



## TO STUDY THE HISTOPATHOLOGICAL EFFECTS OF DIFFERENT DOSES OF METHOTREXATE ON LIVER OF MALE ALBINO RAT WITH SPECIAL REFERENCE TO THE HISTOMORPHOLOGICAL CHANGES.

### Anatomy

**Dr Kamaldeep  
Singh Balowria**

**Dr Sheetal  
Chandel\***

\*Corresponding Author

### ABSTRACT

**INTRODUCTION:** One of the most important roles of cancer chemotherapy is an adjuvant to local treatment modalities such as surgery and radiation therapy. Anticancer drugs are widely used against variety of human tumors. The in vivo system provided the foundation for mass screening of novel compounds. One of the characteristics that distinguish anti cancer agents from other drugs is the frequency and severity of side effects with therapeutics doses. These drugs affect various tissues in a dose dependent manner and also show an inter-individual susceptibility.

**MATERIAL METHODS:** The present study was conceived to evaluate effects of different doses of Methotrexate on liver of rats so that inference drawn from the study could have impact on treatment of cancer and dosage selection.

**RESULTS:** It was found that all the Mtx treated groups (2,3 and 4) showed significant toxic changes where as the control group (1) did not reveal any changes.

### KEYWORDS

Methotrexate, Albino rat

### INTRODUCTION:

The incidence of malignant diseases varies with sex, age, and geographic locations. Most common cancer affecting the females is in the region of breast, colon, cervix, ovary and skin while males are more prone to develop cancer of lungs, gastrointestinal tract, skin, prostate, oral cavity, pharynx, bladder and testis. Cancer in all forms, causes 12% of total deaths throughout the world. The number of new cases is expected to rise by about 70% over the next two decades (Martel C et al., 2012). The systemic treatment of cancer has its roots in the initial work of Paul Ehrlich in early 1900's, who coined the term chemotherapy to the systemic treatment of both infectious diseases and neoplasia. Cancer chemotherapy is a relatively young discipline of oncology. One of the most important roles for cancer chemotherapy is an adjuvant to local treatment modalities such as surgery and radiation therapy. Anticancer drugs are widely used against variety of human tumors. More than half of all people diagnosed with cancer receive chemotherapy regimen that usually include drugs to help support the completion of the cancer treatment at the full dose on schedule (De Graaf H et al., 1996). The use of in vivo rodent model system to develop antibiotics for treating infectious diseases led to the development of inbred rodent lines bearing transplanted tumors that naturally provided a way to screen potential anticancer drugs. This in vivo system provided the foundation for mass screening of novel compounds. One of the characteristics that distinguish anti cancer agents from other drugs is the frequency and severity of side effects with therapeutics doses. These drugs affect various tissues in a dose dependent manner and also show an inter-individual susceptibility. So toxicities are often associated with these tissues. Methotrexate (Mtx) is the common name of 4-amino-N-10-methylpteroylglutamic acid which is an important anti metabolite, anti-folate chemotherapeutic agent that was introduced for therapeutic application in the 1950s. Methotrexate has been used for the treatment of malignancy like breast cancer, epidermoid cancers of head and neck and lung cancer, rheumatic disorders and psoriasis, termination of intrauterine pregnancy and also for ectopic pregnancy. However, the use of Methotrexate is limited due to high incidence of dose dependent toxicity, including hepatotoxicity, renal damage, bone marrow suppression and gastrointestinal mucosal inflammation.

### MATERIAL METHODS:

An extensive investigation has been conducted on hepatotoxicity by using light and electron microscopic study of various organs and biochemical study of liver enzymes. However, there is very limited information available regarding effects of Methotrexate on histomorphology of liver.

