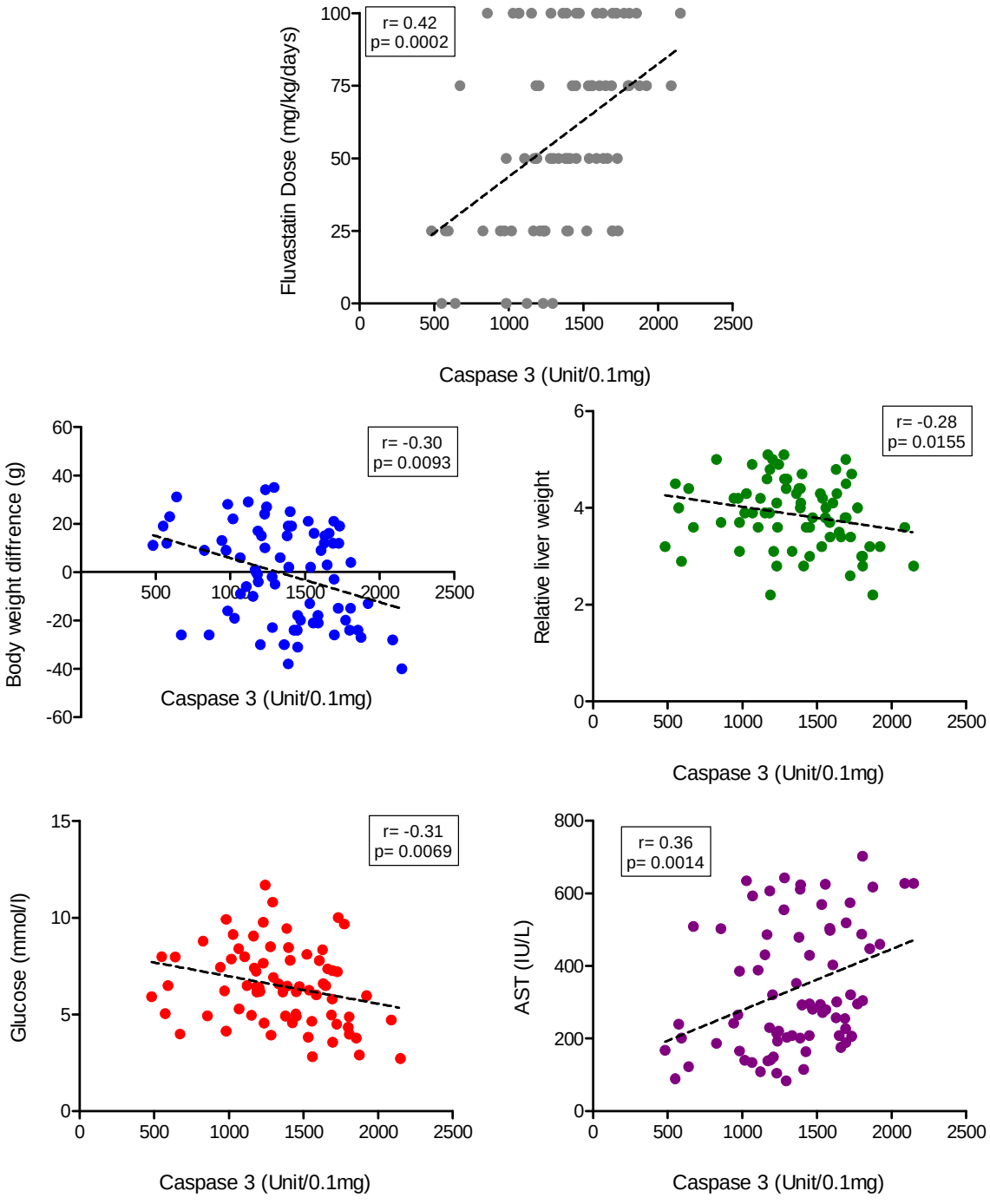
Table 16: The significant nonparametric correlation - Spearman r values (p value) of various parameters

	Flu Dose	Wt diff	Relative Liver wt	Relative kidneys wt	Glu	ALT	AST	GGT	T Protein	Alb	Chol	TG	HDL	Casp-3	Cyto c	P53
Flu Dose		***-0.76***		**+0.44**	***-0.52***	**+0.53**	**+0.61**			***-0.40***			**+0.24**	**+0.42**		***-0.28***
Wt diff	(<0.0001)		**+0.29**	***-0.42***	**+0.57**	***-0.60***	***-0.64***	**+0.26**		**+0.41**			***-0.23***	***-0.30***	***-0.31***	
Relative Liver wt		(0.0102)			**+0.53**	***-0.26***		**+0.28**				**+0.28**		***-0.28***		**+0.23**
Relative kidneys wt	(0.0001)	(0.0001)			***-0.34***	**+0.39**	**+0.35**			***-0.23***						
Glu	(<0.0001)	(<0.0001)	(<0.0001)	(0.0030)		***-0.54***	***-0.50***	**+0.29**				**+0.48**		***-0.31***		
ALT	(<0.0001)	(<0.0001)	(0.0212)	(0.0006)	(<0.0001)		**+0.76**					***-0.26***				
AST	(<0.0001)	(<0.0001)		(0.0019)	(<0.0001)	(<0.0001)				***-0.23***				**+0.36**		
GGT		(0.0304)	(0.0172)		(0.0161)										***-0.25***	**+0.49**
T Protein										**+0.26**						
Alb	(0.0003)	(0.0002)		(0.0440)			(0.0428)		(0.0222)						***-0.25***	
Chol													**+0.45**			
TG			(0.0151)		(<0.0001)	(0.0255)										
HDL	(0.0371)	(0.0492)									(<0.0001)					
Casp- 3	(0.0002)	(0.0093)	(0.0155)		(0.0069)		(0.0014)									
Cyto c		(0.0069)						(0.0402)		(0.0340)						
p53	(0.0152)		(0.0483)					(<0.0001)								

Flu Dose: Fluvastatin Dose, Wt diff: Body Weight difference, Glu: glucose, T Protein: Total Protein, Alb: Albumin, Chol: Cholesterol, TG: Triglyceride, Casp-3: Caspase 3, Cyto c: Cytochrome c

Hepatic apoptotic parameters also showed some correlation with other weight and serum biochemical parameters, but all the correlations were of weak Spearman r values. Caspase-3 correlated positively with fluvastatin dose and AST, and in same time correlated negatively with body weight gain, relative liver weight, and glucose.

Figure 36: Correlation between caspase 3 and fluvastatin dose, body weight difference, relative liver weight, glucose and AST levels.



Cytochrome c showed negative correlation with body weight gain, GGT, and albumin levels. P53 correlated negatively with fluvastatin dose, and positively with relative liver weight and GGT.

Figure 37: Correlation between cytochrome c and body weight difference, albumin and GGT.

