Effect of Methotrexate – induced Hepatotoxicity on Adult Albino Rat and the Possible Protective Role of Folic Acid

Thesis
Submitted for fulfillment
of Master Degree of Anatomy

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2009

ACKNOWLEDGEMENT

First and foremost, deep thanks to "ALLAH "the most merciful" for enabling me to finish this work.

I would like to convey my sincere thanks to ***Professor*** **Dr. Ibrahim Labib Abdalla**, Professor of Anatomy, Faculty of Medicine, Cairo University, for his support, valuable and constructive advice, generous help and sincere encouragement.

I am greatly indebted to **Dr. Sherif Fahmy Arsanyos**, lecturer of Anatomy, Faculty of Medicine, Cairo University, who did a tremendous effort to bring out this thesis in an elegant and scientific form and for his valuable guidance, continuous encouragement and constructive advice.

I owe my deep thanks and gratitude to **Dr. Mohamed Hafez Shaaban**, lecturer of Anatomy, Faculty of Medicine, Cairo University, for his sincere effort, valuable remarks and continuous enthusiasm in supervising this work which have contributed a lot to the delivery of this work.

Finally, I would like to thank all members in the anatomy and histology departments especially **Prof.Dr.Sohir Asaad**, Professor of Histology, Faculty of Medicine, Cairo University for their help to complete this work.

ABSTRACT

Key Words:

(Methotrexate-Methotrexate and folic acid as an antagonist)

Methotrexate is a highly effective drug, so, it is widely used for the treatment of many inflammatory and neoplastic diseases. The treatment of the patients requires a long duration which exposes the patients to the side effects of the drug. This study is carried out to evaluate the possible alterations resulting from the administration of methotrexate on the liver of adult albino rat and the possible protective role of folic acid as an antagonist of methotrexate hepatotoxicity.

List of Abbreviation

AICAR	Aminoimidazole-4-carboxamide ribonucleotide transformylase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
BD	Bile duct
CV	Central vein
CBC	Complete blood cell count
DNA	Deoxyribonucleic acid
FA	Folic acid
gm	Gram
GAR	Glycinamide ribonucleotide transformylase
H δ E	Hematoxyline & Eosin
HMC	Hepatic microcirculatory unit
i.m	Intramuscular
i.v	Intravenous
IL-1	Interleukin-1
KC	Kupffer cells
kg	Kilogram
L	Litre
LPO	Lipid peroxidation
µm	Micrometer
MCV	Mean corpuscular volume
µg	Microgram
mg	Milligram
mL	Millilitre
MT	Masson's trichrome
MTX	Methotrexate
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NO	Nitric oxide
NSAIDs	Non steroidal anti-inflammatory drugs
PAS	Periodic Acid Schiff reagent
PBMN	Peripheral blood mononuclear cell
PV	Portal vein
RA	Rheumatoid arthritis
RNA	Ribonucleic acid

ROS	Reactive oxygen species
S.C.	Subcutaneous
m2	Square meter
DHFR	Dihydrofolate reductase
PN	Pyknotic nuclei
S	Blood sinusoides
D	Degenerating cells
F	Fibrosis
LV	Lymph vessels
HA	Hepatic artery

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Introduction:

Methotrexate (MTX) is an anti-metabolite chemotherapy which is used for the treatment of leukaemias and many neoplasms including hepatic cell carcinoma, bronchogenic carcinoma, breast cancer and chorionic vesicle **(Olsen and Murray, 1989)**.

It is also used in patients with ulcerative colitis or Crohn's disease. Oftenly, oral MTX is not efficient or well tolerated in the presence of gut inflammation, but significant improvement may occur with subcutaneous administration and injectable routes **(Alaricon et al., 1990).**

It is recommended that all patients with rheumatoid arthritis (RA) of longer than 6 months duration are candidates of MTX therapy **(West, 1997)**. It is reported that MTX plays an important role in the chemotherapy of human malignancies including acute lymphoblastic leukemia, lymphoma, breast cancer and osteosarcoma. It is employed in maintenance programs, in the prophylaxis and treatment of meningeal leukaemia. Methotrexate is of value in the treatment of psoriasis. It is also used in bone marrow transplantation **(Changqun et al.**, **2009)**.

