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# The synthesis of methotrexate-loaded F127 microemulsions and their *in vivo* toxicity in a rat model

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**Abstract**

Methotrexate (MTX) has been formulated in nano and micro-emulsions, nominally to address its poor solubility and off-target effects. Nanoformulated MTX is universally reported to be a more efficacious anti-cancer agent than direct-dissolved drug; however, these investigations generally fail to screen for *in vivo* toxicity. This study aims to remedy this oversight. MTX was formulated as a standard Pluronic oil-in-water microemulsion with good drug encapsulation efficiency (73.0 % ± 8.4). Preliminary *in vitro* free radical scavenging studies found that formulation reduces drug oxidation four-fold. The toxic effects of formulated and unformulated MTX were investigated in a Wistar rat model. Rats received 0.05 mg/kg MTX as either the microemulsion or directly dissolved in phosphate-buffered saline. A drug-free microemulsion, PBS solution, and saline solution were used as controls. After 28 days, serum levels of enzymes indicative of kidney and liver damage were quantified. Significantly higher serum liver, and serum kidney enzymes were observed in the rats that received the directly dissolved MTX drug ($P<0.05$) compared to those who received the encapsulated form. Following sacrifice, the levels of catalase and superoxide dismutase (SOD) were significantly lower and the level of malondialdehyde higher, in rats who received either form of MTX relative to untreated

controls. However, the SOD levels were lower in those who received the microemulsion than those who received free MTX. Histology supported the observation that the microemulsion formulation caused no gross structural toxicity to the liver, unlike the free drug. Although toxicity was reduced compared to the free drug, the microemulsion still caused damage to the kidneys. This organ-specific toxicity is consistent with the mode of clearance of the drug. This data demonstrates that the toxicity of formulated drugs must be considered when discussing the relative merits of formulations: encapsulation always improves efficacy but may not always improve safety.

**Keywords**: Pluronic, dynamic light scattering, microemulsion, percolation, Antioxidant activity, DPPH, release rate, liver, kidney, liver enzymes, histopathology

## Introduction

Microemulsions comprising non-ionic surfactants are promising drug-delivery nanocarriers as their encapsulation of pharmaceuticals protects the drugs from physiological degradation, while also assisting with solubility and providing control over the pharmacokinetics of release.[1-6] However, many factors must be considered to ensure appropriate encapsulation and release of a specific drug, including the selection of the oil, surfactant, and co-surfactant.[1-6] In biomedicine, the non-ionic Pluronics, a polymeric surfactant triblock copolymer comprising poly(propylene oxide) terminated with poly(ethylene oxide), is commercially available in a very broad range of compositions, has effective solubilization capacity, sustained release profiles, and good biocompatibility.[1, 2, 7] Most importantly, the Pluronics are already approved for use in both pharmaceutical formulations and medical devices.[1, 2] Pluronic-based microemulsions provide superior colloidal stability compared to those stabilized with classical ionic surfactants, preventing nanoparticle aggregation. F-127, a Pluronic surfactant produced by BASF is a particularly well studied system partially due to its stimuli-responsive behaviour, gelating above 10 ºC at high concentrations.[8, 9]

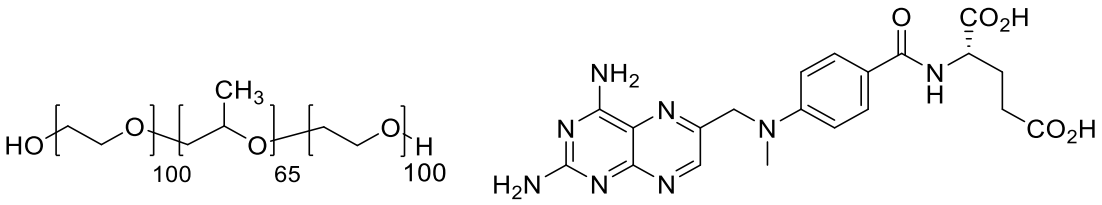


**Figure 1.** Chemical structure of Pluronic F127 (left) and MTX (right)

Methotrexate (MTX), a competitive dihydrofolate reductase inhibitor, was one of the first chemotherapeutics on the market, and is still clinically deployed to treat breast, lung, and head and neck cancers.[10] In addition, patients treated with MTX also show a reduced risk of cardiovascular disease-related hospitalization and death.[11] MTX is also used to treat rheumatoid arthritis, where it likely acts through enhancing adenosine levels.[12] Although inhibiting folate formation increases the risk of systemic oxidative damage, the MTX molecule itself inhibits the formation of reactive oxygen species by directly removing superoxide anions. This allows it to scavenge lipid peroxidation by-products, and diminishes inflammation and oxidative damage.[13, 14] However, the dosage of MTX must be carefully controlled, especially in patients with chronic renal impairment, as it is highly nephrotoxic, and leads to leukopenia and immunosuppression.[10, 13, 15, 16]