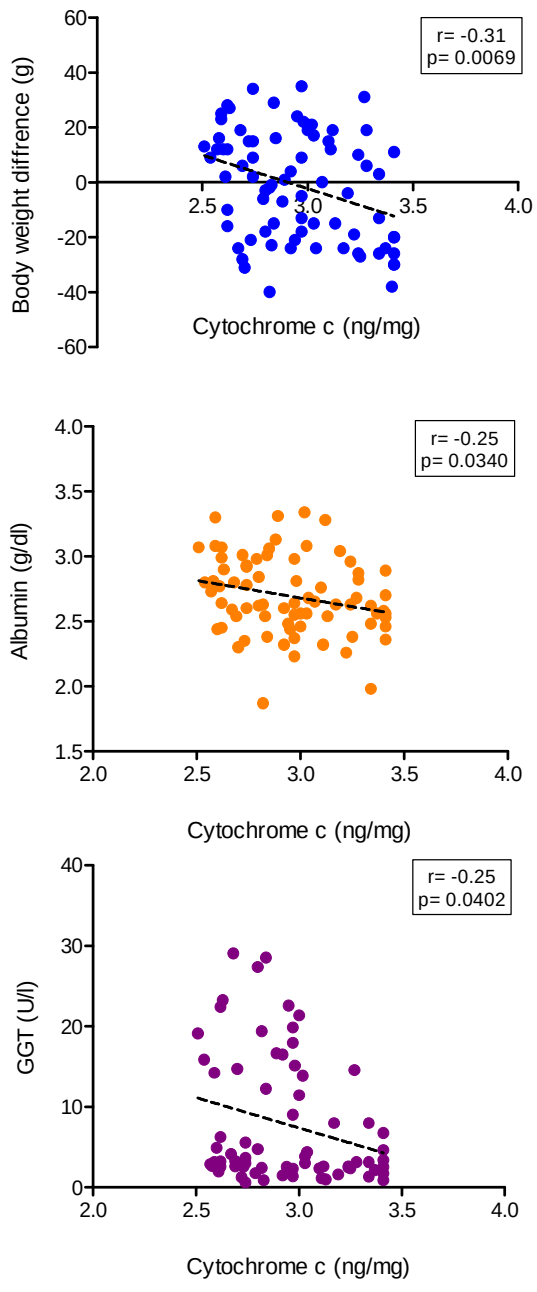
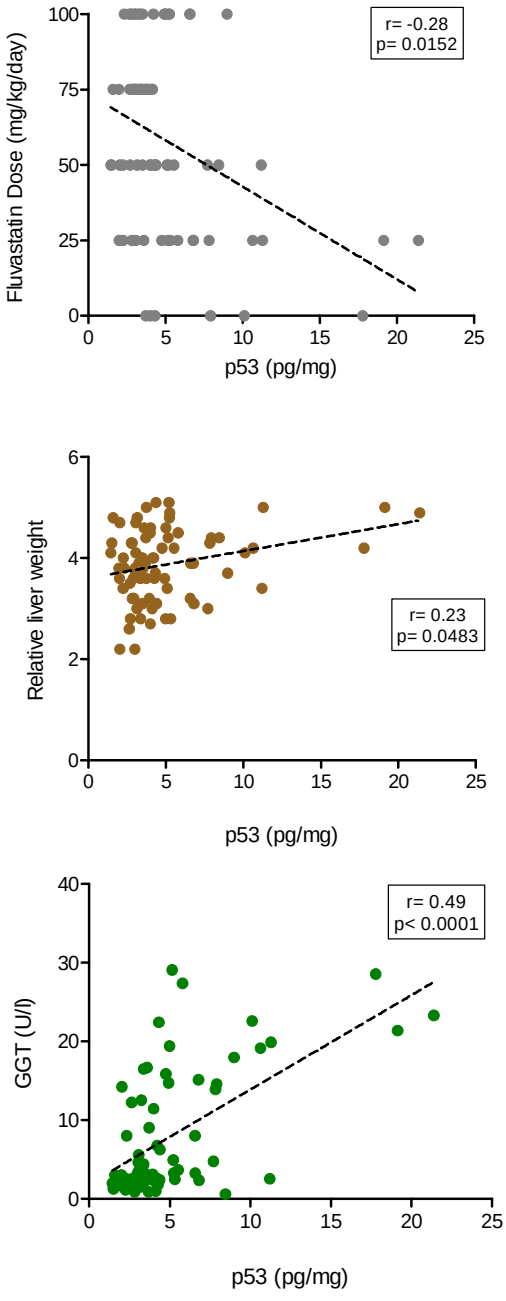


Figure 38: Correlation between p53 and fluvastatin dose, relative liver weight and GGT.



6. Discussion

The liver has the capacity to protect itself against chemical or biological injury by a variety of molecular and enzymatic entities. Nevertheless, with sufficient exposure of this injuring substance exceeding hepatocyte defense system, various processes of macromolecular disruption occur in the liver, leading to loss of cell homeostasis and subsequently cell death (154, 192). Usually, the liver tends to regain its previous healthy condition by metabolizing and eliminating the toxic substance, fighting back the biological insulting factor, adjusting the cell function to over-counter the disturbed homeostasis, and/or removing the damaged cells by apoptosis and stimulating proliferation new healthy cells.

To study such changes in liver condition under stress, a well-defined laboratory model, which can mimic completely the liver impairment, especially viral infection, is still demanded. A reproducible tissue culture model of hepatitis viral B infection does not exist, nor is hepatitis B infectious for immunologically well-defined laboratory animals (202). Although, advances have been made, a reliable *in vitro* cell culture system for hepatitis C is not yet available (16). That is why, in this thesis it was aimed to establish new experimental hepatitis model, where the hepatic damage was gradual and controlled. Thus, it will enable investigating the effect of various drugs on gradual stages of progressive liver impairment.

The new experimental model presented in this thesis was conducted on male Wistar rats. The choice of rat as experimental model was considered because of the following factors: rat's small dimension, lower cost, relative facility of handling, great resistance to infection, short life span, and high scale reproduction. Moreover, in drug liver toxicity studies on rats are preferred, as rat and dog have a longer residence time for xenobiotics, at peak concentrations, compared to humans, making the livers of these species more vulnerable to chemical injury (143).

In this thesis, investigation on the possibility of using fluvastatin as toxic substance for establishing a new experimental model that mimics hepatitis liver condition in experimental animals (Wistar rats) was conducted. Fluvastatin is one

compound that belongs to statin family, and it is potent inhibitor of cholesterol synthesis in liver (30). Despite clinical evidence on efficacy, higher doses of statins are however less prescribed, due to safety concerns, as it was reported one of its major complaint during statin therapy is hepatotoxicity (98). Moreover, Cokca and his colleges (31) reported that high-dose therapy with statins leaded to toxicological situation that mimics viral hepatitis. Thus, statins were considered in establishing the new experimental model of hepatitis.

Fluvastatin was chosen among the statin family, due to its hydrophilic nature. Fluvastatin appears in much higher concentrations in the liver than other statins, where liver is the drug's primary site of both action and one of side effects (155), as marked increase in serum aminotransferases occurred in patients who received fluvastatin for an extended period of time (54, 170). Fluvastatin side action can be explained by the laborious attempt of the liver to counteract the drug action and maintain normal cholesterol synthesis rates. Fluvastatin treatment triggered induction of enzymes of cholesterol synthesis pathway, upstream and downstream of the target enzyme HMG-CoA reductase, and may cause alterations in many enzymes and proteins, which affect cell signalling, membrane trafficking, gene transcription and membrane stability (118, 167). Moreover, HMG-CoA synthase is present in cytoplasm and mitochondria, and both of these enzymes were increased several folds by the statin treatment. While cytosolic enzyme is involved in cholesterol biosynthesis, mitochondrial enzyme is part of the ketone body synthesis pathway (167), which can explain the involvement of statins in various cell functions.

LESCOL Prescribing Information (106) stated the maximum fluvastatin tolerated dose in rats was determined to be 9 mg/kg/day. Steiner et al. (167) showed that 24 mg/kg/day fluvastatin given to male F344 rats for 7 days caused significant changes in 58 liver proteins. This means that 24 mg/kg/day, which represents 34 times the mean human plasma concentration after 40mg oral dose, is the toxic dose at which fluvastatin starts to disturb hepatocytes homeostasis in rats. Thus, in this thesis fluvastatin was chosen because the dose is concentrated in the liver, and the doses were chosen starting from 25 to 100 mg/kg/day for 7 days to insure evidence of fluvastatin toxic effect on the liver.

