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High-dose 7-hydroxymethotrexate: acute toxicity and lethality in a rat model

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Abstract To elucidate mechanisms for methotrexate (MTX)-induced renal and hepatic toxicity, we investigated the acute effects of bolus plus continuous infusion of up to 0.4 g/kg 7-hydroxymethotrexate (7-OH-MTX) in the rat. We demonstrate for the first time in any species the occurrence of acute lethal toxicity within a few hours after 7-OH-MTX administration. Serum concentrations of 7-OH-MTX measured at the time of death were 1.4 m*M* (mean), about one-half of those achieved in some patients after infusion of high-dose MTX (HD-MTX) in the clinic. The data suggest an approximate LD_{50} (the dose lethal to 50% of the study population) of 0.3 g/kg and a steep dose/lethality curve for 7-OH-MTX. Moreover, acute renal and hepatic toxicity occurred as evidenced by severe morphological findings and increased serum levels of creatinine and liver transaminases. In all rats subjected to continuous infusion of 7-OH-MTX, yellow microscopic precipitations were apparent in the kidney tubules. Crystallization was also seen in bile ducts of the liver in some of the rats. These results further support that the formation of 7-OH-MTX is disadvantageous and that reported attempts to prevent its formation during MTX treatment are warranted.

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Introduction

Methotrexate (MTX), the main folate antagonist, is used in high doses in several chemotherapy regimens [22, 41, 56]. Administration of high-dose MTX (HD-MTX) results in significant concentrations of 7-hydroxymethotrexate (7-OH-MTX) in serum and urine [9, 10, 16, 33, 38, 52]. In patients treated for osteosarcoma (12 g/m^2), Ettmann et al. [21] reported 7-OH-MTX levels of up to 2.7 m*M*, whereas Borsi et al. [9] described 7-OH-MTX concentrations of 3.5 m*M* at the end of a 24-h infusion of 33.6 g/m^2 MTX. Continuous production of 7-OH-MTX usually entails prolonged serum levels of 7-OH-MTX at around its peak concentrations [10, 21].

The metabolite was initially considered a product of detoxification [35, 36, 46] that was 200-fold less cytotoxic than the parent compound [27]. However, MTX-induced hepatotoxicity has been associated with this metabolite [14], and intratubular crystallization of 7-OH-MTX is proposed as one mechanism of renal damage after HD-MTX therapy [33, 34, 49]. If renal damage occurs, excretion of the drug is delayed, which may lead to systemic toxicity [50]. The acute lethal dose of 7-OH-MTX (or serum concentrations associated with it) is not known in any species.

To explore directly the toxicity of 7-OH-MTX without interference by MTX, we recently gave a 10-min bolus infusion of 100 mg/kg 7-OH-MTX to rats [53] and demonstrated acute renal and hepatic toxicity without precipitation of the metabolite. However, the 7-OH-MTX serum levels declined rapidly and reached 10 μ*M* within only 2 h [53]. On the basis of these data, we speculated that major toxicity with or without precipitation would be seen when 7-OH-MTX levels of 1 m*M* were maintained for a protracted period. Herein we report that keeping serum 7-OH-MTX levels at about 1 m*M* after bolus plus continuous infusion of

7-OH-MTX caused 100% mortality within 2 h in anesthetized and unanesthetized rats.

Materials and methods

Drugs and chemicals

7-OH-MTX reference standard was a gift from Dr. F. M. Sirotnak (Memorial Sloan-Kettering Cancer Center, N.Y., USA). MTX was kindly provided by Nycomed Pharma (Oslo, Norway). Methanol and tetrahydrofuran [both high-performance liquid chromatography (HPLC)-grade] were obtained from Rathburn Chemicals (Walkerburn, UK). All reagents used for the synthesis of 7-OH-MTX were supplied by Aldrich Chemical Company (USA). Water was distilled on a Milli-Q (Millipore) water purification system. All solutions containing 7-OH-MTX were stored under protection from light at –20 °C.

Preparation of 7-OH-MTX

7-OH-MTX was prepared by a modification of the method of Dawson et al. [17]. Preparation of MTX-dimethylester (compound 1) was performed under an argon atmosphere. Di-Na-MTX (15.0 g, 30 mmol) in dry CH_3OH (100 ml) was added to acetyl chloride (21.3 ml, 0.3 mol) in dry CH_3OH (350 ml) and refluxed for 2–3 h. One-third of the CH_3OH was removed by evaporation under reduced pressure prior to precipitation by the slow addition of cold $NaHCO_3$ (8%, 540 ml). The mixture was refrigerated overnight and the precipitate was filtered, washed, and dried [yield, 11.5 g (79%)].