Hepatotoxicity has been recognized as a potential major adverse effect that can occur with prolonged use of methotrexate especially in patients with pre-existed liver disease**.** Methotrexate can cause liver injury by many mechanisms as methotrexate can activate hepatic stellate cells, which lead to increased collagen deposition. Also it leads to accumulation of metabolites (polyglutamates) resulting in prolonged folate inhibition with subsequent hepatic injury (**Bridges et al., 1989 and Cronstein, 1996**).

Van Ede et al., (2001) noticed that folic acid succeeded to decrease the frequency of methotrexate toxicities, including mucositis, nausea, haematological abnormalities as pancytopenia and, liver enzyme elevations without apparently interfering with the clinical efficiency of

methotrexate**.**

Aim of the work:

The aim of the present work is to study and to evaluate the possible the histological and the histochemical changes resulting from the administration of methotrexate on the liver of adult albino rat and to demonstrate the possible protective role of folic acid as an antagonist of methotrexate hepatotoxicity.

Structure and function of the liver

The liver is formed of complex network of hepatocytes interpenetrated and ensheathed by supportive connective tissue and permeated by a great number of blood vessels perfusing the liver with a rich flow of blood. The hepatocytes, which carry out the major metabolic activities of this organ, are assisted by additional classes of cells which posses potent, phagocytic and mechanically supportive functions (**Motta et al., 1978 and Motto, 1982).**

Hepatocytes are approximately 100 billion in number and constitute 80% of the hepatic cell population. They are arranged in plates of a single cell thickness with intervening sinusoids. Hepatocytes are irregular polyhedrals of variable dimensions and shape, ranging in size from 20 to 30 μm. It is found that the variability in shape and size of hepatocytes depends on the age, the location, the metabolic status and the regenerative activity of the cells. Additionally, it is demonstrated that the shape and size of the hepatocytes are not fixed but adapted to alternations in sinusoidal blood flow and osmotic load **(Wolters et al., 1991 and Feldmann, 1992)**.

Sherlock and Dooley (1997) and Bioulac-Sage et al., (1999) mentioned that the nuclei of the hepatocytes represent about 5-10% of the cell volume. It is spherical in shape and contains one or more prominent nucleoli. Twenty five percent of hepatocytes are binucleated to cope with the extraordinary metabolic activity. All hepatocytes are loaded with cytoplasmic organelles**.**

Dunkelberg et al., (2001) and Marta et al., (2009)defined the hepatocyte as a polarized cell consisting of three functionally specialized membrane surfaces; the basolateral or sinusoidal surface that faces the sinusoids and the peri-sinusoidal space of Disse, the canalicular surface

forms the bile canaliculus in the intercellular space between adjacent hepatocytes. Whereas the lateral surface faces the intercellular space not related to the bile canaliculus. These surfaces differ from each other in protein and lipid composition, fluidity and presence of enzymes, receptors and molecular transport systems.The basolateral and canalicular surfaces contain site-specific transporters of inorganic and organic ions and major endoactive machinery for uptake of macromolecules while, lateral surface contains tight junctions that separate the bile canaliculus from the sinusoids**.**

The peri-sinusoidal space of Disse lies between the hepatocyte basolateral surface and the interrupted sinusoidal endothelium, constituting 2-4% of the hepatic parenchyma. It contains fat-storing hepatic stellate cells, microvilli projecting from the hepatocytes and extracellular matrix proteins. Because neither hepatocytes nor cells lining the sinusoids have basement membranes, movement of fluid occurs freely across the space of Disse. Exchange of substances between blood and hepatocytes is a function of hepatocyte plasma membrane. The space of Disse extends deeply among intercellular spaces between adjacent hepatocytes, creating intercellular recesses, where it is separated from the bile canaliculus by a narrow gap called zone of minimal distance **(Motta et al., 1978)**.

Rogoff and lipsky (1981), Arias (1990) and Macphce et al., (1992) studied the cells that populate the hepatic sinusoids and found that there are four types of cells; endothelial cells, Kupffer cells, stellate cells and lymphocytes. Morphometric analysis demonstrated that the endothelial cells, kupffer cells and stellate cells accounted for the vast majority of hepatic non-parenchymal cells**.** Sinusoidal endothelial cells account for 2-5% of the lobular parenchyma. They have numerous fenestrae and lack a basement membrane.