Results in this thesis showed that, Fluvastatin when given in gradual toxic doses caused a disturbance in many parameters; the first noted parameters were the animals' survival and body weight change during the experiment. Rats tolerated nearly all doses, as the survival rate was 100% in 25, 50 and 75 mg/kg/day fluvastatin for 7 days, although rats showed impairment in liver conditions by serum and liver biochemical analysis. However, when dose reached 100 mg/kg/day the survival rate was 75% by the end of 7^{th} day of fluvastatin administration. These findings agree with LESCOL Prescribing Information (106), as fluvastatin rats' LD50 (for acute toxicity) was 707 mg/kg when taken orally, and 140 mg/kg when taken intra-peritoneal, while the minimal tolerated toxic dose for hepatocellular necrosis is 10 mg/kg/day.

As for the effect of fluvastatin toxic doses on rats' body weight change, results showed a decrease in body weight gained during seven days. This decrease started to appear from lowest toxic dose 25 mg/kg/day, however this decrease was not significantly different from normal rats, and this can mean that rats tolerated this dose. This agrees with Steiner et al. (167) result, as they reported that animals tolerated and survived 24 mg/kg/day fluvastatin for 7 days, and the average weight gain was 43.5 g in control animals and 5.4 g in the 25 mg/kg/day group. In thesis's results, the weight gain in normal control was 27.7 g, while in 25 mg/kg/day fluvastatin group was 17.5g. The difference between results may be due to the difference in drug administration as in this thesis drug was given intragastrically, while in Steiner et al. (167) study drug was dissolved in fed water, and animals consumed it by choice.

As fluvastatin dose increased to 50, 75 and 100 mg/kg/day, the rats' body weight started to show more tendency to loss body weight rather than gain weight. This can be explained by two possibilities. The first assumption is that rats in these groups were more anorexic and their food consumption decreased due to toxic effect of fluvastatin (167). The other assumption is the involvement of rats muscle toxicity in the fluvastatin toxicity causing waste symptom, as LESCOL Prescribing Information (106) stated that fluvastatin minimum toxic dose for skeletal muscle degeneration is 50 mg/kg/day.

This thesis also aimed to challenge two of the common hepatoprotective drugs (silymarin and DDB) against impaired liver function caused by fluvastatin, and note their effect on liver function under stress. Therefore, the effect of both drugs treatment on rats toxicated with gradual doses of fluvastatin was observed.

Silymarin treatment (140 mg/kg/day) while rats toxicated with fluvastatin, showed a protective effect on body weight but only at lowest doses 25 and 50 mg/kg/day fluvastatin. However, silymarin failed to exert this action in higher fluvastatin doses 75 and 100 mg/kg/day. The same pattern was showed in groups challenged with DDB treatment (100 mg/kg/day). However, the weight gain in 50 mg/kg/day fluvastatin treated with DDB was twice the weight gain in same group treated with silymarin, and the weight loss in higher doses was less than silymarin comparative groups.

These results agreed partially with Abdel Hameid (2) study, as he stated that treatment with DDB (100 mg/kg) or silymarin (200 mg/kg) for 14 days improved the body weight loss caused by erythromycin stearate (100 mg/kg) toxicity. He explained this improvement by the fact that these drugs exerted antioxidant and hepatoprotective effects. From above, it can be concluded that silymarin and DDB showed their protective effect whilst the liver damage is still manageable at low toxic doses. Once the liver damage increased drastically, both drugs failed to stop the loss of body weight. However, in weight parameter, DDB showed more efficiency and usefulness than silymarin in minimizing the body weight loss.

Fluvastatin toxic dose increase in this study correlated not only with body weight difference, it correlated also with three of serum metabolic products: glucose, albumin, and HDL, which signify the impairment of these three compounds production and homeostasis in case of fluvastatin toxicity. Body weight difference was found to be also associated with liver transferases level and two of apoptotic parameters (caspase-3 and cytochrome c), indicating fluvastatin toxicity caused loss of cells both by necrotic and apoptotic cell death.

Some authors stated that increase in relative liver weight is a reliable indicator of toxic adaptive effect. Increases in liver weights, especially with chronic toxic exposure, can result from both hypertrophy and hyperplasia of hepatocytes. In results, a decrease in relative liver weight was observed only in group taking fluvastatin lowest toxic dose 25mg/kg/day; however, in rest of doses the relative liver weight increased. This decrease was associated with decrease in body weight gain, which can mean that liver at this dose had not yet started its compensatory proliferating mechanism in order to replace the damaged cells by increasing liver cell mass. In higher fluvastatin toxic

doses, the increase of relative liver weight can be due to hepatic inflammation and increase in liver macrophages infiltration, or the increase in liver capacity to respond to stress, by raising the level of homeostasis for protein catabolism. It can also reflect increased cell proliferation and replacement of damaged cells by apoptotic pathway (192), as an increase in liver apoptotic parameters was also noted.

The negative correlation between body weight gain and relative liver weight was also observed by Abdel Hameid (2), that erythromycin stearate toxicity induced a liver weight increase, which was attributed to the degenerative effects in animals' organs and loss of body weight. However, in his study treatment with DDB or silymarin along with erythromycin stearate failed to restore the liver weight to normal range. In thesis results another outcome was observed, groups treated with silymarin showed nearly stable relative liver weight at low fluvastatin doses (25 and 50 mg/kg/day). This may be because silymarin was found to robust liver growth and the liver tissue weight, as was shown in other studies that used carbon tetrachloride toxicated groups (61, 97). While, in DDB treated groups, the liver weight ratio was normal in all fluvastatin toxic doses, showing that DDB also overcome silymarin protective action in relative liver weight. It can be explained by anti-inflammatory action of DDB, and its effect on whole body weight.

Kidneys usually were not affected until the liver reached the decompensated stage, which affect the kidney function. Such was shown in the group receiving only 100 mg/kg/day fluvastatin and in same group treated with silymarin, as relative kidneys weight had increased. Renal damage with statins use can be explained by kidneys inflammation as a subsequent event of severe liver impairment, or it can be explained by statins rhabdomyolysis that cause acute tubular necrosis (12). Silymarin failed in protecting the kidney, can be due to it lacks the antioxidant silymarin activity in kidneys. As Valenzuela and his colleges (182) stated that silymarin have a role as regulators of the content of GSH in various organs, such as liver, intestine, and stomach, whereas there were no changes in the lungs, spleen, and kidneys GSH content.

Fluvastatin also showed a disturbing effect of on some of metabolic biochemical parameters, which can be also related to hepatotoxicity of fluvastatin high doses. As it is known that hepatocyte functions in controlling various metabolic products such as

serum glucose homeostasis, by controlling glucose uptake, glycolysis, and gluconeogenesis. Hepatocytes are also the main site of cholesterol, albumin, fibrinogen and bile synthesis. In addition, they are responsible for glutathione, amino acids, ammonia and xenobiotics metabolism and conjugation. Unlike other cells, hepatocytes have flexible energy reserves, and can produce energy both for their own needs and for other tissues, which occurs via production of glucose by many pathways such as from carbohydrates via hexokinase and the liver-specific glucokinase, from amino acid via phosphoenolpyruvate carboxykinase, and from glycerol via glycerol kinase. However, in stressful and injurious situations, these cellular adaptive mechanisms do not function properly because of hepatocytes hyper-metabolic state, which increases the needed energy (192).