Preparation of 7-CN-MTX-dimethylester (compound 2) from 7.16 g of compound 1 was done as previously described [17]. The reaction mixture was washed several times with ethyl acetate and saturated $NaHCO_3$. The organic phase was evaporated and stripped with ethyl acetate to remove the remaining water, which was followed by purification by column chromatography (Kieselgel 60; 0.04–0.063 mm, 42 × 3.2 cm). Mobile phase 1 was 5% CH_3OH in ethyl acetate; fraction 750- to 1400-mol contained compound 2, yielding 2.21 g (29%), the melting point being 196.5–197.1 °C. Mobile phase 2 was 10% CH_3OH in CH_2Cl_2 and contained compound 1, yielding 2.29 g (32%).

Preparation of 7-OH-MTX (compound 3) was performed as reported elsewhere [17] with the following exceptions: (1) the reaction was completed after 2 h at 0–4 °C and (2) the precipitate was washed with 30% water in tetrahydrofurane (THF), yielding 2.23 g 7-OH-MTX (60%). Confirmation of the reaction products was done by nuclear magnetic resonance (NMR), and the purity measured by HPLC was more than 99%. A mixture of our synthesized 7-OH-MTX, the 7-OH-MTX standard obtained from Dr. F. M. Sirotnak, and our previously urine-based 7-OH-MTX eluated as one peak on the HPLC column.

Interestingly, the solubility of this synthesized 7-OH-MTX in aqueous solution surpassed 20 mg/ml, which is far in excess of the results previously reported for 7-OH-MTX purified from urine and rabbit liver homogenate [15, 34, 44, 50, 53]. Hence, 7-OH-MTX itself is more soluble than the parent compound in aqueous solutions; however, in vivo, the metabolite may react with unidentified substance(s), forming less soluble complexes. These unidentified substances are probably ions, as both synthetic and urine-based 7-OH-MTX gave similar HPLC chromatograms.

Animals and operation

Nonfasted male Wistar rats weighing 216–290 g (Charles River, WIGA GmbH, Sulzseld, Germany) were used for the experiments. The rats were anesthetized with 0.4 mg/kg fentanyl given i.p. (maintenance, 0.1 mg/kg per hour given i.m.) and had their right external jugular vein cannulated [12]. Body temperature was maintained with a heat lamp. Additionally, one rat was subjected to an operation on 1 day and was allowed to recover from anesthesia prior to the experiment on the following day.

Experiments

7-OH-MTX was dissolved in saline to a final concentration of 20 mg/ml (pH 7.0). Drug and diluent (at identical pH) were delivered through the venous catheter. In all, 2 rats were given a 10-min bolus infusion (100 mg/kg), whereas 16 rats were scheduled for the 100-mg/kg bolus infusion plus a 110-min constant infusion. The infusion rates were calculated by

$$C_{ss}=\frac{mg}{\frac{min\times kg}{Cl_T}}$$

using our previous 7-OH-MTX clearance data [53] to achieve a steady-state serum levels of 1 m*M* for a total of 2 h. 7-OH-MTX infusions were carried out as follows: A, 8.75×10^3 ml min^{-1} kg (n = 4); B, 1.75×10^{-2} ml min^{-1} kg (n = 2); and C, 0.35 ml min^{-1} kg (n = 5). Five control rats were infused with diluent at the highest infusion rate.

Blood samples (250 µl) for 7-OH-MTX analysis were drawn after the bolus infusion at 10 min, followed by samples obtained at 2, 5, 10, 15, 30, 45, 60, 90, 180, 240, 300, 360, and 480 min after the end of the bolus infusion in rats subjected only to the 7-OH-MTX bolus infusion (Fig. 1A) and by samples drawn at 45, 90, 120, 122, 125, 135, 150, 180, 210, 240, 300, 360, and 480 min after the initiation of 7-OH-MTX administration in rats given continuous infusions (Figs. 1B, 2). The samples were drawn from the same line used for drug administration to keep surgical intervention at a minimum. The catheters were flushed with heparinized saline (10 IU/ml) immediately before and after each blood sampling. Blood for enzyme and creatinine analyses was obtained after cannulation of the jugular vein and at 5 and 8 h after infusions. Voided urine was collected, and after the animals had been killed, the urine bladder was aspirated to ensure complete collection. The pH was measured in all urine samples.