Therefore the present study was conceived to evaluate effects of different doses of Methotrexate on liver of rats so that inference drawn from the study could have impact on treatment of cancer and

dosage selection. Albino rats (wistar strain) of either sex weighing 125-160 grams were used for current experimental studies. They were procured from animal house, Government medical college, Jammu. The clearance for the use of animals for experimental purpose was obtained from Animal Institution Ethical Committee constituted for the purpose. Animals were housed in polypropylene cages (6 per cage) with dust free rice husk as bedding material under laboratory conditions with controlled environment of temperature having range between 23 to 27° celsius, humidity (6% to 26%) and 12 hours light/dark cycle as per committee for the purpose of control and supervision of experiment on animal (CPCSEA) Indian guidelines. They were provided standard rodent chaw/feed and water *ad-libitum*. Before subjecting them for experimentation, animals were given a week's time to acclimatize with laboratory conditions. Animals were fasted for 24 hours before experimentation.

The principle of Rationalization, Refinement and Reduction (3 "R's") was strictly followed while undertaking the following experiment.

### Experimental Design:

The animals were divided into 4 groups with each group consisting of 6 animals.

GROUP 1 were administered a daily oral dose of 3 ml of sterile distilled water for 28 days and served as a healthy control.

GROUP 2 were administered a daily low oral dose of methotrexate (Mtx) (0.25 mg/kg b.w) for 28 days.

GROUP 3 were administered a daily therapeutic oral dose of Mtx (0.5 mg/kg b.w) for 28 days.

GROUP 4 were administered a daily high oral dose of Mtx (0.75 mg/kg b.w) for 28 days.

The above dosing schedules were based on study conducted by Patel NN et al (2014).

The animals were sacrificed after 48 hours of the administration of final dose of drugs as per the prescribed methods by CPCSEA. The rats were euthanized by keeping them in an inverted glass jar containing a large piece of cotton soaked in chloroform, without pain or discomfort as recommended by Laboratory Animals Information Service Centre (Hariharan S, 1980).

### Preparation of the tissues for microscopy:

The pieces of dissected out organs were kept in tissue capsules and labelled. The tissue capsules containing the tissue and the labelled slip were kept in 10% formalin solution. (Drury RAB and Wallington EA, 1980).

**Dehydration:**

After fixation, the specimen was washed thoroughly with water and passed through ascending grades of ethyl alcohol ( 50%, 70%, 90% & absolute alcohol). The time of dehydration in each grade of alcohol was 1 hour with two changes in absolute alcohol.

**Clearing:**

Clearing of the tissue was done with Xylene. Two changes of Xylene were given. First change for 5 minutes and second overnight.

**Paraffin embedding:**

The tissues were kept in paraffin bath, the temperature of which was maintained at 58°C (melting point of wax = 58-60° c). Two to three changes after every 2-3 hours were given and tissues were then left overnight. Thus the clearing agent Xylene was replaced by wax in the tissue.

**Block Making:**

This was done by filling fresh filtered molten wax in a mold of suitable size with the help of Lockhart's blocks (L Blocks). Air bubbles were removed with the help of heated spatula. With a warm smooth tipped forceps the specimen was oriented in such a way that it could be cut in a right plane. Label indicating the name of the organ and the group to which it belonged was placed by the side of the mould. It was allowed to cool and solidify.

**Sectioning:**

After trimming and attaching the block to the tissue holders, 5-7 micron thick sections were cut with the rotary microtome. The sections were gently held with the fine forceps and floated in the water bath containing luke warm water, the temperature of which was maintained at 5-10° C below the melting point of wax. This removed the wrinkles in the sections if present.

**Fixation to slide:**

Clean glass slides were labelled and numbered and smeared with a drop of Meyer's egg albumin. These slides were dipped obliquely in water bath so as to take the sections on the slides. The slides were dried again and were kept in an incubator at 37° C overnight.

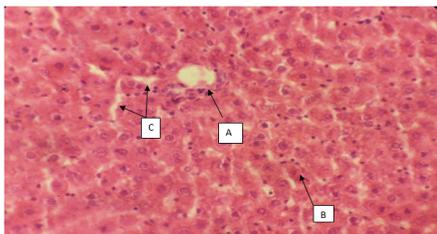
**Staining:** Hematoxylin and Eosin staining was done.