For previous information, it can be concluded that liver toxicity can affect the serum glucose level, especially when liver damage exceeds hepatocytes ability to compensate. However, as noted in results, fluvastatin liver toxicity caused a significant decrease in serum glucose level. There are possible mechanisms that can be the cause of decrease in serum glucose level. Rats' anorexia and body weight loss can cause decrease in glucose production, or the hepatotoxic effect of fluvastatin reflected in the decrease of glucose level, which consequently causes the body weight loss. The second stated reason is most possible explanation, as the influence of fluvastatin on glucose level was explained by Steiner et al. (167) findings. They stated that, fluvastatin altered some carbohydrate metabolism enzymes. It down-regulated fructose-1,6-bisphosphatase, a key regulatory enzyme necessary for gluconeogenesis, down-regulated ketohexokinase that involved in metabolism of fructose, and down-regulated pyruvate kinase that involved in the final step in glycolysis. It is also up-regulated glucose-6-phosphate 1-dehydrogenase, which is the first enzyme in the pentose phosphate pathway. Such alternation in normal hepatocyte enzyme function ay cause a decrease in glucose produced from gluconeogenesis, or fructose metabolism, or tended to be directed more to pentose phosphate pathway.

Silymarin treatment kept glucose level within normal range only at fluvastatin low toxic doses (25 and 50 mg/kg/day); however DDB treatment attenuated the glucose decrease just at 25 mg/kg/day fluvastatin. The effect of silymarin at low fluvastatin doses can be explained by its protective action, as it was found that 140 mg/kg

silymarin treatment for 4 days protected the hepatic structures, liver glucose stores, and enzyme activity in galactosamine models of acute hepatitis in rats (175).

Other metabolic parameters were also affected by the impairment in liver function, such as serum total protein and albumin levels, as a variety of metabolic derangement, as proteins level, was found to occur in patients with chronic liver disease. Moreover, in cirrhotic patients a high protein catabolism and hyper-metabolic state was frequently observed. Many symptoms and markers were related to the impairment of amino-acid turnover, such as muscle wasting, ascites and bleeding due to albumin and clotting factors production impairment, and hepatic encephalopathy by the impairment of urea production (176).

A drastic change in total protein and albumin concentration was not seen in this thesis. This may be due to the liver impairment in this new experimental groups did not reach the cirrhotic decompensated liver condition, which affect cell amino acid turnover. Total Protein concentrations were nearly normal in all groups, except rats toxicated with 25 mg/kg/day fluvastatin showed a significant increase in total protein concentration. This increase may be due to increase in protein production as a feed back of impact of injury of hepatocytes homeostasis or due to increase of immunoglobulin production. Treating rats toxicated with 25 mg/kg/day fluvastatin with silymarin or DDB decreased the raise in total protein to normal limits. This positive effect of silymarin on liver proteins was also noted by other researchers, for example, silymarin (140 mg/kg for 4 days) has abolished the inhibitory effect of galactosamine on liver proteins and glycoproteins biosynthesis (180).

As for the influence of liver diseases on albumin homeostasis, it varies according to the severity and type of the disease. In acute and in chronic persistent hepatitis serum albumin is usually normal or minimally decreased. However, in chronic active hepatitis, serum albumin is often decreased and the immunoglobulin is increased. In alcoholic hepatitis, serum albumin is usually also normal but can be decreased due to poor nutrition (193). In thesis results, albumin concentration in groups toxicated with gradual doses of fluvastatin showed a tendency to increase at low toxic doses (25 and 50 mg/kg/day), then albumin returned to normal values at higher toxic doses (75 and 100 mg/kg/day), weather rats received treatment or not. These fluctuations can be

considered a reflection of liver management to compensate the toxic action of fluvastatin on hepatocytes albumin production and metabolism. However, as the liver condition is deteriorated more drastically under heavy toxic pressure, in addition to animals' anorexia and malnutrition, albumin production started to decrease.

Both of hepatoprotective drugs (silymarin and DDB) did not improved the albumin level. That was in agreement with Abdel Hameid (2), who found that treatment of DDB or silymarin did not restore the albumin level to the control value, and with Buzzelli and his co-workers (22), who stated that silymarin reduced liver aminotransferases levels, but without consistent differences in albumin. Although, silybinin was reported to induce ribosomes formation, DNA synthesis, and protein synthesis in only injured liver cells (165). Moreover, Kim et al. (90) stated that DDB reduces the total proteins levels, and albumin to normal level.

From correlation results, only albumin showed small but significant correlation with hepatic cytochrome c. This implicate that serum albumin concentration, which is usually used to evaluate synthetic function of hepatocytes, is influenced by mitochondrial integrity and function. In addition, this may indicate that when hepatocytes proceed in apoptotic pathway, they also stop synthesizing essential body elements, such as albumin.

Observing the results, serum cholesterol was normal in all groups, although they all received high doses of fluvastatin, which is HMG-CoA reductase inhibitor. Moreover, rats receiving DDB (100 mg/kg/day) with lowest toxic fluvastatin dose (25 mg/kg/day) had an increase in cholesterol concentration. As the fluvastatin dose increased in groups treated with DDB, cholesterol level returned to normal. This means that fluvastatin, which is cholesterol synthesis inhibitor; is successful in lowering elevated cholesterol level more than reducing normal cholesterol level, and its toxic effect does not appear much on this parameter. On the other hand, serum triglycerides values were significantly less than normal in groups toxicated with fluvastatin. Treating with silymarin and DDB managed to normalized triglyceride concentration, but only in groups toxicated with 25mg/kg/day fluvastatin. HDL concentration showed significant increased but starting from 50 mg/kg/day fluvastatin. Moreover, treatment with silymarin and DDB lowered this raise but only at 50 mg/kg/day fluvastatin.

From previous results, it can be concluded that fluvastatin cause no change on cholesterol level, a decrease in triglycerides, and an increase in HDL level in normal rats. The last two parameters (triglycerides and HDL) behavior can be explained by statin mechanism of action. HMG-CoA reductase is the rate-limiting enzyme of the mevalonate pathway, through which cells synthesize cholesterol from acetate moieties, the enzyme inhibition by statins cause a reduction in cholesterol synthesis, induction of LDL-receptors, thus increase in LDL and cholesterol uptake from plasma (59). Thus, statins also increased the level of HDL to facilitate cholesterol uptake, and influence plasma triglycerides and non-essential fatty acids levels (55, 166).

The results showed that silymarin had a normalizing effect on the reduction in triglycerides and the increase in HDL, but only at low doses of fluvastatin toxicity. The influence of silymarin on hepatic lipid profile was in agreement with some researchers findings. Skottova and Kreeman (160) noted that silybinin is able to antagonize partly the increase in total lipids and triglycerides produced in rats' liver by carbon tetrachloride. On the other hand, results did not agree with other researchers, such as Mourelle and Franco (123) who stated that silymarin had an effect on membrane lipids (both cholesterol and phospholipids) and other lipid compartments in the liver; this may influence lipoprotein secretion and uptake. In addition, other scientists stated that silymarin did not appear to be able to normalize the reduction in triglycerides in serum produced by thioacetamide (97). Moreover, silymarin treatment failed to reduce the total lipids significantly during erythromycin stearate induced hepatotoxicity (2).