During the experiment, both experimental and control rats were hydrated with 6 ml kg^{-1} h of 0.06 *M* $NaHCO_3$ in isotonic saline, similar to the alkalinization used in the clinic. Some of the animals exposed to 7-OH-MTX received reduced hydration at some time points due to hyperventilation and, hence, the fear of volume overload. At 8 h, all surviving animals underwent laparotomy and exsanguination. The left kidney and one specified liver lobe were immediately removed for morphological examination. Tissues from the heart and brain were also randomly collected from rats in both groups.

Analytical procedures

Analysis of the 7-OH-MTX concentration in serum and urine was performed by reverse-phase HPLC as previously reported [12, 14]. Levels of serum ASAT (aspartate aminotransferase), ALAT (alanine aminotransferase), and creatinine were determined as described elsewhere [53].

For light microscopy (LM) the rat tissues were immersion-fixed in 4% phosphate-buffered formaldehyde, dehydrated, embedded, cut at 5 µm, and stained with hematoxylin and eosin, van Gieson, Pearl, and reticulin. Since this fixation liquid may dissolve precipitated 7-OH-MTX [43, 57], tissue fixation was also performed with an alcohol-based fixative (Carnoy). McDowell's fixative and 1% aqueous OsO_4 were used to prepare tissue for electron microscopy (EM) as formerly reported [53].

Statistical analysis

Statistical analyses were performed by Students *t*-test. Statistical significance was defined as $P < 0.05$. All results are expressed as mean values ± SD.

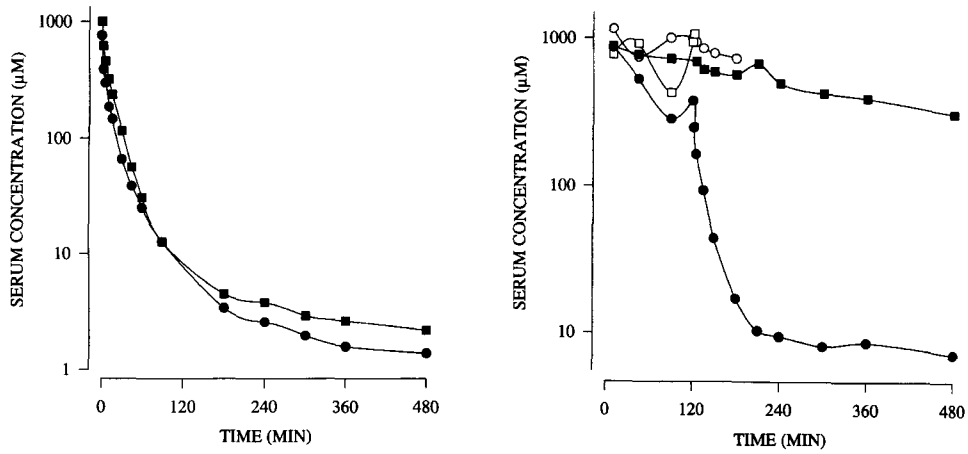

Fig. 1 A, B Serum concentration curves generated for 7-OH-MTX following **A** 10-min bolus infusion of 100 mg/kg 7-OH-MTX to 2 anesthetized rats and **B** bolus infusion followed by continuous infusion at 0.02 ml/min (0.4 mg/min) of 7-OH-MTX to 4 anesthetized rats, of which 2 died (*open symbols*) and 2 survived (*filled symbols*) during the 8-h experiment

