

## COMPARATIVE EVALUATION OF METHOTREXATE TOXICITY AS SOLUTION FOR INJECTION AND LIPOSOMES FOLLOWING A SHORT-TERM TREATMENT IN A MURINE MODEL OF ARTHRITIS: NOTE II. HISTOPATHOLOGICAL CHANGES

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### Abstract

The aim of this study was the evaluation of the histopathological changes induced by methotrexate (MTX)-loaded liposomes, comparatively with MTX injectable solution, following a short-term treatment in a rat model of arthritis (adjuvant Freund induced).

Liposomes with MTX itself ("hydrophobic MTX") and with its disodium salt ("hydrosoluble MTX") have been prepared.

Three different doses of MTX preparations have been intravenously (i.v.) administered, at three doses (0.2 mg/kg b.w., 0.3 mg/kg b.w. and 0.4 mg/kg b.w.), once a week for three weeks. The toxicity of MTX was evaluated in terms of histopathological changes in the liver and kidney.

Histopathological observations revealed more pronounced changes in the kidney, comparatively with the liver. Slight changes in the liver were observed in animals treated with hydro-soluble or hydrophobic MTX-loaded liposomes, (moderate and irregular granular dystrophy, discrete mononuclear cell infiltration, and moderate stasis in *centro lobular* veins), which were not significantly influenced by the type of treatment. The treatment with MTX solution for injection caused the same type of changes, but more pronounced (higher intensity of the dystrophic process, micro-focal points of mononuclear cells infiltrations, moderate steatosis).

Glomerular stases, the increase of the vascular network volume, tubular nephritis and medullary mononuclear cell infiltration have been revealed in the kidney.

Short-term treatment with MTX-loaded liposomes or MTX solution for injection in rats with an inflammatory process of arthritis type, did not induce irreversible histopathological changes in the liver or kidney.

The results of histopathological examinations indicated reduced toxic effects of MTX in the dosage range administered in this study.

### Rezumat

Scopul studiului a fost evaluarea modificărilor histopatologice induse de tratamentul pe termen scurt cu lipozomi cu metotrexat (MTX), comparativ cu soluția injectabilă de MTX, într-un model de artrită la șobolan (indusă cu adjuvant Freund).

Au fost preparați lipozomi cu MTX ca atare ("MTX hidrofoab") și cu sarea sa disodică ("MTX hidrosolubil").

Preparatele cu MTX au fost administrate intravenos (i.v.), în trei doze diferite (0,2 mg/kgcorp, 0,3 mg/kgcorp și 0,4 mg/kgcorp), o dată pe săptămână, timp de trei săptămâni.

Au fost investigate modificările histopatologice la nivel hepatic și renal, în urma tratamentului cu MTX.

Examenul histopatologic a evidențiat modificări mai pronunțate la nivel renal, comparativ cu cel hepatic.

La animalele tratate cu lipozomi cu MTX hidro-solubil sau hidrofoab au fost observate ușoare modificări în ficat (distrofie granulară moderată și neuniform repartizată, infiltrat mononuclear discret, stază în venele centro-lobulare), care nu au fost influențate în mod semnificativ de tipul de tratament. Tratamentul cu MTX soluție injectabilă a provocat același tip de modificări, dar mai accentuate (intensitate mai mare a procesului distrofic, microfocare de infiltrat mononuclear, steatoză moderată).

La nivelul rinichilor, au fost evidențiate stază glomerulară, creșterea volumului ghemului vascular, nefrită tubulară și infiltrat mononuclear în medulară.

Tratamentul pe termen scurt cu lipozomi sau soluție injectabilă cu MTX la șobolani cu proces inflamator de tip artrită reumatoidă, nu a indus modificări histopatologice ireversibile la nivelul ficatului, respectiv rinichiului. Rezultatele examinării histopatologice indică efecte toxice reduse ale MTX în domeniul de doze administrate în acest studiu.