As for the other drug, DDB, Abdel Hameid (2) results showed that it showed protection against plasma total lipids changes induced by erythromycin stearate, while Helal and his colleges (69) found that DDB along with erythromycin stearate treatment induced a significant elevation of plasma total lipids. Other scientists stated that DDB treatment could decrease hepatic (but not serum) lipid levels in various hypercholesterolemia mouse models, however it was also found to cause an increase in serum and/or hepatic triglycerides level in mice. This DDB hypertriglyceridemic action was accompanied by a decrease in hepatic total cholesterol level in normocholesterolemic mice, and it was explained by DDB stimulating esterification and/or inhibits β-oxidation of fatty acids (134). However, this thesis results showed an increase in triglycerides but also in cholesterol levels at least in 25 mg/kg/day fluvastatin toxicated group.

Although the DDB influence on lipid profile was contradictory with others scientists, an interesting finding was observed by Shidoji and Ogawa (158). As they found that natural geranylgeranoic acid was found in Chinese herb, *Schisandra chinensis,* which is the natural source of the schisandrin c that resembles DDB chemically. Geranylgeranoic acid is a product of isoprenoid pathways via the acetate/mevalonate in plants. Mevalonate pathway is the same pathway influenced by statins in hepatocytes. In addition, Geranylgeranoic acid is a derivative of geranylgeranyl diphosphatase GGP, which decreased in fluvastatin-toxicated cells. DDB is chemical mimic of the natural component of Schisandra plant, that means it dose not geranylgeranoic acid. However, whether DDB can be metabolised to a compound resembles geranylgeranoic acid, or has an effect on mevalonate pathway that compensates fluvastatin action, this is a point that needs more investigation.

As fluvastatin was administered in high doses for long period to insure liver cells toxicity, this toxicity showed some change in hepatocytes function and homeostasis. It is most probably that such high doses may also cause cell death, either programmed (apoptosis) in order to remove such damaged cells or un-programmed (necrosis) causing the leak and release of hepatic enzymes in the surrounding area and serum. Thus, in order to investigate the pattern of hepatocytes cell death caused by fluvastatin and the influence of the two hepatoprotective drugs on cell death, parameters of cell necrosis and apoptosis were investigated.

Measurement of aminotransferases levels is considered as the most important laboratory test for hepatitis, because it is an inexpensive and readily available (101). Viral hepatitis and toxic drugs may cause hepatic necrosis, which indicted by 20-50 fold elevation in the serum aminotransferases. Alcoholic hepatitis causes aminotransferases to increase up to 8-10 folds, with AST greater than ALT due to release of m-AST from injured hepatic cell mitochondria (126). The total level of AST usually does not exceed five times normal level, but if it does, concomitant alcoholic rhabdomyolysis should be considered. In viral hepatitis, ALT is higher than AST, however in cirrhosis or chronic persistent hepatitis both are usually normal. Elevations of AST and ALT in cirrhosis indicate continuing hepatocellular necrosis (153).

Nearly all groups, in this thesis, showed AST/ALT ratio exceeding 2:1, which indicate that fluvastatin toxicity cause a hepatitis condition more similar to alcoholic

and drug hepatitis. These also indicate influence of fluvastatin on AST level that may be because of mitochondrial toxicity or muscles rhabdomyolysis. When fluvastatin toxic doses increased, the level of aminotransferases significantly increased in rats toxicated with gradual doses of fluvastatin administered for 7 days. Starting 50 mg/kg/day the increase in ALT was doubled as the dose increased by 25%, while AST increase was slower than ALT.

This increase in aminotransferase release with fluvastatin could be caused by necrotic degeneration of hepatocyte membrane, or by changes in membrane composition induced by reduction of lipid components, especially cholesterol (177). Moreover, some scientists state that elevated aminotransferases levels alone do not usually predict or indicate serious liver injury, it just indicate more hepatocytes cytoplasmic contents leakage or cell necrosis (127, 157).

Silymarin when given with 25 mg/kg/day fluvastatin for 7 days suppressed ALT increase by 75%. As the fluvastatin dose doubled to 50 mg/kg/day, this effect was lost. On the other hand, AST level was not influenced by silymarin treatment. Some authors insisted on silymarin positive impact on liver tests, that silymarin elicit the hepatoprotection by preventing hepatic cell necrosis or by hepatic cell regeneration, also that silymarin tends to normalize liver function test by decreasing the leakage of enzymes, especially in alcoholic liver disease (66).

DDB treated rats, had a different behavior than silymarin treated groups. Although, ALT level increased to nearly twice the normal value, it was nearly steady in all groups in spite of fluvastatin increasing dose. In 75 and 100 mg/kg/day fluvastatin toxicated groups, AST level decreased by DDB treatment. Moreover, Nolan and his co-workers (130) stated that, the increase of aminotransferases less than three times the upper limit of normal might not lead to clinically significant liver damage, because of the liver great capacity to heal injury, with the subsequent development of adaptive tolerance. Although, results did not show normalization of serum ALT by DDB treatment, as it was recorded in many other studies (38, 74, 150), the raise in ALT level in DDB groups is not alarming and indicates DDB stabilize ALT enzyme activity in spite of the increase of the toxic stress on hepatocytes. In addition, Huber et al. (74) stated that DDB did not affect AST and GGT levels, this they suggest that DDB affects the synthesis and/or degradation of ALT in the hepatocytes by a yet unknown mechanism.

In addition to investigating aminotransferases levels in this new experimental model, GGT was also investigated. GGT level signifies the cells membrane integrity; and also glutathione metabolism, and glutamic acid uptake, thus GGT is an extremely sensitive enzyme to identify cholestatic diseases, and it has been taken into account in the evaluation of patients with chronic HCV infection (29, 75). Although GGT level increased as fluvastatin dose increased, but all values were less than normal, which indicating fluvastatin depress enzyme activity at low doses rather than enzyme leakage to plasma. Treating toxicated rats with silymarin and DDB, helped in maintaining their normal GGT level, however only at lowest toxic dose of fluvastatin (25 mg/kg/days).

The silymarin positive impact on GGT was in agreement with Muriel and Mourelle (124) have shown that silybinin preserves the functional and structural integrity of hepatocyte membranes by preventing alterations of their phospholipid structure produced by carbon tetrachloride and by restoring alkaline phosphatase and GGT activities. On the contrary to this thesis finding concerning DDB positive effect on GGT, Botros et al. (19), Huber et al. (74), and Salama et al. (150) stated that although aminotransferases improvement after DDB treatment, yet GGT did not show any significant improvement at any of the time periods examined.

Observing the correlation results, liver enzymes differed in their correlation to apoptotic parameters. As, ALT level showed no correlation, AST correlated with caspase-3, and GGT correlated with cytochrome-c and p53 levels. Such data notes that AST is associated with the end stage of apoptotic elimination, may be through mitochondrial and cell membrane leakage rather than cell burst, while GGT was associated with intrinsic apoptotic pathway, which related to cell recognizes itself damaged beyond repair and starts its own elimination from the surrounding environment.

As some necrosis parameters were investigated, some apoptosis parameters were also investigated. Depending on cell ability to adapt to the toxic stress, necrosis and apoptosis pathways may sometimes intercross. When cell tend to inhibit the necrosis in order to overcome the inflammation and toxic damage, it may have to precede more toward apoptotic pathway in order to remove the same damaged cells. This change in cell death pathway depends on cell energy, as apoptosis depend on cellular ATP level. Apoptosis can be considered the last mechanism of liver defense against toxic or

insulting pressure that disturb hepatocyte function beyond compensate or adaptive mechanisms. Apoptosis enables the elimination of damaged cell without inflammatory consequence, restoring the functional liver cells mass number after induction of proliferation, or sustaining cell cycle to enable DNA repair systems.

