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The pharmacokinetic profile of mitomycin C released from an injectable supramolecular hydrogel in a rodent model☆

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ABSTRACT

Objective: This study evaluates the pharmacokinetics of an ureido-pyrimidinone poly(ethylene) glycol (UPy-PEG) hydrogel loaded with mitomycin C (MMC) in rats. The hydrogel aims to enhance the intraperitoneal residence time of MMC, potentially improving therapeutic outcomes for peritoneal metastases (PM) patients.
Methods: Rats were divided into two groups: h-MMC ($n = 8$), receiving MMC encapsulated in hydrogel, and pbs-MMC ($n = 6$), receiving MMC in PBS. Blood samples were collected from 5 min to 48 h post-administration. MMC concentrations were measured using LC-ESI-MS. Systemic and local adverse effects were assessed through blood analysis and post-mortem histopathology.
Results: The hydrogel prolonged detectable plasma MMC levels: 24 h for h-MMC vs. 4 h for pbs-MMC. h-MMC had a Cmax of 120 ± 21 μg/L and a Tmax of 52.5 ± 8.2 min; pbs-MMC had a Cmax of 358 ± 24 μg/L and a Tmax of 37.5 ± 8.2 min. The area under the curve ratio of h-MMC/pbs-MMC was 87 %. Platelet counts were significantly lower in h-MMC at 24- and 48 h and in pbs-MMC at 48 h. No liver or kidney damage was observed, though vacuolated macrophages were noted in the hydrogel-treated groups.
Conclusion: The hydrogel effectively prolonged MMC presence in plasma, suggesting extended intraperitoneal residence time and supporting previous findings of therapeutic effectiveness in a PM rat model.

1. Introduction

Peritoneal metastases (PM) of colorectal origin refer to the spread of colorectal cancer cells from the primary tumor to the peritoneum. It occurs when cells from the primary tumor detach and migrate to the peritoneum, forming metastatic deposits [1,2]. Generally, the presence of PM is associated with advanced-stage colorectal cancer (CRC) and patients diagnosed with PM have the poorest survival prognosis compared to CRC patients with metastases to other sites [3].

As PM are considered as being a regional disease rather than systemic

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disease, treatment should preferably also be loco-regional [4]. The intraperitoneal treatment efficacy of PM is most importantly based on the direct exposure of tumor tissue to the therapeutic agent; via passive diffusion, the cytostatic enters the cell where it subsequently can perform its anticancer mechanism of action [5]. Gold standard treatment of PM involves both cytoreductive surgery (CRS) followed by hyperthermic intraperitoneal chemotherapy (HIPEC). CRS aims at removing the macroscopic tumor deposits within the peritoneal cavity, whereas during HIPEC, heated chemotherapy is delivered directly to the affected area to destroy remaining cancer cells. Mitomycin C (MMC) is the most frequently chosen chemotherapeutic agent for HIPEC. It is a selective DNA synthesis inhibitor [6], that has a relatively long half-life and high peritoneal-plasma area under the curve (AUC) ratio compared to other cytostatic agents [7], although its intraperitoneal use is off-label as the drug is not registered for intraperitoneal application [8]. During HIPEC, the MMC solution is heated to 41–43 °C and flushed through the abdomen for 90 min at a cumulative dose of 35 mg/m^2 [9]. This dose has been shown to balance regional efficacy over systemic toxicity that occurs at higher doses [10]. Systemic toxicity also limits HIPEC duration to maximally 90 min for MMC, although preclinical experiments suggest that longer exposure to the drug results in more tumor cell death [11].

To extend the intraperitoneal residence time of chemotherapeutic agents while restraining systemic toxicity, drug delivery systems (DDSs) are currently developed [12]. Prolonged exposure to chemotherapy might result in improved tumor penetration and hence increased apoptosis, thereby potentially enhancing treatment efficacy. This becomes particularly crucial for eliminating micro-metastases, such as those that might be present in the very early onset of local peritoneal spread or those remaining in the abdominal cavity after cytoreductive surgery.