Reducing the impact of these off-target effects has been a focus of recent effort as MTX itself has a long track record of efficacy. Complicating matters, MTX is a poorly lipid-soluble drug and its water-insolubility makes delivery challenging. Microemulsive dispersions increase bioavailability, and by sequestering the drug, reduce its side effects.[17, 18] In an early example, Karasulu *et al.* designed a microemulsion of MTX to evaluate its *in vitro* suppressive effects on MCF-7 human breast cancer cells.[19] The team employed soybean oil, a mixture of Cremophore EL and Span 80, isopropyl alcohol and 0.2 N NaOH respectively as the oil phase, surfactants, co-surfactant and the aqueous phase. More recently, Amarji and colleagues, interested in psoriasis therapies, used a microemulsion comprising a polysorbate 80 surfactant, phospholipon 90G and ethanol as co-surfactants, and isopropyl palmitate as the oil, using an analysis of an experimentally-derived pseudo-ternary phase diagram to identify appropriate ratios. They characterized the *in vitro* release kinetics, *ex vivo* permeation and drug distribution of their microemulsion. [18]

Pluronics have seen some use in MTX formulation, although normally as a bulk hydrogel rather than as part of a colloidal solution.[9] The amphiphilicity of MTX that make it poorly soluble in both hydrophobic and hydrophilic environments do limit its loading as the drug tends to sit at the interface between the particle and water. This property makes small nanoparticles with high surface area a useful system for its formulation.[20, 21]

A recent study by Prakash and coworkers used a hydrogel formed from F-127 (20-22% w/v) in water to deliver MTX as an injectable gel; however, as F-127 is not metabolized, this high level of loading is non-ideal.[22] Pluta and Karolewicz formulated similar MTX-F127-based hydrogels for potential direct injection into tumours, although they only studied the release kinetics and rheology of the material,[23] as did Lu and Jun in a similar F-127 hydrogel.[24] Venkatesh and co-workers likewise studied a potential MTX-F127 hydrogel formulation for proposed use to treat rheumatoid arthritis, and showed that the formulation reduced inflammation in a mouse model.[22, 25, 26]

In the earliest example of nanoparticle MTX formulation, Law and Lin prepared a microsphere emulsion using bovine serum albumin as the core and Pluronic F68 as an emulsifier. The group showed that formulation slowed MTX release in an animal model.[27] Abdelbary and Haider studied the growth inhibition on cancer cell lines of F127-MTX nanostructured lipid carriers with diameters of 250-350 nm with various high concentrations of MTX.[28] The Sha group prepared a 22 nm mixed micelle consisting of both F127 and the more hydrophobic P105 to physically encapsulate MTX. This formulation showed good efficacy against a human cancer xenografted into a murine model.[29] The Sha group followed up this report with a covalently-immobilized Pluronic-MTX conjugate that they formulated into micellular nanoparticles. The material also showed efficacy in a patient-derived xenograft mouse model. However, in neither study was the toxicity of the formulations investigated.[30] Gao and Jiang showed that nanoparticles employing Pluronic F68 as an emulsifier were effective at transporting MTX across the blood-brain barrier.[31] The Zhang group at Liaoning used a nanoparticle derived from a physical mixture of a Pluronic shell on a chitosan core to deliver MTX into cancer cells and examined intracellular release. It demonstrated higher efficacy than the free drug.[32] Katare and co-authors developed a simple synthesis of a lipid-polymer nanoparticle using F-87 as the Pluronic. This formulation showed excellent

entrapment efficiency and promising steady release kinetics with a moderate initial burst release of the drug.[33]

Ren and colleagues formed a Pluronic-drug conjugate using an ester linkage, attaching MTX to a Pluronic polymer of undisclosed molecular weight. The ester hydrolyses slowly to release the drug under physiological conditions.[34] While we were preparing this manuscript, in an effort to better control drug release, Loureiro and coworkers formulated a nanoparticle where MTX was immobilized on the P407 through a comparably stable amide bond. These systems showed slower release in the presence of enzymes necessary to cleave the linkage, and also showed higher efficacy against cancer cell lines than the free MTX.[35]

Building on all these results, Meshkini and Oveisi immobilized F127 to a mesoporous zinc hydroxyapatite and then to a covalently conjugated MTX to the free terminus of the polymer to make a hard nanoparticle. The amide bond between the drug and the polymer was selected to be enzymatically cleavable at low tumour-relevant pH. Like the other results described here, they were effective against cancer cell lines.[36]

What most studies have overlooked to date is the toxicity of MTX; the efficacy of nanoformulated MTX therapy is not in doubt, but one of the goals of a formulation is to reduce the side effects and the toxic profile of the drug. As part of our program in targeted drug delivery, we wish to emphasize the need to consider systemic toxicity early in the discovery process as an essential endpoint.[37] Consequently, using a Pluronic microemulsion related to those discussed above, we needed to investigate the safety of physically-encapsulated MTX in a mammalian model.

## 2. Material and Methods

### *2.1 Materials*

Pluronic F127 was purchased from BASF Inc. (Mount Olive, NJ). MTX, sodium caprylate, sodium deconoate, ethyl butyrate and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO). Potassium phosphate monobasic, potassium phosphate dibasic, sodium chloride, and potassium chloride which were purchased from Fisher Scientific Inc. (Suwanee, GA). Doubly distilled, deionized Millipore water was used. All chemicals were used as received with no further purification.