Apoptosis was found to proceed through three possible pathways; intrinsic pathway, which may depend on p53 gene and protein, extrinsic pathway that signaled from death receptor and ligand on cell membrane, and the third pathway which is mediated immunological through CTLs. These pathways may interact with mitochondrial role in apoptosis, especially if extrinsic and CTL apoptotic signal need amplification. However, such mitochondrial role is not essential, and apoptosis may find other ways to activate caspase-3, and proceed to dissemble molecular and nuclear cell components. As macrophages and CTLs may result in caspase-8 activation which trigger two signalling pathways; mitochondria-dependent activation of caspase-9 via cytochrome C release, or direct activation of executioner caspases (caspase-3, -6, -7) (48).

Several studies have shown that statins induce apoptosis in a variety of cell lines (18, 81). Moreover, many authors noted that statins have shown to activate caspases (23). Regarding the results, an agreement is noted. Although, fluvastatin lowest toxic dose (25 mg/kg/day) did not affect hepatic caspase-3 activity, high fluvastatin doses 50, 75 and 100 mg/kg increased caspase-3 activity by nearly 50-70%. Moreover, Caspase-3 level was correlated just with glucose level, and there was no significant correlation with biochemical activity (aminotransferase levels), in agreement with Kountouras et al. (95) results. In addition, the negative correlation with glucose may be because caspase-3 and apoptosis consume ATP, which produced mostly by glucose metabolism. When rats were treated with silymarin and DDB while toxicated with fluvastatin, caspase-3 activity increased even at 25 mg/kg/day, and the increase in silymarin was more pronounced than in DDB treated groups. This implicate that both drugs induced apoptosis final step in order to help the liver remove the damaged cells with less inflammatory consequence to the surroundings.

As for hepatic cytochrome c concentration, it did not show any significant difference between different groups, or from normal values. However, these results did not agree with Samson et al. (151) results, may be due to difference in cell model and fluvastatin dose. As they treated cell culture of both resting and activated CD^{4+} T cells

with 10 µM fluvastatin, which caused an increase in the release of cytochrome c from the mitochondria to the cytoplasm. Cytochrome c parameter indicate that apoptosis proceed through mitochondrial changes, as the increase in mitochondria cell permeability cause the release of cytochrome c, which start activating caspase-9 to initiate the executioner caspases (caspase-3). Thus in this new model such role of cytochrome c was avoided may be by other pathways.

Concerning hepatocytes DNA damage, which may be induced by toxic stress, p53 plays the main role in leading to cell cycle arrest allowing damaged DNA to be repaired. Moreover, if DNA repair is not possible, p53 may initiate apoptosis, preventing the replication of abnormal genetic material (173). However, results showed a decrease in p53 by nearly 50% the normal mean value in groups toxicated with fluvastatin from 25 to 100 mg/kg/day. In addition, when rats were treated with silymarin and DDB, p53 decreased except in 25 mg/kg/day fluvastatin groups. At such low toxicity, silymarin managed to raise p53 by 150% the normal value and DDB raised p53 to just 70% the normal value. From results, it can be concluded that these hepatoprotectives may try to help the cells in repairing their DNA, and induce intrinsic cell signalling to commit suicide quietly.

Many mechanisms were postulated to explain the induction of apoptosis in lipophilic statins such as fluvastatin. One of these mechanisms, the apoptosis can be mediated by the inhibition of mevalonic acid pathway products, such as isoprenoids, granylgeranyl and franesyl pyrophosphate and ubiquinone (99). In the case of an additional insult, e.g. a large increase in the statin plasma and tissue concentrations, there may be a massive increase in apoptotic cell death, possibly leading to organ damage (184). Isoprenoids molecules required for the activation of signaling G-proteins, which are important for cell survival (129). Granylgeranyl pyrophosphate has the ability to inhibit apoptosis (196). Ubiquinone is an essential part of mitochondrial respiratory chain, and is responsible for ATP production (32, 35); however, the impact of statins on ubiquinone content depends on statin site of action and statin dose. Simvastatin at 50 mg/kg did not affect skeletal muscle ubiquinone content, but reduced ubiquinone content in the liver and cardiac muscle (52).

Another mechanism was stated by Kaufmann and his co-workers (85); the induction of apoptosis by statins may be initiated by inhibition of protein prenylation, which affect mitochondrial function. That is why it was stated that lipophilic statins are

mitochondrial toxins, leading potentially to energy depletion and oxidative stress, damaging muscles and other tissues. In addition, statin-mediated apoptosis was found to proceed via some mitochondrial apoptotic factors e.g. up-regulation of proapoptotic protein expression (e.g., Bax, Bim) combined with decreased antiapoptotic protein expression (e.g., Bcl-2) (36). Then again, fluvastatin effect on Bcl-2 may depend on its dose, as Xu and his colleges (200) found that fluvastatin at nanomolar concentrations cause up-regulation of Bcl-2, while at high (micromolar) concentrations fluvastatin is pro-apoptotic and has no protective effects. Third mechanism can be that statins autoimmune responses, such as autoimmune hepatitis (8). In addition, statins were reported to lead to growth arrest at the G1/S phase boundary of the cell cycle, where apoptotic cell death follows later. As the presence of cells at sub-G1 peak, induction of caspase-3 activity, and the presence of extensive DNA fragmentation were observed to be induced by fluvastatin (129).

In thesis's new model, fluvastatin apoptotic action may be favoured by the results to be initiated by extrinsic or CTL pathways more than intrinsic pathway, as p53 level decreased, and cytochrome c level did not change in highly toxicated groups with fluvastatin. However, caspase-3 was increased, indicating that proceed of apoptotic signalling avoided mitochondrial damage. Hence, apoptosis in fluvastatin toxicity most probably was initiated by immunological cells (macrophages or CTL) as a reaction of autoimmune hepatitis.

As for the effect of silymarin treatment on cell apoptosis, silymarin was reported to modulate an imbalance between cell survival and apoptosis through interfering with cell cycle regulators and proteins expressions that involved in apoptosis (63, 200). However, there is a debate concerning whether silymarin inhibit apoptosis or induce apoptosis. Results in this thesis showed that silymarin induce apoptosis, as shown in the increase in caspase-3 level, and p53 level. Indicating that silymarin tend to direct the liver cells to be removed by programmed death quietly with less inflammatory condition, and especially using intrinsic pathway by inducing p53 action, however such pathway did not affect cytochrome c and mitochondrial integrity. Such conclusion was in agreement partially with the findings stating that silymarin induce apoptosis by up-regulating expression of pro-apoptotic proteins (such as p53, Bax and caspase-3) with decrease in anti-apoptotic proteins (Bcl-2), while decreasing mitochondrial trans-

membrane potential, thereby increasing levels of cytosolic cytochrome c (146). Moreover, silymarin-induced apoptosis in JB6 C141 cells primarily mediated through a p53-dependent pathway (145).

On the other hand, results did not consent with the statement that silymarin inhibit hepatocyte apoptosis, and normalize the increase in apoptotic proteins in order to protect the genomic DNA (122, 138). In addition, Li et al. (107) found that silibinin treatment eliminate the expression of Fas-associating protein with death domain, followed by inhibit cleavage of procaspase-8 and decrease the release of cytochrome c from mitochondria.

Although, there was no documentation showing the impact of DDB on cell apoptosis, Park et al. (137) noted that schisandrin C induce apoptotic events. This was mediated by inhibition of the expression of anti-apoptotic proteins Bcl-2 and Bcl-xL, and proteolytic activation of caspase-9, leading to the activation of caspase-3, which, in turn, results in PARP degradation. Thesis results agreed with such statement, as results showed increase in p53 level and caspase-3 activity in experimental model induced by fluvastatin toxic doses; however, the influence of DDB on apoptotic pathway was weaker than silymarin in this new model.