Kupffer cells account for about 2% of the lobular parenchyma which belong to the macrophage monocyte system and represent fixed macrophages of the liver. They are considered as the largest population of macrophages any where in the body. They are more numerous in periportal sinusoids but can migrate along the sinusoids into areas of liver injury with and against the blood flow **(Ramadoril et al., 1993; Burt, 1999 and Eng and Friedman, 2000).**

Stellate cells, para-sinusoidal cells or fat storing cells are capable of storing vitamin A. They are stellate in shape, account for 1.4% of lobular parynchyma and lie between hepatocytes in the space of Disse. The liver contains populations of several types of lymphocytes that provide it with humoral immunity (**Doherry and Farrelly, 2000 and Greets, 2001)**.

Reddy (2001) confirmed the work made by **Groothuis et al., (1982) and Jungermann (1986)** and reported that, the hepatic acinus, which is the hepatic parenchyma present between two central veins, has three zones; zone (1) close to the entry of blood, zone (3) about the terminal central hepatic vein, and zone (2) is between the previous zones. Despite the utility of the acinar concept, lobular terminology is still profoundly used to describe regions of pathologic lesions of the hepatic parenchyma. Fortunately, the three zones of the acinus roughly coincide with the three zones of the lobule. Acinar zonation is of considerable functional consequence regarding gradients of components both in blood and hepatocytes.They also postulated that another well-documented acinar gradient is that of bile salts. They are efficiently extracted by zone (1) hepatocytes with little bile salts left in blood that flow to zone (3) hepatocytes. In addition to the metabolic heterogenicity of the acinus, structural differences are apparent between the various acinus zones. The sinusoids are narrower and more tortuous in zone (1) as compared to zone

(3), thereby facilitating uptake of the solutes because of the greater surface to volume ratio. However, higher number of fenestrations in zone (3); endothelial sieve plate; results in greater porosity in this acinar region**.**

Concerning the function of the liver, the liver is the maestro organ supplying optimal nutrition for all the body cells. It consumes 12-20% of the total body energy. Liver disease is usually a profound life changing disease as the liver's function affects almost every other organ in the body**.**The basic functions of the liver can be divided into vascular function for storage of blood, metabolic function concerned with the majority of metabolic systems of the body as the liver is the main site for carbohydrate, protein and lipid metabolism and furthermore, secretory and excretory functions that are responsible for forming the bile (**Aponte and Petrelli, 1988 and Michalopoulos and Defrances, 1997).**

Jungermann and Katz (1989) found that, 60-70% of blood entering the acinus consists of low-oxygen blood and derived from the portal vein, while the remaining 30-40% of the hepatic blood flow is oxygenated and derived from the hepatic artery.

Regarding the terminal hepatic venule, they added that oxygen rapidly leaves the blood to meet the high metabolic demands of the parenchymal cells. Approximately oxygen concentrations in zone (1) are 9-13%, compared to only 4-5% in zone (3). Therefore, hepatocytes in zone (3) are exposed substantially to lower concentration of oxygen than hepatocytes in zone (1). In comparison to other zones, zone (3) is hypoxic.

Jungermann and Katzmann, (1997) found that the variability in protein levels and enzymes of hepatocytes caused different metabolic functions; hepatocytes in the periportal and perivenous zones of the liver parenchyma differed in their enzyme content, subcellular structures and plasma membrane channels and thus have different metabolic capacity.

The periportal zone contains predominantly oxidative energy metabolism with beta-oxidation, amino acid catabolism, urea genesis, gluconeogenesis for synthesis of both glucose and glycogen, cholesterol synthesis, bile formation and protective metabolism. While, the perivenous zone contains glycolysis, glycogen synthesis from glucose, lipogenesis, ketogenesis, glutamine formation and xenobiotic metabolism. Moreover, the input of the humoral and nervous signals into the two zones is different **(Reddy, 2001).**

The Biotransformation of non-polar substances to polar i.e. water-soluble products; that can be excreted in either bile or urine; occurs in two phases**:** Phase (I) metabolism binds the compound with polar groups, preparing it for conjugation by phase (II) enzymes. The primary enzyme system involved in phase (I) reactions is the mixed-function oxidase system or cytochrome P450 system. They are oxygen-binding enzymes and catalyzed by the flavin mono-oxygenase system and (NADP/NADPH) **(Smith and Williams, 1970 and Parsons and Neims, 1978)**.