### 2.2 Preparation of MTX-containing F127 nanometer-sized microemulsions

Pluronic oil-in-water microemulsions (10 mL samples) encapsulating MTX were prepared by vigorously stirring the required amounts of fatty acid sodium caprylate (SC), phosphate buffered saline (PBS at pH 7.4), and at a fixed oil-to-surfactant molar ratio (Ow = [ethyl butyrate]/[Pluronic] = 1), and MTX-to-pluronic molar ratio of Z=[MTX]/[F127]=0.23. This is according to the protocol developed by Varshney and coworkers.[6] The MTX was introduced by being pre-dissolved in the ester. Once formed, these Pluronic microemulsions were diluted with PBS to obtain the desired mass fraction of droplets (MFD). All operations were conducted at room temperature.

### 2.3 Dynamic Light Scattering (DLS) characterization of drug-loaded microemulsions

DLS characterization was conducted on an ALV-5000F (ALV-GmbH, Germany) Compact Goniometer System under vertically polarized incident light ($\lambda$ = 488 nm, 2W) supplied by a diode pumped Nd:YAG solid-state laser (Coherent Inc., CA) combined with an ALV SP-86 digital correlator with a sampling time of 25 ns to 100 ms. Measurements were made at an angle of $\theta = 90°$ to the incident beam. The intensity scale was calibrated against scattering from toluene. Prior to analysis, solutions were filtered through Millipore Millex filters (Triton free, 0.22 μm porosity) directly into cleaned scattering cells and allowed to equilibrate at the required temperature for 10 min before measurement. Each experiment was repeated at least three times. Sampling time was 5-10 min as required to obtain a fitted correlation function.

### 2.4. Determination of the entrapment efficiency (EE)

The protocol was adapted from standard published approaches.[38-40] MTX concentration was determined spectrophotometrically using a UV-Vis spectrophotometer (Agilent Technologies, Cary 60, USA) at a wavelength of 320 nm. Briefly, the spectrum of MTX was recorded between 200 and 700 nm by a UV-VIS Spectrophotometer to determine the characteristic peaks of MTX. Our MTX solution in PBS pH 7.4 had two characteristic peaks at 290 and 320 nm. We chose 320 nm to avoid interfering of

polymeric backbones with absorption of free MTX. MTX was quantified against a calibration curve prepared from fresh MTX solutions.

After synthesis, the microemulsions were centrifuged for 20 min at 15000 rpm (model 5415D, Eppendorf, Germany) to separate the free MTX in the supernatant from the nanodroplets [38, 40-42], followed by filtration with 0.22 μm filters. The MTX content of the supernatant was quantified at 320 nm. The encapsulation efficiency (EE%) was calculated as the difference between the total MTX content in the microemulsion (430 μg/mL) and the free MTX content obtained from the supernatant (Equation 1):

$$EE\% = \frac{Total\ Content\ of\ MTX - Free\ MTX}{Total\ Content\ of\ MTX} \times 100 \qquad (1)$$

### 2.5. Antioxidant activity of oil-in-water F127 microemulsions

The interaction rate of the DPPH free radical with bulk and microemulsion forms of MTX was measured as an indicator of the rate of drug release. The absorbance of methanolic solutions including 1 ml of various forms of MTX (400 μg/ml) and 4 ml of DPPH (0.004% w/v) was read every 5 min for 30 min at 517 nm. A graph of inhibition percentage (([absorbance of blank – absorbance of sample] / absorbance of blank) × 100) vs time was plotted. The slope of the straight-line equations was considered as the release rate.[5] All tests were repeated three times and the results were expressed as the average of three independent experiments.

### 2.6. Animal studies

All animal treatments were carried out in a humane fashion in compliance with both institutional (University of Zabol) and the NIH guideline for Care and Use of Animals (NIH publication No 85-23, revised 1996).

In the current experimental study, fifty male adult rats (Wistar breed), were obtained from the laboratory animal centre of University of Zabol and were divided into five groups. The first group (control group) received normal saline for four weeks; the second group received the PBS vehicle alone; the third group received the microemulsion without the presence of MTX; the fourth group received an unformulated 0.05 mg/kg dose of MTX in PBS; and the fifth group received the microemulsion formulated MTX with a dose of 0.05 mg/kg. Animals were kept at standard conditions 20-23 °C and 12 (h)

light/dark cycles and were fed a standard laboratory diet (Javaneh-Khorasan, Iran). After 28 days, the rats were anesthetized, and blood samples were collected by heart puncture and immediately centrifuged (3000 rpm for 5 min) in order to separate the serum. The serum samples were immediately frozen at -80 °C. After euthanasia, liver specimens were carefully removed and preserved in formalin until examined.