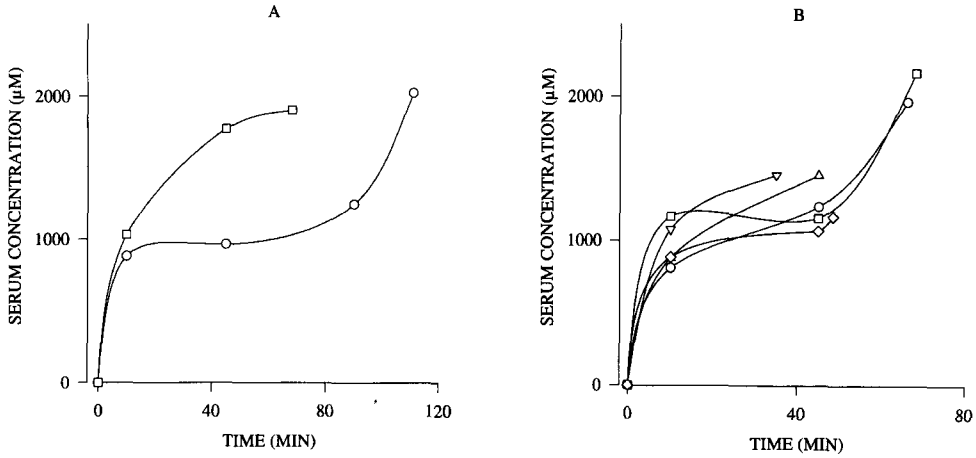

Fig. 2 A, B Serum concentrations of 7-OH-MTX plotted versus time after short-time bolus infusions of 100 mg/kg 7-OH-MTX followed by continuous infusions in anesthetized rats. **A** Infusion at 0.04 ml/min (0.08 mg/min). **B** Infusion at 0.08 ml/min (1.6 mg/min). Data represent single rats; the end points of the curves represent the time of death

Results

Survival

Control rats (n = 5) survived the 2-h infusion at the highest infusion rate and were killed at the termination of the experiment after 8 h. Autopsy revealed no sign of pathology. Hematocrit analysis was achieved in 5 rats, providing results comparable with those of our earlier studies using the same model [12–14]. Furthermore, venous blood-gas analysis was performed [53], showing no difference between experimental and control rats.

The 2 rats receiving the 100-mg/kg 10-min bolus infusion and 2 of 4 rats exposed to the lowest infusion rate survived the 8-h experiment. All other rats receiving 7-OH-MTX died after 125 and 178 min (lowest infusion rate), after 68 and 111 min (medium infusion velocity) and between 35 and 68 min (53 min, mean) after infusion at the highest rate. The unanesthetized rat treated at the highest infusion rate died after 56 min. The events observed prior to death were the same in all rats. About 15 min before death, their peripheral blood circulation was perturbed, and both the heart and the respiration rate decreased until the rats died with spasms.

The rats that survived appeared to be rather unconspicuous at autopsy, whereas all the rats that died presented almost the same macroscopic picture. The livers were swollen and dark brown. The kidneys were also enlarged and had a spotted appearance. In some of the rats the vessels in the lungs were distended.

Serum levels of 7-OH-MTX

The two rats given only the bolus dose of 100 mg/kg revealed respective peak serum 7-OH-MTX concentrations of 0.8 and 1.0 mM (Fig. 1 A), similar to the overall peak concentration observed after the 10-min bolus infusion (0.9 ± 0.1 mM, n = 13). After infusion at the lowest rate, 7-OH-MTX serum levels were 0.7 and 1.1 mM, respectively, at the time of death for 2 of the rats, whereas the rats that survived exhibited peak 7-OH-MTX concentrations of 0.9 mM (Fig. 1 B). The total dose of 7-OH-MTX delivered to these rats was 0.3 ± 0.004 g/kg, the approximate dose lethal to 50% of the animals (LD_{50}).

The results obtained in animals infused at intermediate rate are presented in Fig. 2 A. Serum concentrations of 7-OH-MTX measured at the point of death were 1.9 and