**Keywords:** methotrexate toxicity, liposomes, adjuvant Freund induced arthritis

### Introduction

Methotrexate (MTX) is a folic acid antagonist that belongs to the class of antineoplastic agents known as antimetabolites. It acts by competitive inhibition of the enzyme dihydrofolate reductase, and thus diminishes reduced folate pools, which are essential cofactors, particularly for DNA synthesis, but also for purine and protein synthesis. MTX has also immunosuppressive and anti-inflammatory effects and is used at low doses, in certain autoimmune diseases [3]. However, it has been shown that in particular circumstances, such as a high number of activated peripheral monocytes subjected to low concentrations of drugs, MTX may sustain the systemic inflammatory process in rheumatoid arthritis [5].

The efficacy of MTX is often limited by its hepatotoxicity. With the increasing use of MTX in rheumatoid arthritis (RA), the interest in liver pathology in patients with RA receiving MTX has grown. Most liver biopsies show minor damage such as fat loading, nuclear variability, portal inflammation and small focal points of hepatocellular necrosis. However, liver fibrosis and cirrhosis have been reported in the patients during the first years of treatment [4]. In rat studies, it has been demonstrated that histopathologically hepatocyte necrosis induced by MTX can be prevented by ursodeoxycholic acid [8]. The vacuolation and necrosis, mainly manifested in the peripheral zones of hepatic lobules were also reported after MTX administration in the male rats [2]. It is suggested that melatonin may be beneficial in minimizing MTX induced renal damage in humans, as it has been shown that melatonin attenuates MTX-induced oxidative stress and renal damage in rats [1].

Liposomes have been proposed as drug carrier systems in the treatment of many diseases. It has been proved that the encapsulation of the drug substance in the carrier systems leads to increasing of the concentration at the site of action and a targeted therapy with optimized benefit-toxicity ratio is achieved.

The study aimed to evaluate the histopathological changes induced by methotrexate (MTX)-loaded liposomes, comparatively with MTX injectable solution, following a short-term treatment in a rat model of arthritis (adjuvant Freund induced).

### Materials and Methods

Liposomes with MTX itself ("hydrophobic MTX") and with its disodium salt ("hydrosoluble MTX") have been prepared [3].

Male Wistar rats, 12 weeks old were used in the experiment. The animals were brought from an authorized breeding farm and were housed for 7 days in the new environment with a 12-hr light/dark cycle and free access to special rat food provided twice a day (at 8.00 in the morning and again at 17.00 in the evening) and water *ad libitum* all day long. The temperature was maintained between 21-24°C, while the humidity oscillated between 45-60%. All researches were conducted in accordance with The European Directive 86/609/EEC/24.11.1986 and The Romanian Government Ordinance 37/30.01.2002 regarding the protection of animals used for experimental and other scientific purposes. The study was approved by the Ethics Committee of the Carol Davila University of Medicine and Pharmacy Bucharest.

After induction of arthritis, 21 days after the injection of the Freund adjuvant in the left rear paw of the rats, the animals were assigned to 10 groups, each of five animals, which received the following treatments: groups 1;2;3 – hydro-soluble MTX-loaded liposomes, at a dose of 0.2;0.3;0.4 mg/kg b.w; groups 4;5;6 – hydrophobic MTX -loaded liposomes, at a dose of 0.2;0.3;0.4mg/kg b.w; groups 7;8;10– MTX solution for injection, at a dose of 0.2;0.3;0.4 mg/kg b.w; group 9 – control group, treatment with liposomes, 0.1 mL/100 g b.w.

The doses were intravenously administrated once a week for 21 days.

At the end of the experimental period (7 days and 14 days after the last MTX dose, respectively), after decapitation, the animals were quickly dissected and their liver and kidney were removed and weighed. The tissue samples were prepared by the usual technique used for the histopathological examination [6, 7]. Thus, the samples were fixed in 10% formalin solution, and after 24 hours were passed in a series of graded ethanol, and embedded in paraffin. Paraffin sections were cut at 5  $\mu$ m thickness, by using a MicroTec CUT 4055 microtome, and were stained with hematoxiline-eosine-methylen blue (HEM) for the light microscopic examination. The sections were examined on an Olympus BX 40 light microscope, equipped with an automatic system for microphotography.