### 2.7. Determination of serum biochemical parameters and statistical analysis

The activity of catalase in liver tissues was assayed by the Aebi method.[43] This method was based on measuring the rate of decomposition of $H_2O_2$ at 240 nm using a commercial spectrophotometer (UNICO UV/VIS- 2100 Spectrophotometer). In addition, the hepatic superoxide dismutase (SOD) activity was assayed based on the method of Kakkar.[44]

The liver malondialdehyde levels, a marker of lipid peroxidation, was assayed spectrophotometrically at 532 nm according the method of Okhawa. The Okhawa method is based on the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde.[45]

Commercial kits (Pars Azmoon. Co., Tehran, Iran) were used to determine the serum levels of AST, ALT, BUN and creatinine. All the serum biochemical parameters were measured in the biochemistry laboratory of University of Zabol using the Selectra Pro M auto analyser, (Vital Scientific, SpanNeren, Netherlands).

Statistical analysis was conducted using the SPSS software (version 20.0). Multiple comparisons between the experimental groups were performed by ANOVA using Tukey tests. Statistical significance was accepted at $P<0.05$.

### 2.8. Histopathological investigations

For histopathological examinations, rats were euthanized using diethyl ether and tissue samples from liver and kidney were chopped and preserved in 10% formalin. After two days, the formalin was changed, and the samples were processed for histopathological staining. After paraffin embedding and block making, the tissue sections were prepared with the hematoxylin-eosin staining method and were examined under a light microscope (Olympus Optical Co., Tokyo, Japan) at 40x magnification.

## 3. Results and Discussion

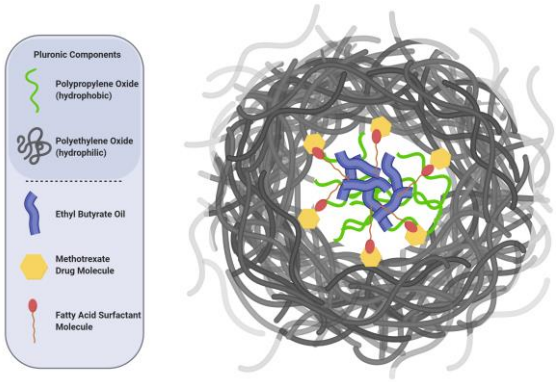


**Figure 2.** Cartoon representation of the morphology and content of the Pluronic microemulsions used in the current study.

### 3.1. Determination of Microemulsion Formation Behavior

The microemulsions were synthesized by mixing the Pluronic polymer with a solution of MTX, dissolved in ethyl butyrate as the "oil" phase, and fatty acids as the surfactant (Figure 1). We started by investigating the dynamic behaviour of the F127-based oil-in-water microemulsions as a function of the mass fraction of the nanodroplet (MFD). The auto-correlation function of the microemulsions versus time for different MFD values was plotted (Fig. 3a). The decay rate is, as expected, directly proportional to the MFD; inter-droplet repulsive interactions increase as the number of droplets increases. At a constant MTX concentration, more particles will mean a greater surface area is available to host the MTX at the oil-water interface rather than being enclosed in the core of the particles. The charged carboxylates on the water-facing MTX molecules induces a charge density on the surface of the particle, leading to an increase in repulsion as has been noted by us and others.[6, 17, 46, 47] From the experimental diffusion data, the hydrodynamic diameters of the MTX -loaded microemulsions were obtained as a function of MFD and demonstrated that size is inversely proportional to MFD. This further supports the observation that surface area increases as the MFD increases due to the reduction in particle clustering, consistent with the reports of others in similar systems (Fig.3b). In other words, an enhancement of the electrostatic repulsion between nanodroplets as the MFD increases (as more nanodroplets leads to a greater possibility of inter-nanodroplet interactions) can lead to a reduction in the nanodroplet size. This occurs because the PEO layer collapses inwards due to this effect. [1, 10, 17, 46] However, the changes are small. In all cases, the average size of the particles is under 10 nm, and there

is likely little clinical difference between these various formulations. For this reason, the particular formulation is unlikely a critical factor, and an MFD of 0.01 was selected as the basis for the materials used in the other studies outlined here as it uses the minimum amount of material, limiting off-target effects arising from the Pluronic polymer.

Although particle size plays a key role in nanobiointeractions in the physiological milieu, as well as in particle biodistribution, targeting, cell uptake and internalization, toxicity, and particle degradation; we want to stress that the size ranges of our nanodroplets, 5-9 nm, is limited, and renal excretion is still readily possible for all of the microemulsions studied here.