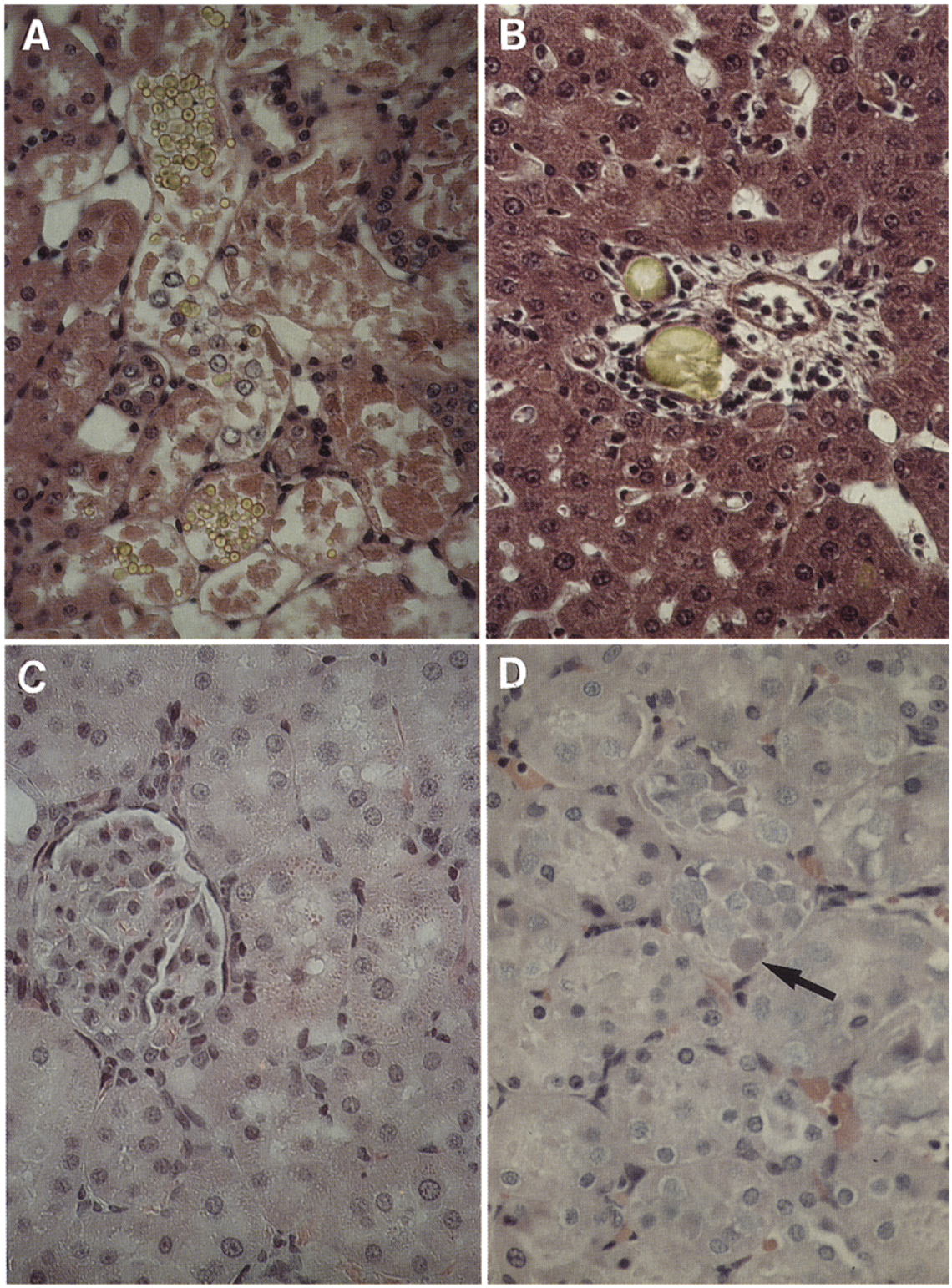

Fig. 3A–D Light micrographs obtained at the original magnification of × 40. **A** Acute tubulotoxic injury found following bolus plus constant infusion of 296 mg/kg 7-OH-MTX at the end of the experiment, 8 h after the start of 7-OH-MTX administration. Rat kidney showing vacuolation and disintegration of tubular epithelial cells, with denuded basement membrane being visible in the tubules. Yellow crystalloid granules consistent with 7-OH-MTX precipitates can be seen in the lumina of numerous tubules. **B** Liver from the rat described in Fig. 4A. Section of liver parenchyma at the periphery of a lobule, with a central typical portal triade, consisting of branches of the hepatic artery and portal vein, and small bile ducts occluded by yellow crystalloid material (see Fig. 4A, B; Carnoy alcohol-based fixation). **C** Paraffin-embedded, formalin-fixed section of rat kidney at the time of death, 66 min after the initiation of bolus + continuous infusion of a total of 399 mg/kg 7-OH-MTX. The tubular epithelium is swollen and contains small intracellular eosinophilic granules. The glomerulus in the upper central area is congested but shows no evident structural change. **D** Same conditions described in **C**, but the rat died after only 50 min. Visible area marked tubular changes involving congestion and vacuolization of the tubular epithelium, some hyalinized necrotic cells (*arrow*), and nearly occluded tubular lumina