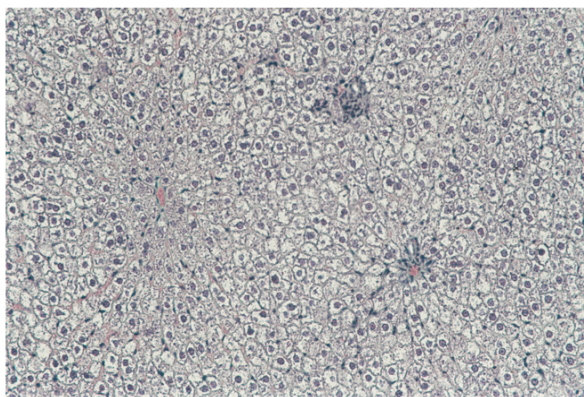
## Results and Discussion

### *Histopathological changes in the liver*

In the control group (treated with MTX free liposomes), no particular changes were observed, except for a discrete granulo-vacuolar dystrophy uniformly distributed in space and discrete porto-biliary mononuclear infiltrate (Figure 1).

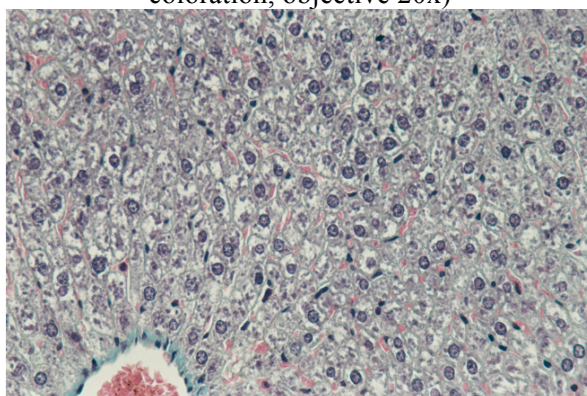
In the rat groups treated with "hydrosoluble MTX" liposomes variable changes depending on the dose were shown:

- moderate granulo-vacuolar lesions, irregularly distributed, stasis in centrilobular veins (VCL) and portobiliare spaces (PB), rare hepatocytes with karyolysis and moderate activity of Kupffer cells (Figure 2) – at a dose of 0.2 mg/kg bw
- granulo-vacuolar degeneration and moderate stasis in VCL (Figure 3) - MTX at a dose of 0.3 mg/kg bw
- discrete mononuclear infiltrate in the PB space and small focal points of hepatocyte necrosis - at the highest dose administered (0.4 mg/kg bw)



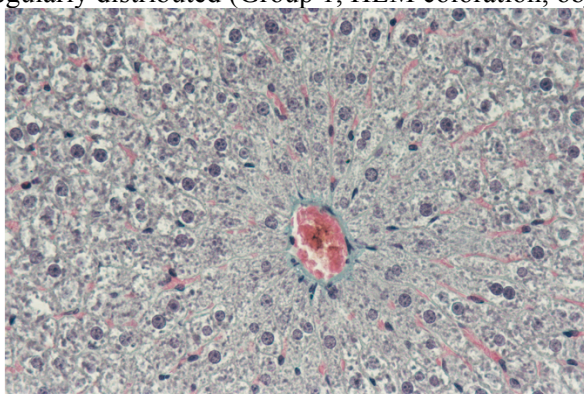
**Figure 1**

Liver with discrete porto-biliary mononuclear infiltrate (Control group, HEM coloration, objective 20x)