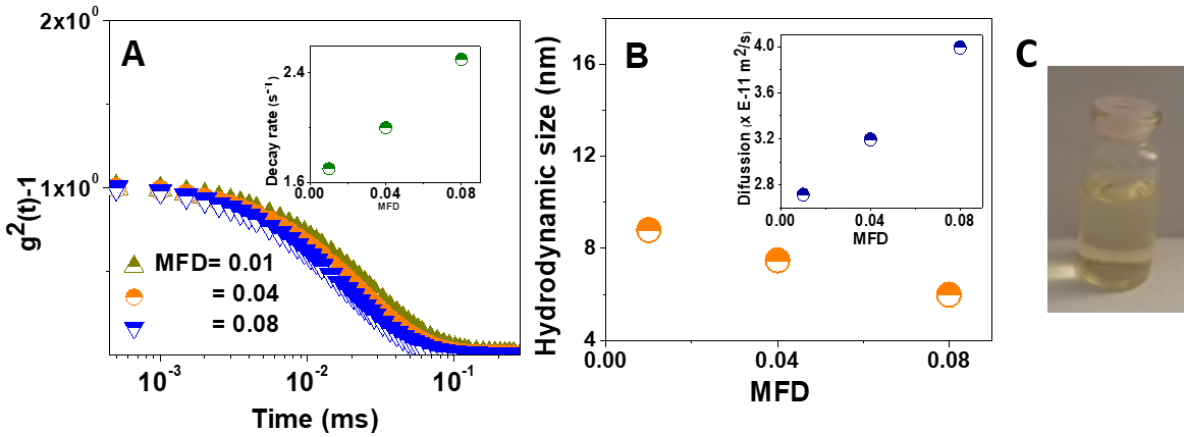


**Figure 3.** Dynamic light scattering behaviour as a function of MFD. A) Auto-correlation function of microemulsions versus time for different MFD values (inset: decay rate vs MFD); B) Hydrodynamic diameter as a function of MFD (inset: the Diffusion Coefficient versus MFD for microemulsions); C) Digital photograph of the microemulsion solution after standing for 6 months.

### 3.2. Entrapment Efficiency

Ideally, as with any pharmaceutical intervention, the lowest possible dose of all components of the formulation are desired. As the MTX dosage is the limiting factor, we want to maximize the encapsulation efficiency of the nanocarrier to minimize the amount of nanocarrier that must be used. This is determined by the entrapment efficiency (EE%). The components of the formulation were selected to maximize this parameter: high loading was predicted to be facilitated due to the molecular interactions

between MTX and F-127. Using sodium caprylate as the surfactant further reduces inadvertent burst release as it helps to rigidify the outer walls of the nanoparticles due to its favorable apparent packing factor [48] coupled with the higher oil/water interface area on the surface of the particles [49]. These factors, along with favorable economics, are the reasons that it is commonly used as a surfactant for stabilizing microemulsions [50]. In the EE% study, 320 nm absorption wavelength was chosen to avoid interfering of polymeric backbones in UV-Vis spectrum of free MTX. In this case, loading the MTX into the MFD 0.01 formulation described above resulted in a good EE % of 73.0 % ± 8.4 [57]. With the loading established, the stability of the microemulsion was investigated.

### 3.3. Stability of F127 microemulsion

To achieve a stable microemulsion, one must incorporate a repulsion mechanism that counteracts thermodynamically favourable Ostwald ripening. Such phenomenon arises from the mutual attraction of particles and the decrease in energy that results from the minimization of the surface area: volume ratio. This can be accomplished using either electrostatic or steric repulsion. The former results from the incorporation of a double layer of charged components in the particle's shell; the latter is achieved by incorporating non-ionic surfactants or polymers that interfere with membrane fusion. Steric repulsion is very useful for preventing aggregation of colloidal solutions. As is usual, our formulations combine both strategies as the caprylate provides a negative surface, while the hydrocarbon chains of the Pluronic provide the steric blocking. Upon standing for three months, no visible turbidity or sedimentation was observed (Figure 3c), suggesting that no meaningful aggregation was observed. Similarly, the zeta potential of microemulsion immediately after preparation and after three months remained similar: they were -35 mV and -30 mV, respectively. Many authors have reported similar results: the Pluronic polymers and their mutual steric repulsion defines the high stability of these systems.[1, 5, 17, 20, 50] The size distribution of the materials also remained stable over 12 months; average particle size remained the same, and the dispersity index (PDI) only

changed from 0.21 to 0.26. This is consistent with similar F127 microemulsions from our research group which were also completely stable for very long periods of time.[5]

### 3.4. Antioxidant activity of MTX-loaded microemulsion

MTX induces oxidative stress by interrupting the folate pathway, but is itself an antioxidant. To quantify the relative antioxidant activity of the nanoformulations as a proxy for exposed MTX, we employed the standard 2,2-diphenyl-1-picrylhydrazyl assay in which the colour of a methanolic DPPH solution changes from purple to yellow. The challenges in using this assay for predicting absolute antioxidative activity are many, and it is not a precise tool; however, under proper control, it can be used to estimate the relative behaviour of systems.[51, 52] We measured the reaction rate of the DPPH free radical with both bulk and microemulsion forms of MTX (final concentration of 80 μg/ml). Under parallel measurements, initial reaction rates of 0.873 and 0.238 μg/ml × min were respectively measured for both the bulk solution and the microemulsion. This approximate four-fold difference demonstrates that the microemulsion protects the drug from metabolism and keeps it encapsulated. As it is located at the surface, some reactivity is to be expected.