Hydrogels represent a promising DDS for chemotherapy due to their unique physical and chemical properties. These three-dimensional polymer networks are highly hydrophilic, capable of absorbing and retaining significant amounts of water or biological fluids, which can be loaded with chemotherapeutic agents [13]. Due to this property, the gels are suitable to prolong the exposure time of cytostatic agents to tumor cells. Previously, we showed that intraperitoneal administration of MMC loaded on a ureido-pyrimidinone poly(ethylene) glycol (UPy-PEG) supramolecular hydrogel (h-MMC) improves therapeutic outcome in terms of increased overall survival in a rat model of PM-CRC compared to animals that had MMC dissolved in PBS administered (pbs-MMC) [14]. We demonstrated that h-MMC significantly improved survival outcomes compared to pbs-MMC. Specifically, after 120 days, the overall survival rate was 78 % in the h-MMC group versus 38 % in the pbs-MMC group. Although the difference in survival curves showed a trend toward significance ($p = 0.087$), these results suggest a clinically relevant benefit of the hydrogel in enhancing therapeutic outcomes. In that therapeutic effectiveness study, we were unable to verify whether the observed survival benefit was explained by differences in the pharmacokinetic profile of MMC, as extensive blood sampling could have interfered with the primary outcome (survival) of the experiment. Therefore, this study was designed to investigate whether administering MMC via the hydrogel has a favorable pharmacokinetic profile that could explain the observed increased survival.

Specifically, the current experimental study aims to assess the pharmacokinetic profile of a single intraperitoneal injection of h-MMC compared to a single injection of pbs-MMC within the first 48 h post-administration. We seek to demonstrate that MMC has an increased residence time in the peritoneal cavity in the h-MMC group and to evaluate the (local) toxicity resulting from this prolonged release.

2. Materials and methods

2.1. Ethics

The study protocol for this experiment was approved by the Ethical Committee of Animal Experiments (license number AVD1070020198765), which complied with the Dutch Animal Experimental Act, and was approved by the Animal Experimental Committee of Maastricht University (license number 2019–001). Data reporting of this experiment followed the ARRIVE 2.0 (Animal Research: Reporting of In Vivo Experiments) guidelines.

2.2. Animals and housing

Male adult WAG/Rij rats (weighing 250–270 g) were purchased from a registered breeding company (Charles River Laboratories, Calco, Italy). The animals were socially housed in filtertop cages (two animals per cage) in a temperature- and humidity-controlled room with 12-h light/dark cycles. Access to acidified water and food (10 mm Sniff rat/mouse sterilized food compressed into pellets) was ad libitum. After an acclimatization time of at least one week, the experiment was started. All animal procedures were conducted at the animal center of Maastricht University. We previously investigated the therapeutic efficacy of the MMC-loaded hydrogel in a CRC-PM model induced with CC531 cancer cells derived from the WAG/Rij rat [14]. To maximize comparability between our experiments, the WAG/Rij strain was chosen.

2.3. Formulation of MMC-loaded hydrogel (h-MMC) and MMC dissolved in PBS (w-MMC)

The UPy-PEG polymer was synthesized under aseptic conditions in a similar way as described before with a pH of 8.5 [15–17]. Hereafter, MMC was loaded into the hydrogel (h-MMC) or dissolved in sterile PBS (pbs-MMC) as previously described [14]. Formulations were freshly prepared on the day of administration. The h-MMC and pbs-drug solution were administered at a volume-to-weight ratio of 20 mL/kg, corresponding to 5 mL per animal. The solutions were administered at room temperature with a pH of 8.5 in the h-MMC group and pH neutral in the pbs-MMC group. The pH of the h-MMC formulation of 8.5 was determined in previous experiments based on the sol-gel properties, as at lower pH values, the UPy-PEG components aggregate, forming a viscoelastic network that is no longer injectable [18]. Pbs-MMC was prepared at a neutral pH to align with clinical MMC formulations. The MMC was administered at a dose of 10 mg/m^2 (= 1.36 mg/kg), which had demonstrated therapeutic efficacy in our previous studies [14].