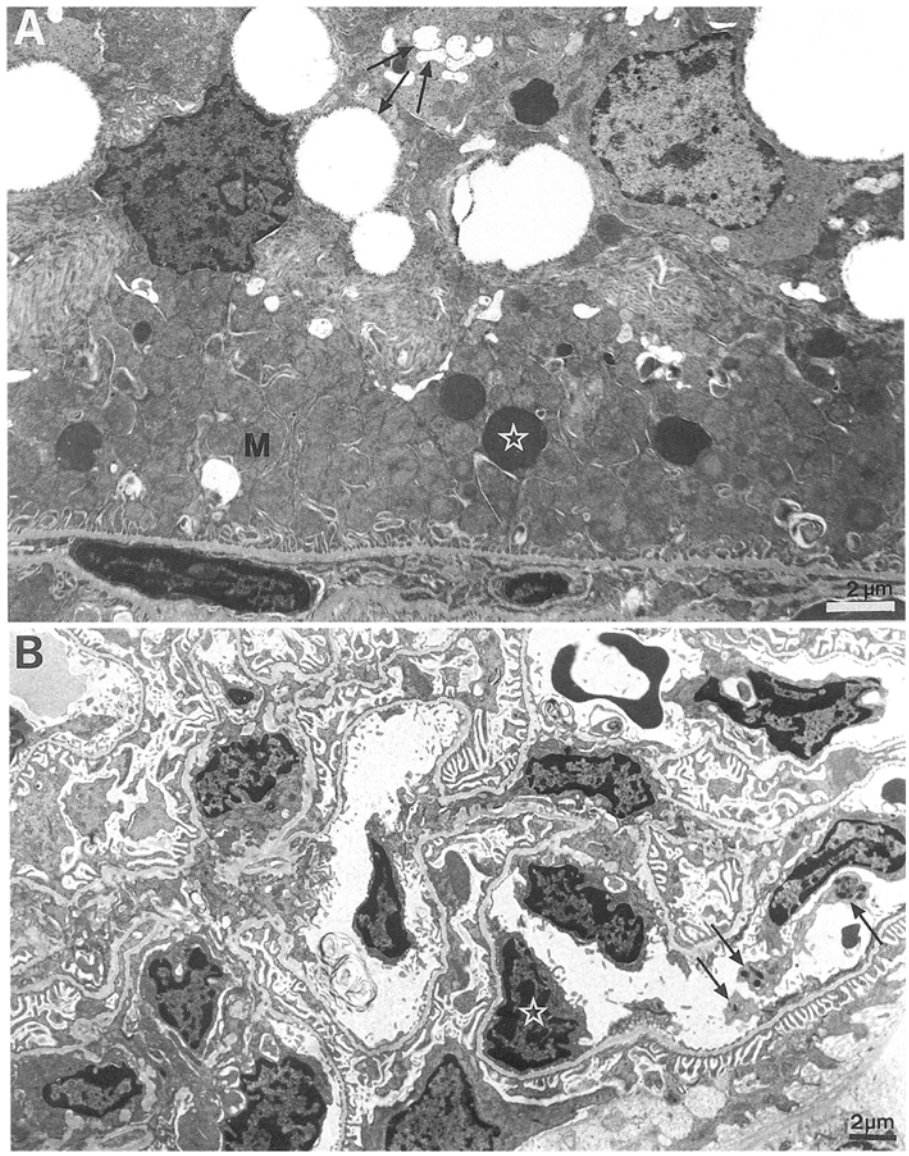

Fig. 4A, B Electron micrographs of a rat kidney obtained at the time of death, 50 min after the start of bolus dosing and continuous infusion of a total of 399 mg/kg 7-OH-MTX. **A** Transverse section of a proximal tubulus, with large amounts of electron-lucent vacuoles without a limiting membrane being gathered mainly in the apical part of the cells (*arrows*). The basal part of the tubular cells contains aggregates of mitochondria (*M*), with increased numbers of electron-dense granules representing abnormal accumulation of lipid (*star*). **B** Well-preserved glomerular capillaries with normal endothelial lining, basal lamina, and foot processes. Some of the endothelial cells are swollen and show a loss of pinocytic vesicles; the nuclei exhibit condensation and margination of chromatin (*star*). The capillary lumen contains small aggregates of platelets (*arrows*)

2.0 m*M* following the infusion of a total of 0.38 g/kg 7-OH-MTX. The mean serum level found at the time of death in rats exposed to the highest infusion rate was 1.6 ± 0.4 m*M*; a 7-OH-MTX dose of 0.4 ± 0.1 g/kg had been infused (Fig. 2B). There was no statistically significant difference between these last two groups with respect to the serum level or the total dose given.

Morphological changes

The microscopic specimens were demonstrated in a blind fashion to the pathologist. Even in rats surviving for only 35–45 min, there were major findings in both liver and kidney tissues by light microscopy (LM) examinations. The liver exhibited steatosis, edema, incipient and manifest single-cell necrosis (piecemeal necrosis), and bleeding. The kidneys presented with swollen tubules, vesicular nuclei, and small eosinophilic inclusion bodies inside the tubular epithelium. Pearl staining with respect to hemosiderin was negative. Furthermore, in all Carnoy-fixed preparations from the kidneys, yellow material was apparent in the tubules, especially in the renal cortex (Fig. 3A). These precipitations were absent in formalin-fixed kidney specimens, whereas the dense red inclusion bodies observed in formalin-fixed preparations were lacking in kidney tissue fixed in Carnoy (Fig. 3C). Yellow precipitations were also seen in bile canaliculi (Fig. 3B), especially in rats living for more than 2 h, although treated at the lower infusion rate. No pathology was observed in specimens from the heart and brain.