**OBSERVATIONS:**

The current study was undertaken to evaluate the effects of different doses of Methotrexate(Mtx) on liver of albino rats. The study was conducted in the department of Anatomy, GMC Jammu from November, 2016 to November, 2017. The animals were divided into 4 groups with each group consisting of 6 animals as:-

- 1) Control group
- 2) Low dose group
- 3) Therapeutic dose group
- 4) High dose group

Changes observed on macroscopic and microscopic examination were classified as per the severity and were graded as:- Mild(+), Moderate(++), Severe(+++)



**Fig: Photomicrograph of hepatic lobule showing Central vein(A), Plates of hepatocytes(B) and sinusoidal spaces (C) {Control group 400x}**

**OBSERVATIONS IN NORMAL CONTROL GROUP I**

**MACROSCOPIC CHANGES:** No gross changes seen in liver.

**MICROSCOPIC CHANGES**

Sections of liver stained with a standard hematoxylin and eosin (H&E) procedure revealed a hepatic lobular pattern demarcated in the form of portal areas embedded in thin connective tissue containing smaller branches of portal vein (venule), a branch of the hepatic artery

(arteriole) and a small bile duct (ductule) along with lymphatic vessels. The presence of portal areas along with central venules provided evidence of a lobular structure. Liver cells were arranged in plates or cords and were seen radiating from the regions of the central venules and extending to the portal areas. The plates or cords of cells were separated by hepatic sinusoids. They also contained bile canaliculi.

**OBSERVATIONS IN ALL RATS OF GROUP 2 (LOW DOSE GROUP) SHOWED FOLLOWING:**

**MACROSCOPIC CHANGES:** No gross changes seen in liver.

**MICROSCOPIC CHANGES:**

**LIVER:** Cut section of liver showed mild dilatation (+) of hepatic sinusoids. Central vein showed mild congestion (+). Hepatic cord pattern was preserved. No dissolution of cords was seen. Portal tract showed chronic inflammatory infiltrate. Focal fatty change (+) present. Occasional hepatocytes showed fatty change. Pyknosis of nuclei seen (+). Apoptosis not seen.

**OBSERVATION IN ALL RATS OF GROUP 3 (THERAPEUTIC DOSE GROUP) SHOWED FOLLOWING:**

**MACROSCOPIC CHANGES:** No gross changes were seen in liver.

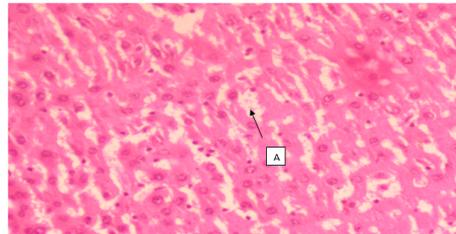
**MICROSCOPIC CHANGES:**

**LIVER:** Hepatic cord pattern was preserved but showed marked sinusoidal dilatation (++) . Fatty changes (++) seen with vacuolization of hepatocytes. There was marked congestion (++) in central vein with feathery degeneration of hepatocytes. Also, there was presence of focal spotting necrosis.

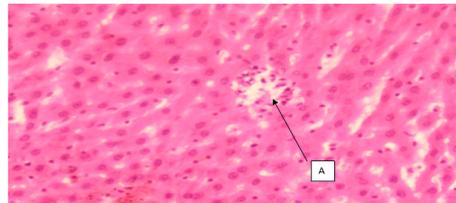
**OBSERVATION IN ALL RATS OF GROUP 4 (HIGH DOSE GROUP) SHOWED FOLLOWING:**

**MACROSCOPIC CHANGES:** No gross changes seen in liver.

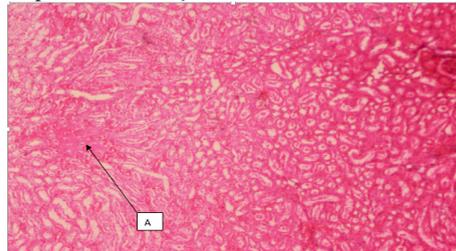
**MICROSCOPIC CHANGES:**



**Fig: Photomicrograph of liver showing Feathery Degeneration of Hepatocytes (A) {Therapeutic dose 400 x}**



**Fig: Photomicrograph of liver showing Focal Spotting Necrosis (A) {Therapeutic dose 400x}**



**Fig: Photomicrograph of Liver showing Dissolution of Hepatic Cord Pattern (A) {High dose 400x}**

**LIVER: -** There was marked sinusoidal dilatation (+++). Central vein showed congestion (+++). Portal tract showed chronic inflammatory infiltrate. Also, there was presence of fresh interstitial haemorrhages. There was disruption distortion of hepatic cords along with vacuolation of hepatocytes (+++). Centrizonal necrosis was also seen. It was found that all the Mtx treated groups (2,3 and 4) showed

significant toxic changes where as the control group (1) did not reveal any changes.