### 3.5. *In vivo* toxicity

Systemic MTX toxicity arises in the form of kidney or liver damage. Along with histopathology, measuring the serum content of specific kidney and liver enzymes can provide information about damage to these organs in an *in vivo* model.[53, 54] One of the proposed benefits of microemulsions is protection from this toxicity: a formulated MTX dose should be less toxic than a direct dissolved equivalent dose. To investigate toxicity, ten healthy male Wistar rats were assigned to each of 5 groups. The first was a control group on a saline; the others received intraperitoneal administration of a treatment. The second received PBS, identical to the buffer used in the treatments. The third received the F127 microemulsion without any MTX, the fourth received MTX in PBS (no microemulsion) and the fifth received the formulated MTX. Liver enzyme levels (ALT and AST) remained statistically unchanged for the PBS, F127 microemulsion, and MTX (0.05 mg/kg)-microemulsion formulations (Fig. 4a and 4b). The 0.05 mg/kg direct dissolved treatment did show a statistically significant increase in the enzyme levels ($P<0.05$). The mean of the microemulsion is higher than the control, but the variance in

the systems prevents it from reaching the threshold for significance. Serum BUN and creatinine levels also followed the same exact pattern (Fig. 4c and 4d). Combined, these results indicate that formulating MTX as a microemulsion renders the drug less toxic than direct dissolution.

MTX-derived oxidative damage will occur outside of these organs and can be measured by the upregulation of catalase to counteract the increased number of reactive oxygen species. However, no significant increase in catalase activity was observed for either MTX formulation (Fig. 4f). Curiously, the F127 microemulsion did elicit an increase. Superoxide dismutase activity dropped in both the MTX-treated groups (Fig. 4e). This is possibly because MTX itself specifically interacts with ROS, decreasing the need for enzyme upregulation. In contrast, malondialdehyde (MDA) levels were higher in both MTX-treated groups. MDA is an excellent marker of the presence of ROS, resulting directly from oxidative stress (Fig 4g). In both cases, the microemulsion-formulated MTX moderated the effects, with the directly dissolved MTX showing greater changes.

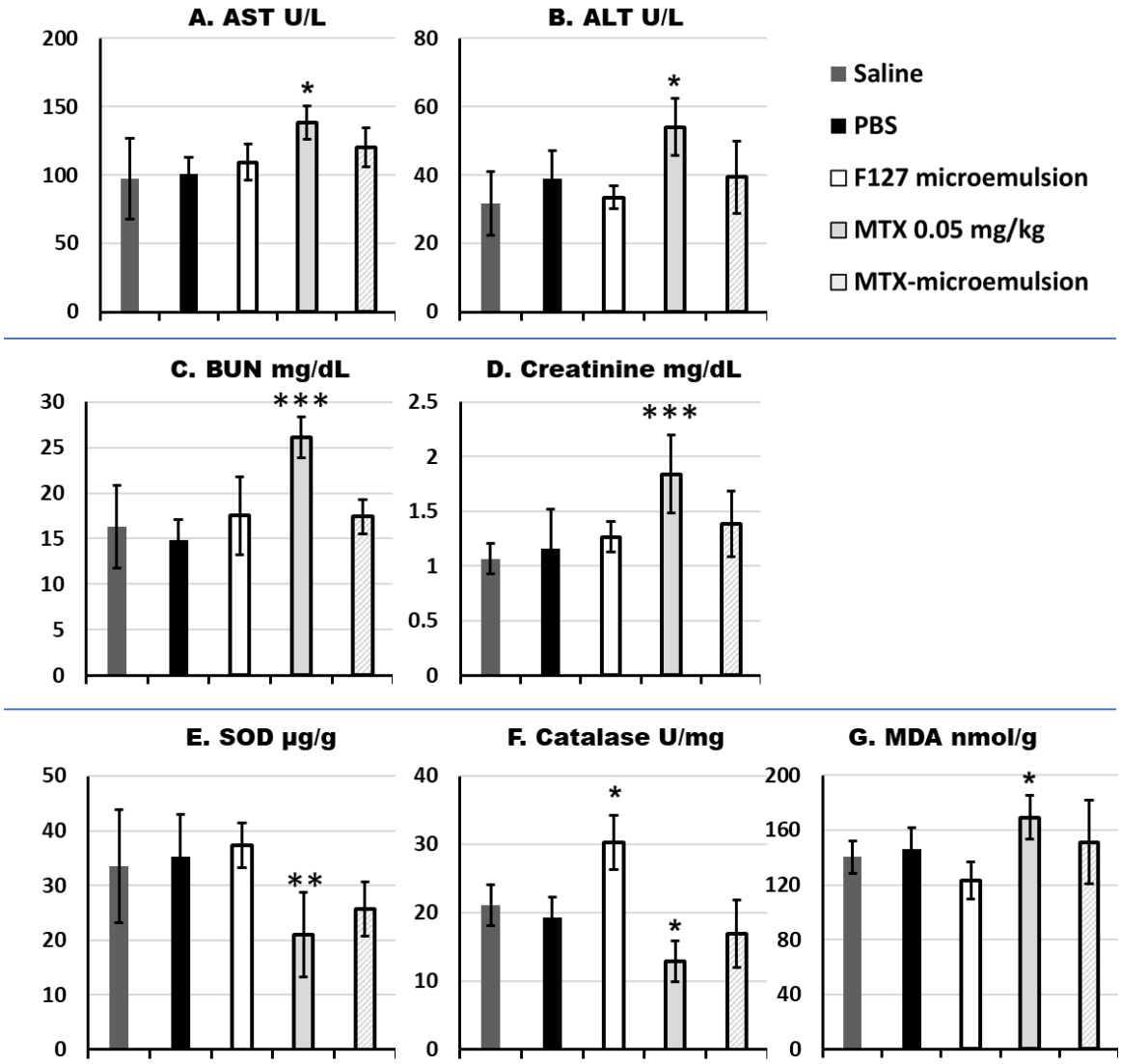


**Figure 4.** Serum AST (**A**), AST (**B**), BUN (**C**), and creatinine (**D**) levels in male Wistar rats, Mean ± SD, N=10. *P < 0.05, compared to the saline control group. Corrections made for multiple comparisons. Enzyme levels in liver homogenates of SOD (**E**), catalase (**F**), and MDA (**G**)