Phase (I) metabolism may result in production of reactive species which are called free radicals that include reactive oxygen species (ROS), carbon centered compounds or nitric oxide (NO).These free radicals are ionically unstable metabolic species; each one possesses one or more unpaired electrons. Such unstable molecules seek charge stability by either donating or acquiring electrons from adjacent molecules. Consequently, sometimes Phase I metabolism referred to be the toxification phase**.** Phase II has been called the detoxifying phase because its products are generally non-toxic, polar substances that are readily excreted **(Cheesman and Slater, 1993)**.

Estabrook (1996), **Vermeulen (1996) and Park et al., (1996)** added that, phase (II) reactions further increase the water solubility of a drug or

toxin, even beyond that caused by phase (I) reactions. If the reactive molecules formed in phase I reactions are not further metabolized by phase (II) conjugation reactions, they may cause damage to certain proteins e.g. DNA and RNA within the cells. Therefore, the succession of phase (I) and phase (II) reactions has a major effect on the detoxification and execretion of foreign compounds. However, many compounds can be metabolized only by phase (II) reactions without phase (I) reactions.While the detoxification systems share in the management of exposure to exogenous compounds, the body exerts several endogenous mechanisms to regulate detoxification activity. Additionally, numerous exogenous compounds can change the detoxification system enzymes.

Feldman (1997) and Guengerich (2001) clarified that; many substances can induce the detoxification system enzymes such as aryl amines from charbroiled meats. Other inducers include many of the flavinoid molecules found in fruits and vegetables. Phase (I) and phase (II) enzyme activities can also be inhibited by competition between two or more compounds for the same detoxifying enzyme. Increased toxic load may lead to inhibition of detoxification of number of compounds by simply overwhelming the system and competing for detoxification enzyme activities. A common mechanism of inhibition for some enzymes of phase II is the depletion of necessary cofactors.

Regarding the effects of some drugs on some organs, **Halliwell and Chirico (1993) and Hall (1994)** reported that some organs are more vulnerable to the adverse effects of drugs than others. The liver is often a target organ for the injurious effect of many drugs as it detoxifies most of them. Moreover, the liver has the ability of concentration, biotransformation and execretion of chemicals, irrespective to the route of the exposure as it has the highest concentration of drug metabolizing enzymes.

Side effects of drugs and chemicals can mimic any recognized liver disorder, both clinically and pathologically. Some chemicals cause one type of injury but with individual variation in the severity of damage. Other substances can cause a variety of injurious patterns depending on a number of factors such as the dose and the duration of the treatment**.** Oftenly, these chemicals can cause liver injury by the accumalation of free radicals. These free radicals can cause heptatoxicity through destruction of cell membranes **(Benzie, 1996)**.

Lipid peroxidation (LPO) is thought to be the primary mechanism by which free radicals can cause death of the different body cells**.** Lipid peroxidation decreases membrane fluidity and increases permeability of membranes of organelles, plasma membrane and endoplasmic reticulum and initiated by both oxygen and carbon centered radicals **(Larry and Pageaus, 1997)**.

Steatosis is the accumulation of lipid droplets within hepatocyte cytoplasm which is a common expression of hepatotoxicity for many chemicals. Steatosis usually has ill-defined zonal distribution due to impaired cellular metabolism of fatty acids. The lipid droplets are composed primarily of triglycerides and phospholipids **(Guigui et al., 1988).**

Acute hepatocellular or cytotoxic injury is manifested by necrosis and apoptosis of hepatic parenchymal cells. Hepatocellular necrosis may occur in zonal or non-zonal pattern and may overlap **(Marquardt et al., 1999)**.

Inflammatory and granulomatous reactions can be secondary to hepatocellular injury, which may be caused by bacterial or viral infections, necrosis or directly by a toxin. Generally, the degree of inflammatory infiltrates following toxic hepatocellular injury or necrosis tends to be less severe than that seen subsequent to viral-induced hepatitis **(Kalara et al.,**