2.4. Study design and procedures

A total of 14 male rats were randomly divided by a computer-based random order generator into either the h-MMC group ($n = 8$) or the pbs-MMC group ($n = 6$). Group sizes were based on a conservative sample size estimation. The h-MMC group included an extra animal per time point to account for potential tolerability issues, while three animals per time point were sufficient for the pbs-MMC group. Animals received either solution via a single intraperitoneal injection as previously described [18]. In short, under isoflurane anesthesia, the solution was administered intraperitoneally in the lower right quadrant. Twenty-four hours after administration, half of the animals were euthanized, followed by the other half after 48 h. The rationale behind the 48 h is that an in vitro experiment studying the h-MMC demonstrated that >95 % of MMC was released from the hydrogel within 48 h [19]. For euthanasia, in anesthetized animals, a midline laparotomy was made followed by macroscopic evaluation of the abdominal organs. Animals were euthanized via a terminal cardiac puncture and the remaining blood was collected. The diaphragm was punctured to ensure the death of the animal. Due to practical constraints, only male animals were used in this experiment, as their larger body weight and greater circulating blood volume allowed for multiple blood withdrawals from the same animal across different time points, reducing the total number of animals required.

2.5. Plasma sample collection

Blood samples were collected at predetermined, fixed time intervals. At baseline prior to administering pbs-MMC and h-MMC at $t = 0$ min, and after administration at $t = 5$, 15, 30, and 45 min and 1, 2, 2.5, 3, 4, 6, 12, 24, and 48 h, 300 μL whole blood was collected in a 300 μL lithium-coated heparin capillary tube (Sarstedt, Nümbrecht, Germany). On each time point, three pbs-MMC blood samples and four h-MMC blood samples were collected. To limit both animal discomfort and risk of circulatory failure, we took maximally six blood samples from every individual animal (about 10 % of the total blood volume). Each blood sample was collected through a single transversal tail incision. The first tail incision was made with a disposable surgical blade nr. 22 (Swann Morton, Sheffield, United Kingdom) when the animal was under anesthesia. For every consecutive blood sample, the original incision was reopened by rubbing with a sterile gauze. At the time points within 20 min after administration, the animals were still under anesthesia and thereafter the animals were restrained in a towel. After collecting the animals' final sample at the first day of the experiment, 5 mL saline with 3 % glucose solution was administered subcutaneously to all animals to prevent hypovolemia. Directly after collection, the blood samples were centrifuged at 2000 xg for five minutes at 20 °C. Subsequently, at least 100 μL plasma was separated and stored at −80 °C.

2.6. Chromatographic analysis

The following chemicals and reagents were used: MMC was obtained from Beta Pharma (Shanghai, China). The MMC derivative (metabolite) porfiromycin (PMC) of a purity of 99 % was used as an internal standard and kindly synthesized by the Laboratory for Organic and Bio-organic Synthesis of Ghent University (Ghent, Belgium). High-performance liquid chromatography (HPLC)-grade acetonitrile (CAN, LC-MS grade HiPerSolv) was purchased from VWR International (Fontenay-sous-Bois, France). Milli-Q water (0.005 μS/cm, Sartorius Arium Pro UV) was used for the preparation of all standards and sample solutions.

The Liquid Chromatography-Electrospray Ionization-Mass Spectometry (LC-ESI-MS) system consisted of a Thermo/Dionex Ultimate 3000 system equipped with a Dionex 3000RS autosampler, pump, column oven, and an LCQ-Fleet Iontrap MS system. Chromatographic separation was performed on a Kinetex® C_{18} 100 Å analytical column (200 × 2.1 mm, 2.6 μm, Phenomenex) at a column temperature of 30 °C. The mobile phase for the plasma analysis consisted of H_2O in 0.1 % acetic acid (HAc) and acetonitrile (ACN) in 0.1 % HAc and runned with a flow rate of 0.3 mL/min. The injection volume was set at 20 μL. Several internal validation samples of MMC were injected in each run. The column was rinsed with H_2O after a series of samples representing all time points were analyzed. For MMC and PMC detection, multi-stage (MS^n) were implemented to increase selectivity. More specifically, for MMC *m/z* at 335 in MS^1 (molecular ion) and m/z 242 in MS^2 (fragment ion) were used for quantification, whereas m/z 349 (molecular ion) and m/z 256 (MS^2 fragment ion) were monitored for PMC.