Electron microscopy (EM) detailed alterations seen by LM. After the extensive preparation process, the intracellular inoculation bodies were visible only as empty shells, consistent with lysosomes (Fig. 4A). In addition, platelet aggregation was apparent in both kidney (Fig. 4B) and liver tissues.

Serum levels of ALAT, ASAT, and creatinine

Animals receiving only the 100-mg/kg bolus displayed no significant rise in serum levels of ALAT. Rats treated at the lowest infusion velocity that died (after 152 min, mean) revealed mean serum ALAT values of 1422 units/l, whereas those treated at the highest infusion rate (after 53 min, mean) presented serum ALAT levels of 668 ± 203 units/l as compared with the control value of 59.6 ± 12.3 ($P < 0.001$). Animals exposed to bolus infusion only (100 mg/kg) presented ASAT elevations, albeit not statistically significant, whereas a mean ASAT value of 1962 units/l was demonstrated in the rats dying after infusions at the lowest rate. At the highest infusion rate, serum ASAT levels reached 458 ± 162 units/l (after 53 min, mean), whereas control rats displayed ASAT values of 85 ± 31 ($P < 0.001$). There was no statistically significant increase in the serum ALAT or ASAT values of the controls during the 8-h experiment.

With respect to serum creatinine levels, the 4 rats living through the experiment attained creatinine values of 150.5 ± 11 μmol/l. Furthermore, rats receiving the highest-velocity 7-OH-MTX infusion displayed serum creatinine levels significantly higher than those seen in controls at the time of death, after a mean period of only 53 min (39.6 ± 6.5 and 30.4 ± 3.8 μmol/l, respectively; $P < 0.05$). At the start of the experiments there was no statistically significant difference in serum levels of ALAT, ASAT, or creatinine between controls and rats subjected to 7-OH-MTX treatment.

Renal excretion of 7-OH-MTX

Only 1 rat receiving 7-OH-MTX voided urine during the experiment. All rats living for more than 1 h presented some precipitation of 7-OH-MTX in the urinary bladder. However, punctate urine was limited, with the mean being 0.34 ml (range 0.0–1.4 ml; pH 6.5–7.7) Alkalinization of the urine with 0.1 *M* NaOH prior to HPLC analysis was probably not sufficient to dissolve all the 7-OH-MTX, as the sediment was light yellow in color after centrifugation. Hence, the 7-OH-MTX concentrations found in urine to be 0.7 ± 0.4 m*M* are underestimated. Voided urine in the control rats amounted to 7.68 ± 2.2 ml, with the mean pH being 6.49 ± 0.3.

Discussion

This is the first report demonstrating doses and concentrations of 7-OH-MTX causing acute lethal toxicity within a few hours in any species. With a limited supply of 7-OH-MTX, an approximate LD_{50} of 0.3 g/kg could be estimated, suggesting a steep dose/lethality curve for 7-OH-MTX.

The pharmacological and toxicological importance of 7-OH-MTX remains a matter of discussion [4, 51], but the formation of 7-OH-MTX appears to be undesirable. First, 7-OH-MTX interferes with MTX entry [24, 38] and polyglutamation [25, 40], thereby reducing MTX toxicity toward dividing cells [30, 39]. Some clinical studies in patients receiving HD-MTX therapy support this view [11, 19, 20]. Second, the renal and hepatic toxicity reported previously [14, 53] and herein is attributable to 7-OH-MTX.

Renal damage remains a highly feared side effect of HD-MTX treatment [29, 32, 45, 48, 55, 58]. Reduced renal function has an effect on MTX plasma levels and the rate of MTX clearance and could therefore induce systemic toxicity [48, 50]. The specific mechanism of renal MTX toxicity is not yet clear [1, 2, 18, 31, 49, 50]. Of several postulated mechanisms, tubular precipitation of MTX and/or 7-OH-MTX, producing obstructive renal failure, is the most widely accepted concept [1, 44, 50, 57]. The present study demonstrates severe morphological findings and elevated serum creatinine levels. In the kidney tubules of the rat, we detected microscopic crystallization of (presumably) 7-OH-MTX, consistent with the 7-OH-MTX precipitations in monkey kidneys reported after HD-MTX administration by Jacobs et al. [33, 34]. Moreover, EM examination indicated that intracellular 7-OH-MTX could be accumulated in lysosomes. Lysosomal changes have been reported after MTX administration [8, 37, 60], and Barrueco et al. [6, 7] have described transport of MTX polyglutamates into lysosomes. A direct toxic effect of 7-OH-MTX on the cells of the kidney as previously indicated [53] cannot be excluded.