#### DISCUSSION:

The purpose of the present study was done to evaluate the toxicity profile of the drug methotrexate(Mtx) histologically in wistar albino rats in order to suggest whether to modify the present therapeutic dosage of this drug for avoiding its toxicity on liver. The wistar albino rats were selected as they are very close species among mammals with humans and toxicity in them with this drug may reflect the similar toxicity pattern in humans with per kg dosage formula.

There are many chemotherapeutic drugs available. The selection of Mtx was done for the present study as it is amongst the most widely used agent against the variety of cancers like breast cancer, epidermoid cancer of head and neck and lung cancer.

Hepatotoxicity is main undesirable side effect of Mtx, however little work has been done for evaluation of hepatotoxicity. Therefore present study was under taken to evaluate hepatotoxicity with different doses of Mtx ( 0.25mg/kg b.w, 0.5 mg/kg b.w and 0.75mg/kg b.w ) orally for 28 days.

Efforts were made to elucidate gross (macroscopic) and microscopic (histopathological) changes in detail of the liver.

Hall PD et al. (1991) showed hepatotoxicity in a rat model by orally administered methotrexate(Mtx) at 300 microgram per day for variable length of time resulted in liver injury in the form of focal necrosis, cell lysis and enlarged kupffer cells. Mild sinusoidal ectasia was seen in the region of a terminal hepatic venule. These results are in accordance to our observations.

Al-Ali SY et al. (2005) observed ultrastructural changes in rat livers

#### Comparative hepatotoxic effect of different oral doses of Methotrexate given in rats for 28 days

Site and effects	GROUP 1 ( CONTROL)	GROUP 2 0.25mg/kg	GROUP 3 0.5 mg/kg	GROUP 4 0.75 mg/kg	REMARKS
Hepatic cord pattern	normal	Normal	Mild disruption of hepatic cord pattern	Marked disruption of hepatic cord pattern	No effects seen in Mtx treated group (1&2) except group3& 4
Sinusoidal dilatation	Not seen	+	++	+++	Sinusoidal dilatation was more in group 4
Focal fatty change	Not seen	+	++	++	
Vacuolization of hepatocytes	Not seen	++	+++	+++	vacuolization of hepatocytes was more marked in groups 3&4
Pyknosis of hepatocytes	Not seen	++	+++	+++	Hepatocytes damage was seen more in group 3&4
Apoptosis	-	-	-	-	No apoptosis observed in any group

#### SUMMARY AND CONCLUSION:

Present study was done for the purpose of studying the histopathological effects of Methotrexate(Mtx) on liver of albino rats, so that appropriate dose adjustments and combination chemotherapy regimens can be suggested for cancer chemotherapy.

Hepatic changes were seen in all three Methotrexate treated groups manifested as sinusoidal dilatation, vacuolization with pyknotic nuclei and fatty infiltration. Apoptosis was not observed in these groups and also hepatic cord pattern remained unaffected except high dose group which showed marked disruption of hepatic plates.

We conclude from the present work that experimental administration of methotrexate(Mtx) in rats was greatly associated with histological changes in the form of hepato toxicity in all the three groups. The liver were involved in all three groups where Methotrexate was administered but the magnitude of changes in group 4(0.75mg/kg body weight) were more marked than other groups.

The results obtained from present study clearly demonstrated the hepatotoxic effects of Methotrexate and this outcome may have clinical utility in patients who are on Methotrexate therapy.