The histopathological observations align well with the biomarker measurements. The livers of the rats who received saline, PBS, or MTX-free F127 microemulsion had a normal hepatic architecture with distinct hepatocytes, central vein, and sinusoids (Fig. 5a-c). Conversely, liver sections of rats treated with unformulated MTX (0.05 mg/kg; Figure 5d) showed signs of cell necrosis and a disarrangement of the sinusoids. The administration of MTX microemulsion at a dose of 0.05 mg/kg (Fig 5e) caused no pathological changes in liver sections compared to the rats not treated with MTX (Fig 5a-c).

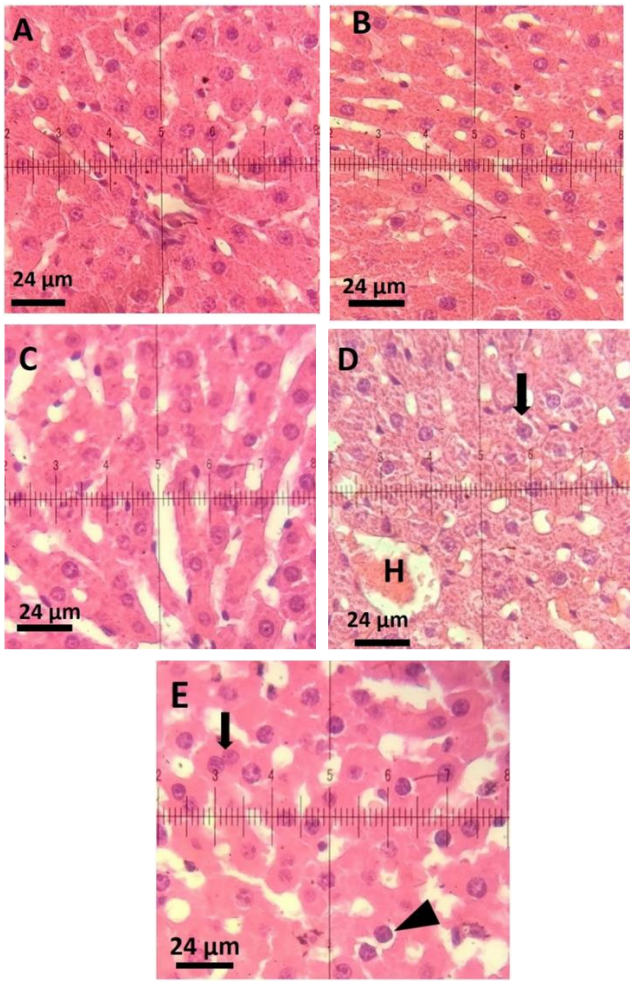


**Figure 5.** Liver sections stained by hematoxylin and eosin of rats: A) Saline; B) PBS; C) F127 microemulsion with no MTX; D) MTX 0.05 mg/kg; E) MTX in microemulsion formulated 0.05 mg/kg MTX. Alterations in liver tissue including congestion of central vein (H), sinusoidal dilatation (arrow) are highlighted for the liver obtained from the liver subjected to unformulated MTX. All images at 40x magnification.

There were similar changes in the kidney. Intraperitoneal injections of unformulated MTX induced prominent histological changes in renal tissues, such as decreased proximal tubule diameter (Fig. 6d), compared to healthy kidneys in the saline control and the PBS and F127-treated groups (Fig. 6a-c). These same histopathological changes were less prominent in the kidney micrographs of rats treated with the microemulsion form of methotrexate (Fig. 6e); however, they are still present with clear mild congestion of the proximal tubules.