DNA fragmentation can be a consequence of induced caspase activity and apoptosis and can also be a consequence of drug-induced hepatotoxicity and lipid peroxidation. DNA fragmentation and lipid peroxidation observed is the normal consequence of oxidative stress that was demonstrated through decreasing GSH and superoxide dismutase activities in liver (10). The DNA laddering technique was used to visualize the endonuclease cleavage DNA products of apoptosis (100, 199). In this thesis, DNA electrophoresis showed no DNA ladder pattern in fluvastatin toxicated groups just fragmentation, and a 700bp band is distinctively shown. Thus, it can be concluded from both caspase-3 results and DNA electrophoresis, that when rats received fluvastatin toxic doses both necrosis and apoptosis happens, but necrosis is dominating, that DNA ladder is not detectable at this stage.

While treating toxicated rats with silymarin, fragmentation, and necrosis appear at low toxic doses, (25 and 50 mg/kg/day) but as dose increase to 75 to 100 mg/kg/day a more distinctive DNA ladder start to appear. At DDB treatment, mixed DNA pattern are shown, fragmentation with a band at 700bp, and DNA ladder. Such observation

confirms that these two drugs tend to induce cellular apoptosis more that the DNA ladder is more detectable as the cells are more stressed by higher toxic fluvastatin doses; however, silymarin is more influential than DDB in directing cell towards apoptosis.

Liver cell injury and cell death is a prominent feature in all liver disease processes. Moreover, apoptotic cell death is found to be present in various patterns of liver diseases, e.g. alcoholic liver injury have shown to induce apoptosis in vivo and in vitro (100). Moreover, the increase in TUNEL- positive cells number, Fas receptor, and activated caspases confirm the apoptotic cell elimination in viral infected cells (76, 201). Apoptosis is essential for the control and elimination of viral infections, as hepatocytes induce apoptosis as a host defence against viral infections. However, despite enhanced hepatocyte apoptosis, viral persistence may be observed (33). In addition, CTL are known to play a crucial role in the clearance of viral infection through apoptosis, and in the immuno-pathogenesis features of viral hepatitis (135).

From all of this, the induction or inhibition of apoptosis by drugs in hepatitis diseases may help or worsen liver condition, especially in viral hepatitis. Therefore, more detailed investigations are still needed; regarding drugs role in apoptosis pathway. As Panasiuk et al. (135) stated that, the pharmacological inhibition of apoptosis may favor chronic viral infection persistence and oncogenesis and even intensify its replication. Thus, the antiviral effect of interferon may be mediated through neutralizing antibodies, prevent virus cell entry, and/or induction of apoptosis, which lead to viral infected cells elimination. Others support the notion that apoptosis may represent a mechanism for viral shedding rather than for viral elimination, thereby inhibition of apoptosis could improve hepatitis C, moreover induction of apoptosis upon viral infection may contribute to liver failure (95).

In this thesis, a new model of experimental hepatitis on rats using toxic doses of fluvastatin was tested. From the results, fluvastatin showed to cause a liver damage pattern in dose dependent. While, rats tolerated 25mg/kg/day fluvastatin, they started to suffer when this dose was doubled. Rats showed signs and symptoms of toxicity, especially in body weight, and their blood parameters showed a decrease in glucose, albumin, triglycerides, at same time they showed an increase in HDL and necrotic markers (ALT, and AST). Moreover, the AST to ALT ratio was higher than 2:1. As for liver apoptotic parameters, they showed a correlation between fluvastatin dose and

caspase-3, indicating increase in apoptosis. Nevertheless, from DNA electrophoresis, necrosis and DNA fragmentation were more dominating then DNA ladder pattern. Thus, it can be concluded that fluvastatin can be used to induce a hepatitis model in experimental Wistar rats, and liver cell reached high degree of damage by dose increasing that both cell death pathways (necrosis and apoptosis) were noted, although necrosis was more pronounced. Also from apoptotic parameters, the induced apoptosis may mediate by external immunological pathway rather than intrinsic pathway, resembling the immunopathological pattern of hepatitis.

As for challenging this new model with two known hepatoprotective drugs, the changes in liver homeostasis, production, metabolism, as well as liver cell death markers were observed. The first drug, Silymarin, is the most popular natural drug that is used over the world as hepatoprotective. The hepatoprotective action of silymarin was reputed to its regulatory action on cellular and mitochondrial membrane permeability, neutralization and scavenging of free radicals (40), anti-inflammatory properties (84), and/or protective effect in minimizing induced mitochondrial membrane perturbations and lowering the level of mitochondrial reactive oxygen species production (33). Silymarin use among patients with advanced hepatitis C related liver disease is associated with reduced progression to cirrhosis, but has no impact on clinical outcomes. Although, silymarin does not affect viral replication, it might play a beneficial role in viral hepatitis by its inhibitory action on the inflammatory cascade induced by viral infection (4). However, Wellington et al. (190) stated that silymarin was largely ineffective in patients with viral hepatitis.

Results showed that silymarin has managed to ameliorate the toxic symptoms of fluvastatin, such as body weight, glucose, triglycerides, albumin, and liver enzymes, however just in groups subjected to the lowest toxic dose of fluvastatin 25 mg/kg/day. In addition, it stimulated apoptotic parameters: caspase-3 and p53. Thus, the conclusion is silymarin may be efficient hepatoprotective drug but under stressful challenge, it showed that its helpful effect is limited to the timing and degree of liver damage, and its effect was more pronounced in parameters of liver apoptosis than in liver metabolism and necrosis.

Thesis results agreed with Freedman et al. (50) statement that in order to exert an effect, silymarin must be used early in the disease progression process. The sooner

the drug was given, the more effective was its protection. The importance of silymarin administration timing was shown in silymarin therapeutic activity against death cap (*Amanita phalloides*) mushroom poisoning. When silymarin (15 mg/kg) was administered intravenously 60 minutes before lethal dose of phalloidin, silymarin protected all animal species tested. When, silymarin was injected 10 minutes after phalloidin, it still afforded similar protection but only at 100 mg/kg. The longer the time that has elapsed after administration of the toxin, the less effective the drug becomes, and after 30 minutes, it is no longer effective even at high doses (97).

The other drug investigated in this thesis, DDB, is a Chinese drug used also as semi-natural safe hepatoprotective and sometimes even known as anti-hepatitis. The hepatoprotection of DDB may attribute to its' stimulation of hepatic mitochondrial GSH antioxidant system (78), its' decreasing effect on lipid peroxides (38, 42), its' inflammatory responses inhibition (89, 150), and/or its' directly protection of hepatocyte DNA from oxidative damage (53).

Results showed that DDB was effective in helping the animals toxicated with Fluvastatin, in decreasing the loss in body weight, tending to normalize glucose, triglycerides, HDL, and albumin. In addition, it showed pronounced effect in maintaining normal liver aminotransferases activities, especially ALT, in low level. However, most of these actions were effective only in rats minimally toxicated with 25 mg/kg/day fluvastatin, same as silymarin. As for apoptosis, DDB was less effective than silymarin in inducing the cells towards intrinsic apoptosis pathway. From above, it can be concluded that DDB may be efficient hepatoprotective drug for stressful liver condition also when administered at the beginning of liver impairment. In addition, DDB effect was more pronounced in adjusting parameters of liver metabolism and necrosis rather than liver apoptosis.