**Figure 2**

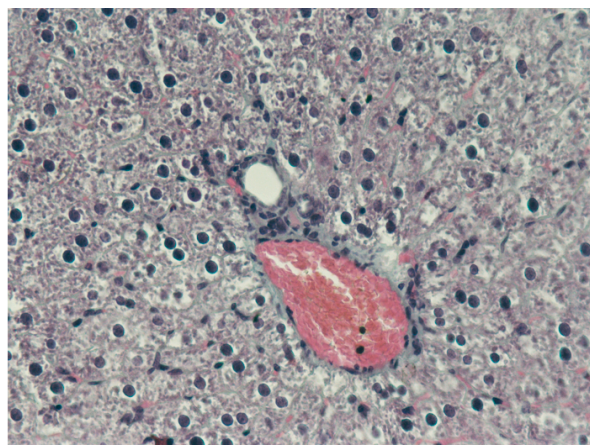
Liver with moderate activity of the Kupffer cells and moderate granulo-vacuolar lesions, irregularly distributed (Group 1, HEM coloration, objective 40x)



**Figure 3**

Liver with stasis in the *centri lobular* veins (Group 2, HEM coloration, objective 40x)

In animals treated with hydrophobic MTX liposomes, changes in the liver are similar to those in the groups treated with hydrosoluble MTX liposomes, except that their intensity is even lower. The MTX solution for injection treatment caused the same type of changes, only more accentuated (a higher intensity of the protein dystrophic process, medium mononuclear cell infiltration, occurrence of lipid dystrophy – steatosis) (Figure 4).



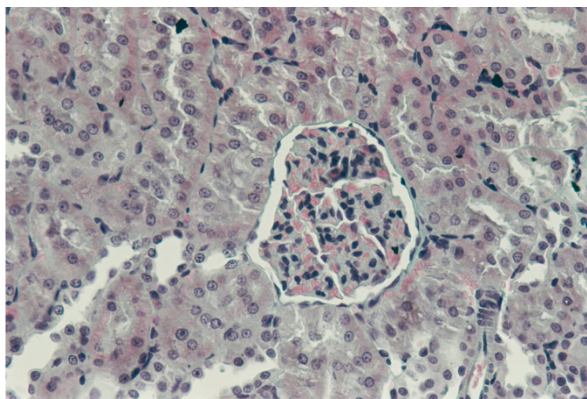
**Figure 4**

Liver with granular dystrophy with perinuclear cytolysis and discrete stasis in the porta-biliary veins ( Group 7, HEM coloration, objective 40x)

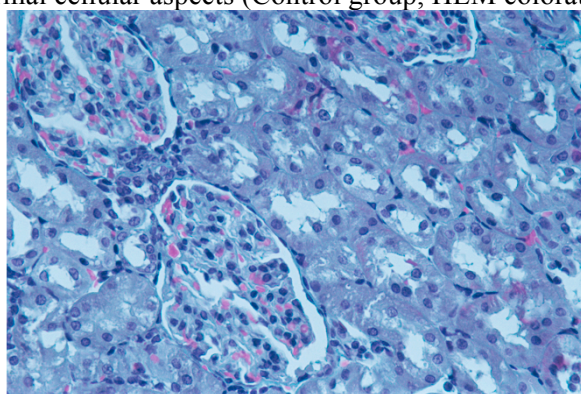
#### *Histopathological changes in the kidney*

The results of histopathological examination of the kidney samples from the control group were within normal limits (Figure 5). However, isolated cases of the interstitial mononuclear infiltrate, and unfrequent vacuolar dystrophy of the tubular epithelium were observed