Samples were thawed from −80 °C and immediately processed. To 100 μL plasma, 900 μL of 75 μg/L PMC in ACN was added. To allow protein precipitation, the samples were thoroughly mixed on a vortex. The mixture was then centrifuged for 20 min at 4400 rpm at 20 °C. The supernatant was transferred into a clean amber glass vial. The sample was evaporated to dryness under a steam of N_2 at room temperature. The residue was dissolved in 250 μL H_2O and again centrifuged for 20 min at 4400 rpm at 20 °C. The supernatant was transferred to a glass vial with an insert for small volumes for subsequent LCMS injection. The total runtime of each plasma sample was 20 min.

Stock solutions of MMC and PMC were prepared in ACN. Standard solutions for MMC were freshly prepared from the stock solution in milli-Q water at 200, 160, 80, 40, 20, 16, 8, 4, and 2 μg/L for MMC. The PMC internal standard solution used for spiking was freshly prepared in ACN at a concentration of 75 μg/L and added to all standard solutions and samples. A linear calibration curve ($R^2 = 0.997$) for MMC could be obtained between 4 ppb and 2 ppm, whereas the relative standard deviations (RSD) for the internal PMC standard were limited to 2.11 %. The detection limit of MMC was 0.05 mg/L.

2.7. Outcome measures

The primary outcome of this experiment was to investigate and compare the in vivo pharmacokinetic profile of MMC when released from a hydrogel versus as pbs-MMC after intraperitoneal administration in rats. This was achieved by creating a concentration versus time curve of MMC, comparing MMC plasma concentrations of both experimental groups at predefined time points. Key pharmacokinetic parameters that were analyzed included peak plasma concentration (Cmax), time to reach the peak concentration (Tmax), area under the concentration-time curve (AUC), and half-life. These parameters facilitate a comprehensive comparison of the drug's absorption, distribution, metabolism, and excretion characteristics between the two delivery methods. Ideally, peritoneal fluid would (also) have been collected to determine the MMC concentration intraperitoneally. However, the total volume of peritoneal fluid in rodents is low [20], making it difficult to collect. Furthermore, collecting this fluid would have required sacrificing several animals at each time point, which was not feasible due to considerations related to the 3 R's principle (Replacement, Reduction, and Refinement) in animal research.

Secondary outcome measures included identifying the acute systemic and local adverse effects of MMC exposure such as observation of adverse reactions, histopathological examination of tissues for signs of toxicity or inflammation, and routine blood analysis to evaluate organ function as described below. Data for histological and routine blood analysis was collected at 24 and 48 h after administration of h-MMC or pbs-MMC.

2.8. Histological analysis

Histological analysis focused on both the local and systemic effects of MMC exposure released from the hydrogel or administered as pbs-drug solution. The liver, kidney, and duodenum were collected and carefully inspected after euthanasia, after which they were fixed in a 4 % formaldehyde solution. For the animals in the h-MMC group after 48 h, the urine bladder and seminal vesicle was collected as well. After embedding in paraffin, 5 μm thick tissue sections were cut and subjected to hematoxylin-eosin (H&E) staining. A specialized laboratory animal pathologist (M.G.), blinded for the assigned group, evaluated the stained sections microscopically. During histopathological examination, special attention was given to the expected effects of prolonged exposure to MMC, such as apoptosis, inflammation, and regeneration.

2.9. Plasma and hematological parameter analysis

Several plasma and hematological parameters were determined in the blood to assess organ function and/or damage. The presence of hepatotoxicity was assessed by determining the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma-glutamyltransferase (GGT). The liver function was assessed by albumin levels. The kidney function was assessed by the estimated glomerular filtration rate (eGFR). In addition, we determined the levels of electrolytes including sodium and potassium, inflammation parameters including C-reactive protein (CRP) and haptoglobin, and parameters for bone marrow functioning including hemoglobin, white blood cell, and platelet count. The analyses were performed by lab technicians blinded to the assigned group. Reference values were obtained from the breeding company and relevant textbooks [21]. However, comparable values for the WAG/Rij rat strain were not available. Since specific reference values for WAG/Rij rats were unavailable, values from Wistar rats—the most closely related

strain—were used for comparison. Additionally, reference values derived from control animals in previous experiments ($n = 2$) were included for select parameters, which fell outside the Wistar reference range, such as albumin and hemoglobin.