#### REFERENCES

- 1) Al-Ali SY, Hassan IM, Sadek S. Ultrastructural Changes in Rats Livers Perfused in Vivo with a High Dose of Methotrexate. *Histo Histopathol.* 2005; 20 (4):1131-1145.
- 2) Al-Motabagani MA. Histological and Histochemical Studies on the Effects of Methotrexate on the Liver of Adult Male Albino Rat. *Int J Morphol.* 2006; 24 (3):417-422.

perfused in vitro and in vivo with high dose of Mtx(2000 mg) for each liver. On light microscopy, there was disruption of normal hepatic plates and swelling and vacuolation of hepatocytes. These findings are also similar to our study.

Vardi N et al. (2010) observed radial arrangement of hepatocytes from central vein were abnormal. Foci of apoptotic cells were detected inside the lobule. Shrinkage observed in nuclear contour and condensation of chromatin also noticed. In the sinusoid lumen of some sections, dilation was observed. However, we could not demonstrate apoptosis in our study.

Ozogul B et al. (2013) showed apoptotic bodies with condensed cytoplasm, peripheral and pyknotic nuclei in the hepatocytes. These findings are not in accordance to our observations as we could not demonstrate apoptosis in our study.

Tawfeeq AA and Taifoor SM (2014) found changes in the hepatic tissue like lymphocyte infiltration, necrosis, liver tissue fatty degeneration and congestion and dilation of the hepatic vein. Histological examination of liver tissue showed a significant increase in giant cells, necrosis and cellular infiltration of lobules with no fibrosis. These observations are in concurrence with Toussen E et al. (2014). They observed severe loss of liver architecture as disturbance of the hepatocytes, radially arranged cords, moderate vacuolated hepatocytes with condensation of nuclear chromatin and congestion of central vein. In the present study we found that liver gets damaged even more with small dose of Methotrexate if given for longer period. However, liver changes were more marked with higher doses (0.75mg/kg body weight) indicating that hepatic damage with methotrexate(Mtx) is proportional to the dosage administered. The outcome of study suggested that caution should be exercised while giving prolonged and high dose therapy with Methotrexate.

- 3) De Graaf H, Willemsse PH, Bong SB, Piersma H, Tjabbes T, Van VH, et al. Dose Intensity of Standard Adjuvant CsF with Granulocyte Colony Stimulating Factor for Premenopausal Patients with Node Positive Breast Cancer. *Oncology.* 1996;53:289-294.
- 4) Hall PD, Jenner MA, Ahern MJ. Hepatotoxicity in a Rat Model Caused by Orally Administered Methotrexate. *Hepatology.* 1991; 14(5):906-910.
- 5) Hendel J, Poulsen H, Nyfors B, Nyfors A. Changes in Liver Histology During Methotrexate Therapy of Psoriasis Correlated to Concentration of Methotrexate and Folate in Erythrocytes. *Acta pharmacol toxicol.* 1985;56:321-326.
- 6) Martel C, Ferlay J, Franceschi S, Vignat J, Bray F, Forman D et al. Global Burden of Cancers Attributable to Infections in 2008: a Review and Synthetic Analysis. *lancet oncol.* 2012;13(6):607-615.
- 7) Ozogul B, Kisaoglu A, Turam MI, Altuner D, Sener E, Cetin N, et al. The Effect of Mirtazapine on Methotrexate Induced Toxicity in Rat Liver. *Sci Asia.* 2013; 39:356-362.
- 8) Samuel J and Marsh R. General Principle in Cancer. In: *Essential of Clinical Oncology.* 1st ed., New Delhi: Jaypee Brothers Med Pub; 2005. pp 3-10
- 9) Toussen E, Zaki ZT, Abu-Shaier WA, Hassan h. Methotrexate Induced Hepatic and Renal Toxicity: Role of L- Carnitine in Treatment. *Biomed Biotechnol.* 2014; 2(4):85-92.
- 10) Vardi N, Parlakpinar H, Cetin A, Erdogan A, Ozturk C. Protective Effect of Beta-Carotene on Methotrexate Induced Oxidative Liver Damage. *Toxicol pathol.* 2010;38(4):592-597.