Acute hepatotoxicity, detected as significantly elevated levels of transaminases, is frequently reported in the clinic after the administration of MTX doses surpassing 12 g [5, 23, 47, 59]. Besides, we have previously demonstrated acute hepatotoxicity after HD-MTX treatment in rats [14]. However, the observed enzymatic and morphological hepatic alterations were more severe after treatment with 0.3–0.4 g/kg 7-OH-MTX than after therapy with 1 g/kg MTX [14]. Morphologically, we demonstrate herein for the first time biliary precipitations in non-bile-drained rats receiving MTX or 7-OH-MTX. Single-cell necrosis and steatosis were also evident. Additionally, platelet aggregation was seen in both liver and kidney tissues. The significance of the latter finding is unclear.

The rats died with a few spasms after a period of reduced peripheral and central circulation. Acute renal and hepatic damage requires more than 1–2 h to develop into life-threatening effects [28]. Cerebral affection and/or a direct toxic effect on the heart by 7-OH-MTX may be possible explanations. However, morphological findings supporting such a hypothesis were not detected. Side effects of fentanyl seem unlikely to be the cause, as the unanesthetized rat died in the same way as the others.

A total of 0.4 g/kg continuously infused 7-OH-MTX killed all rats exposed to this dose. In contrast, 1 g/kg of the parent compound MTX given as a bolus over 10 min was well tolerated in studies lasting up to 10 h in rats [13, 14]. Moreover, we have given 5 g/kg MTX over only 10 min to 2 rats, which survived a 6-h experiment (unpublished data). These data suggest that the approximate acute LD_{50} for

MTX in rats will greatly surpass the approximate value for its major metabolite (0.3 g/kg). With increasing observation time (up to 24 h), the LD_{50} for MTX in rats decreases to 14–300 mg/kg [3, 54]. Dose-limiting bone marrow suppression could be prevented by leucovorin therapy. Further studies will show whether 7-OH-MTX-mediated toxicity, if initiated by a direct toxic event, can be prevented by leucovorin.

Additionally, serum levels of MTX measured in rats after the administration of 1 g/kg MTX reached peaks of approximately 10 m*M*, declining to about 1 m*M* after 6 h [13, 14]. Initial serum MTX levels determined following the administration of 5 g/kg in surviving rats were about 20 m*M* (unpublished data), whereas 12-fold lower serum concentrations of 7-OH-MTX killed all the rats. Taken together, these doses and concentrations strongly suggest that the 7-hydroxylated metabolite has a greater potential for acute toxicity than does MTC, at least in the species investigated in the present study.

The response to different infusion rats following the 10-min bolus infusion of 7-OH-MTX may give some indication as to whether the critical issue is the serum level or the total dose of 7-OH-MTX delivered. However, both parameters exhibit a wide interindividual variation. With respect to MTX, preclinical and clinical studies have indicated that toxicity is determined more by the duration of MTX exposure above a critical time threshold than by the magnitude of the MTX concentration above this threshold [32]. Whether the 7-OH-MTX peak concentration or the AMC is critical for the metabolite's toxicity is the subject of a larger series of experiments.

Serum 7-OH-MTX levels measured in rats receiving the highest dose reached 1.6 m*M* at the time of death. Since 7-OH-MTX serum concentrations as high as 3.5 m*M* have been reported after HD-MTX therapy in surviving human patients [9], the toxicity/lethality potential of 7-OH-MTX in humans is intriguing. Furthermore, renal excretion is the major elimination pathway for MTX and 7-OH-MTX in humans [34, 42], whereas biliary elimination is the main excretion route in rats [12, 13, 26]. Shortly before death during treatment at the highest infusion rates, serum concentrations of 7-OH-MTX increased sharply in some rats showing reduced clearance (Fig. 2). Reduced biliary excretion due to acute liver failure seems to be the most likely reason for this increase in 7-OH-MTX serum concentration.

In conclusion, our data show lethal renal and hepatic toxicity for 7-OH-MTX after continuous infusion in the rat. Moreover, 7-OH-MTX has been reported to decrease MTX entry and polyglutamation [24, 25]. To reduce the toxicity and improve the antineoplastic effect of MTX, efforts should be made to prevent 7-OH-MTX formation.

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