DDB may have a more direct repairing effect on fluvastatin melvonate pathway, which is a probable cause for statin toxicity, thus it showed a better hepatoprotection than silymarin in this new model especially in body weight parameters. However, as blood and hepatic parameters were studied, both drugs showed nearly the same action. Even though there was a difference in hepatitis model insulting agent, Abdel Hameid (2) also stated that DDB showed a better hepatoprotective effect compared with silymarin preventing the erythromycin stearate induced liver damage.

Both drugs tended to restore hepatocytes homeostasis function, decrease necrotic markers, and induce more apoptosis cell elimination. However, they succeeded only at the lowest toxicity, which means that they may be helpful at the beginning only. However, this does not eliminate that these drugs may represent a burden to the impaired liver when the injuring stress overcome cell ability to compensate, thus a caution in such drug use is very much advised.

7. Summary

Liver is unique organ, it has a very important role in maintaining body metabolic homeostasis, and it has its own energy substrates and defense mechanism, which enable it to withstand most of chemical or biological injurious insults. If these injurious insults, due to high concentration or prolonged period of exposure, exceed hepatocytes ability to adapt and compensate, impairment in liver condition and functions start to appear and subsequently start to affect the whole body. Thus, more trials and experimental models are needed to understand fully the molecular and nuclear changes in hepatocytes, subjected to various grades of liver damage.

In this thesis, a new experimental model for hepatitis was set using fluvastatin as a toxic agent, to cause a gradual and progressive liver impairment. Fluvastatin, was given in gradual toxic doses, starting from 25 to 100 mg/kg/day for 7 days. Such prolonged toxicity was found to cause disturbance in many parameters during the experiment. As fluvastatin dose increased, animals' body weight gain, serum glucose, and triglycerides levels were decreased. While relative liver weight and aminotransferases levels were increased, indicating a hepatitis condition in the liver.

Interestingly, investigating the liver enzymes levels, fluvastatin hepatitis represented typical drug hepatotoxicity hepatitis as AST to ALT ratio exceeded 2:1. In addition, fluvastatin toxic doses had an inducing effect on apoptotic pathway, as shown on caspase-3 activity, however on same time it inhibited p53 production. Moreover, DNA electrophoresis did not detect DNA ladder fragmentation pattern. This means that fluvastatin toxicity causes necrosis cell deaths that overwhelm apoptosis cell elimination.

Such experimental model induced by fluvastatin, can be used to investigate the effect of various drugs on liver subjected to continuous stressful condition, especially drugs claimed to be hepatoprotective. To investigate the effect of such drugs on a hepatocytes function, parameters reflecting liver metabolic and synthetic function and markers indicating cell membrane, mitochondrial and nuclear integrity, and the inhibition or change in cell death pathways under the influence of such drugs must be tested.

In this thesis, two popular hepatoprotective drugs were challenged with the new experimental model. The first drug is European popular natural hepatoprotective drug

"Silymarin" that extracted from *Silybum marianum*. The second drug is Chinese popular hepatoprotective drug "DDB" that is a chemical synthetic product mimic one ingredient (shisandrin C) of *Schisandra chinensis* plant.

At first, DDB had shown more protective effect than silymarin in preserving rats' body weight. However, after investigating rest of liver biochemical and apoptotic parameters, both drugs showed nearly the same protective action. Both drugs tended to preserve hepatocyte membrane, as indicated by the decrease in necrotic markers especially ALT. In addition, both tended to preserve glucose and triglycerides normal homeostasis level. Both silymarin and DDB induced caspase-3 activity, which can be explained that they tended to direct the liver cells more towards apoptosis cell elimination rather than necrotic cell death. However, both drugs efficiency was observed only at the lowest fluvastatin toxic dose (25 mg/kg/day) which was the maximum tolerated dose by rats.

From all of this, it can be concluded that hepatoprotective efficiency of these drugs depends mostly on their dose and time of administration. When they used at the beginning of liver impairment, they tend to help the liver regain its previous healthy condition, as hepatocytes also tend to recover and repair itself by its protective mechanism. Such outcome directs minds to think over the true value of all hepatoprotective drugs, natural or chemical. As their true efficiency is debatable, their mechanism of action are still not fully understood, they may have a negative impact of natural repairing process of hepatocytes, and they also may represent a burden to the impaired liver, and hinders its tendency to regain its function.

Accordingly, it is recommended that all hepatoprotective drugs to be more thoroughly investigated prior release in market for patient use, as they may disturb the balance of liver cells proliferation and elimination. Such action can be masked by the natural progress of liver disease. Moreover, advice hepatic patients to avoid consuming various hepatoprotective drugs under the claim they are natural and non-toxic, and they should always notify their physician or medical staff if they consumed such drugs to enable the medical staff of rescuing them in emergencies from liver toxicity by such drugs.

8. Abstract

As experimental models are always needed to investigate the changes in hepatocytes under various liver damage conditions, fluvastatin was used in this thesis as a toxic agent in order to develop gradual and progressive liver damage. Fluvastatin was administered orally to male Wistar rats in doses: 25, 50, 75, and 100 mg/kg/day for 7 days. These toxic doses caused a condition resembles hepatitis, which disturbed rats' body weight, serum glucose, serum triglycerides, and aminotransferases levels. In addition, fluvastatin toxicity directed injured hepatocytes elimination by two pathways necrosis and apoptosis, however, necrosis pattern accompanied with inflammatory liver condition was more predominant than apoptosis.

Such challenging new experimental model helped in investigating the effect of two hepatoprotective drugs (Silymarin 140mg/kg/day and DDB 100mg/kg/day) on damaged hepatocytes. In addition, from results, both drugs showed a tendency to preserve hepatocyte membrane, and induce apoptotic cell elimination. However, they succeeded only at the lowest fluvastatin toxic dose (25 mg/kg/day), whereas at higher doses necrosis dominated.

It can be concluded that fluvastatin can be used to establish rat experimental hepatitis model, and hepatoprotective drugs can be beneficial only if given as early as possible at the beginning of liver disease.

Streszczenie

Wykorzystując modele doświadczalne można badać zmiany w hepatocytach różne stadia uszkodzenia wątroby. W przeprowadzonych badaniach zastosowano fluwastatynę jako toksyczny środek, powodujący stopniowy oraz postępujący rozwój uszkodzenia wątroby. Fluwastatyna była podawana doustnie samcom szczurów Wistar w dawce 25, 50, 75 i 100mg/kg mc/dobę przez 7 dni. Toksyczne dawki uszkadzały wątrobę powodując zmiany w parametrach takich jak masa ciała, stężenie glukozy i triglicerydów oraz zwiększając aktywności enzymatyczne aminotransferaz. Toksyczne działanie fluwastatyny wywołało śmierć hepatocytów na drodze zarówno apoptozy jak i nekrozy, jednakże nekroza była procesem bardziej dominującym niż apoptoza.

W pracy badano wpływ dwóch leków hepatoochronnych (Sylimarin 140mg/kg/dzień i DDB 100mg/kg/dzień) wykorzystując nowy model eksperymentalny. Oba leki wykazały ochronny wpływ na stabilność błon komórek wątrobowych. Wobec uszkodzonych komórek indukowały apoptozę, ale tylko przy najniższej toksycznej dawce fluwastatyny (25 mg/ kg/ dzień) jednak przy większej dawce występowała nekroza..

Można stwierdzić, że fluwastatyna może być wykorzystana do wyjaśnienia rozwoju zapalenia wątroby w szczurzym modelu eksperymentalnym oraz wykorzystanie leków hepatoochronnych może być korzystne tylko wtedy, gdy zostaną podane w jak najwcześniejszym stadium choroby wątroby.

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