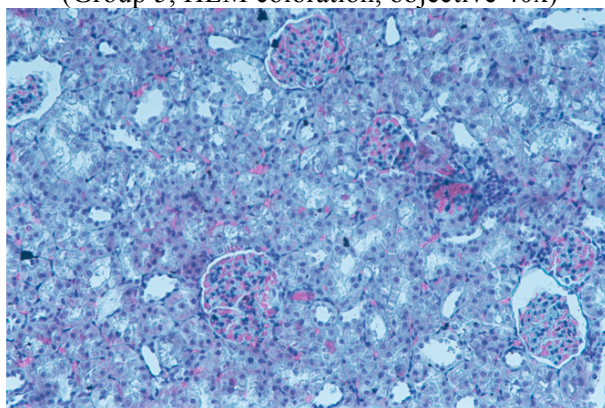
Histopathological observations identified more pronounced changes in the kidney, compared with the liver of the treated animals. However, the observed changes in the kidney are reversible. The recorded lesions ranged from the glomerular stasis and the increase of the vascular network volume (due to circulatory impairments, in the group treated with hydrofobic MTX liposomes, Figure 6) to tubular nephritis and medullar mononuclear cell infiltration (distinct effect observed with MTX injection solution at the highest dose administered) (Figures 7 and 8), and oxyphilie of the renal tubular epithelium, tubular degenerative changes, without cell necrosis (Figure 9). The tubulointerstitial inflammation, more prominent at the corticomedullary junction, induced by MTX was also reported in a recent study on Wistar rats [9].

**Figure 5**

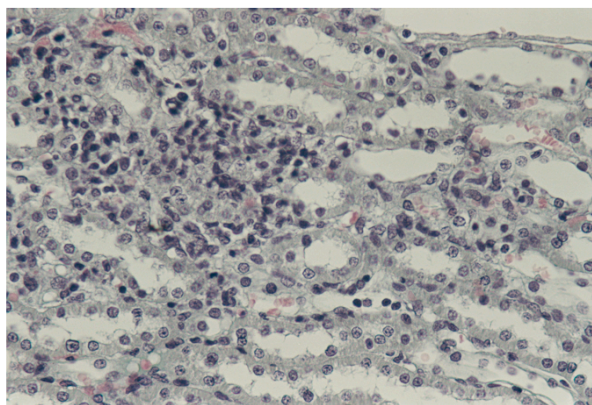
Kidney with normal cellular aspects (Control group, HEM coloration, objective 40x)

**Figure 6**

Kidney with glomerular stasis, and the increase of the vascular network volume (Group 5, HEM coloration, objective 40x)

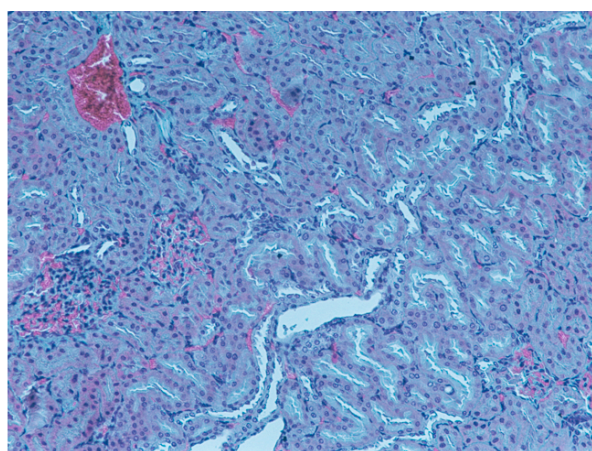
**Figure 7**

Kidney with glomerular stasis, and the increase of the vascular network volume; non-uniformly distributed tubular nephritis and a microfocus of interstitial mononuclear cell infiltration (Group 7, HEM coloration, objective 20x)



**Figure 8**

Kidney with microfocus of interstitial mononuclear cell infiltration in the renal medulla (Group 7, HEM coloration, objective 20x)



**Figure 9**

Kidney with glomerular and interstitial stasis, and oxyphilie of the renal tubular epithelium (Group 3, HEM coloration, objective 20x)

### **Conclusions**

Short-term treatment with MTX-loaded liposomes or MTX injectable solution in rats with an inflammatory process of arthritis type, did not induce irreversible histopathological changes in the liver or kidney.

Overall, the observed changes were discrete to moderate, slightly differentiated by the type of treatment (MTX-loaded liposomes or MTX solution for injection) and the administrated doses. The results of histopathological examinations indicate reduced toxic effects of MTX in the dosage range administered in this study.

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