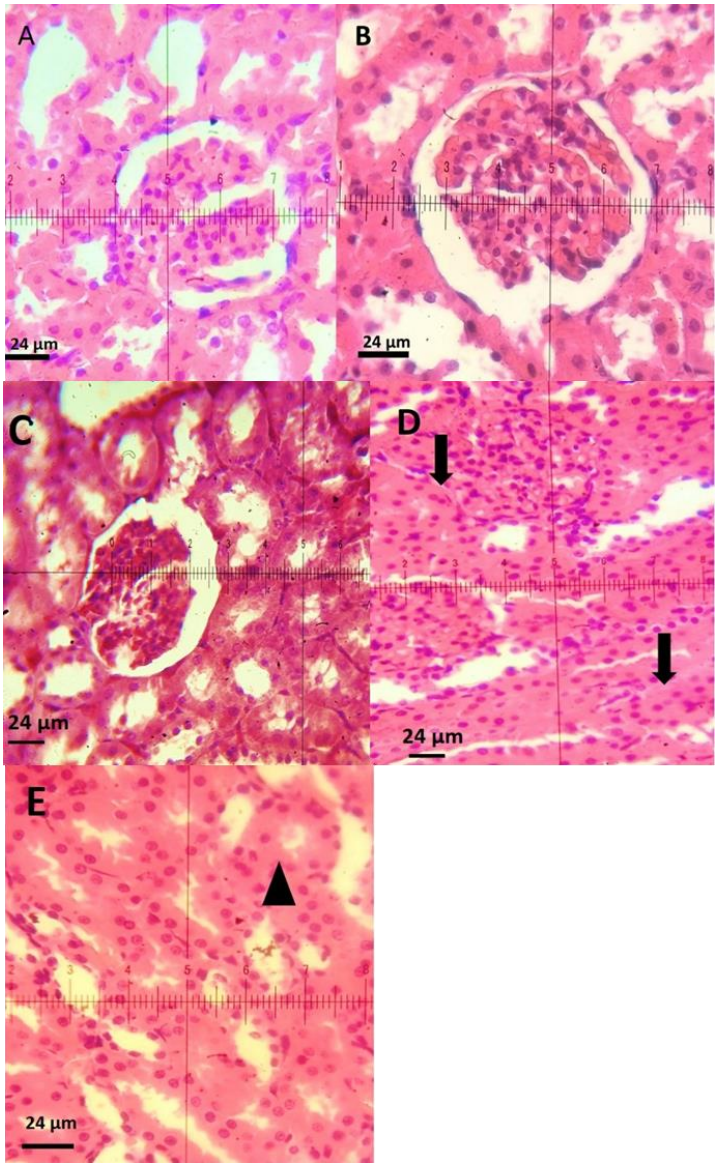


**Figure 6.** Rat kidney sections stained by hematoxylin and eosin: A) Saline; B) PBS; C) F127 microemulsion no MTX; D) MTX 0.05 mg/kg; E) MTX in F127 0.05 mg/kg. Congestion of proximal tubules is highlighted in both MTX-subjected kidneys using arrows. 40x magnification was used for all images.

Rats subjected to the microemulsion-MTX suffered no hepatotoxic effects as determined by the serum presence of elevated liver function biomarkers, or liver histopathology. On the other hand, kidney histology was affected in the group treated with the microemulsion forms of MTX. MTX is nephrotoxic, so the lower threshold on this organ is to be expected.[55, 56] Unformulated MTX produced pronounced toxic

effects on both the liver and the kidney according to both serum titres and histopathology. Similar doses of MTX have shown similar nephrotoxicity, and hepatotoxicity.[57, 58]

Different MTX formulations have distinct biological activity, leading to the development of a series of nanoemulsion delivery systems. The best-studied drug delivery systems are solid-lipid nanoparticles, magnetic nanoparticles, carbon nanotubes, human serum albumin, and polymeric nanoparticles.[59, 60] Since most of the studies have been pre-clinical trials, at now, it is difficult to evaluate the benefits of one system over the others. In the case of nano-emulsions synthesized in the present study, there is still a lack of clinical trials to evaluate the advantages of methotrexate-loaded F127 microemulsions over other formulations. However, the laboratory animal models could give us a better understanding of the newly formulated microemulsions. Many experiments have been conducted on biological effects of methotrexate nanoformulations. The results of Mello and coworkers demonstrated that MTX-lipid nanoemulsions at 0.2 mg/kg were effective intravenously.[61] MTX-microemulsions based on Cremophore EL have also showed potent antitumor activities on prostate, ovarian, and human breast carcinoma cell lines at 0.1 mg/mL and was more potent than direct dissolution of the same concentration.[62]

The concentration tested in our study and our microemulsion formulation is comparable to these systems. The results of this study clearly show that the microemulsion formulation protects the rat from the off-target toxicity of MTX, and the low cost and ease of synthesis of these F-127-based microemulsions might prove to make them promising delivery formulations for both anti-cancer and anti-inflammatory applications.

## 4. Conclusions

Encapsulation in oil-in-water microemulsions is well established to universally improve the efficacy of MTX, but the effect on toxicity of these systems has not been previously investigated in the literature. In this study, oil-in-water Pluronic F127-based microemulsions containing the MTX drug were prepared at an equimolar oil-to-surfactant molar ratio at nano-droplet mass fractions (MFD) of between 0.01 to 0.08. The droplet size increased and the attractive interactions between nano-droplets increased as the MFD rose. MTX encapsulation efficiency (EE%) of inside the microemulsions was ca. 73.0 %

± 8.4 indicating a high loading of the drug into the nanocarrier. Finally, the microemulsions remain stable for up to a year after synthesis, demonstrating their ability to be stored long term and made in bulk.

F127-formulated MTX showed no liver toxicity, and only limited kidney toxicity in comparison to an equivalent dose of the unformulated MTX in a Wistar rat model. This formulation, and other similar approaches, is promising for the future of MTX clinical deployment to improve outcomes for patients. However, measuring toxicity should be an essential component of the study of chemotherapeutic microemulsions in this growing literature.

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**Table of Contents Graphic**