2.10. Statistical analysis

All statistical analyses and visual representation of data were computed by GraphPad Prism version 8. The level of significance was set at $p < 0.05$. Data on the pharmacokinetic parameters and biochemical parameters was displayed as mean with standard deviation (SD). For the concentration versus time curve, we also showed per time point mean with SD. Furthermore, a bar chart of the 3-, 4-, and 6-h time points was made, demonstrating the most relevant differences. Tmax and Cmax were calculated based on individual curves of each animal, whereas AUC was calculated based on the mean curves of the h-MMC and pbs-MMC groups. The half-life was calculated for the pbs-MMC group. For the analysis of the biochemical parameters, means were compared between the four groups (h-MMC 24 h, h-MMC 48 h, pbs-MMC 24 h, pbs-MMC 48 h) with a two-way analysis of variance (ANOVA) with post-hoc Tukey's multiple comparison test.

3. Results

3.1. Pharmacokinetic MMC profile

A total of fourteen animals were included in this experiment, with eight receiving h-MMC and six receiving pbs-MMC. One sample from the h-MMC group was lost due to a preparation error during analysis. The concentration versus time curve of MMC in plasma is shown in Fig. 1. MMC concentrations in plasma were quantified at baseline and between 5 min and 48 h after administering h-MMC or pbs-MMC using LC-ESI-MS.

In the h-MMC group, MMC was detected up to 24 h after administration, whereas in the pbs-MMC group, MMC was detectable only up to 4 h after administration. The pharmacological parameters in both groups are displayed in Table 1. For h-MMC, the Tmax was 52.5 ± 8.2 min and the Cmax was 120 ± 21 μg/L. The AUC was 42.76 (μg/L)min.

Table 1
Pharmacokinetic parameters in h-MMC and pbs-MMC group

Parameter	h-MMC ($n = 7$)	pbs-MMC ($n = 6$)
Tmax (minutes)	52.5 ± 8.2	37.5 ± 8.2
Cmax (μg/L)	120 ± 21	358 ± 24
AUC ((μg/L)min)	42.76	49.25

For pbs-MMC, the Tmax was 37.5 ± 8.2 min The Cmax was 338 ± 24 μg/L with an AUC of 49.25 (μg/L)min. The half-life of MMC was 25 min in pbs-MMC group. Due to the plateauing effect of h-MMC, it was not possible to calculate the half-life in this group. The AUC h-MMC/pbs-MMC ratio is 0.87.

These findings indicate that h-MMC allows for a more prolonged detection of MMC in plasma compared to pbs-MMC, which could be indicative of sustained release and extended exposure intraperitoneally.

3.2. Plasma markers for organ- and bone marrow functioning

Electrolytes, markers for kidney-, liver-, and bone marrow functioning, indicators of hepatotoxicity, and inflammation were assessed and shown in Table 2. The mean platelet counts in the pbs-MMC 48 h, h-MMC 24 h, and h-MMC 48 h was significantly lower compared to the pbs-MMC 24 h group ($p < 0.0001$). For all other parameters, no significant differences were found between the groups. Most parameters, except for albumin and hemoglobin, fell within the reference ranges. For albumin, the values were comparable to those observed in our own control data. However, the hemoglobin values in the current experiment were lower than the reference values, but not different between groups.

3.3. Local and systemic effects of MMC exposure

The effects of MMC exposure on organ level were further evaluated using histological analysis, as shown in Fig. 2 and Table 3. In liver tissue, vacuolated (resident) macrophages (Kupffer cells) were found after both 24 and 48 h in animals that were administered h-MMC, whereas this was not found in animals of the pbs-MMC groups (Fig. 2A, vacuolated macrophages indicated with black arrows). No steatosis or other abnormalities were found in the hepatocytes. No abnormalities were found

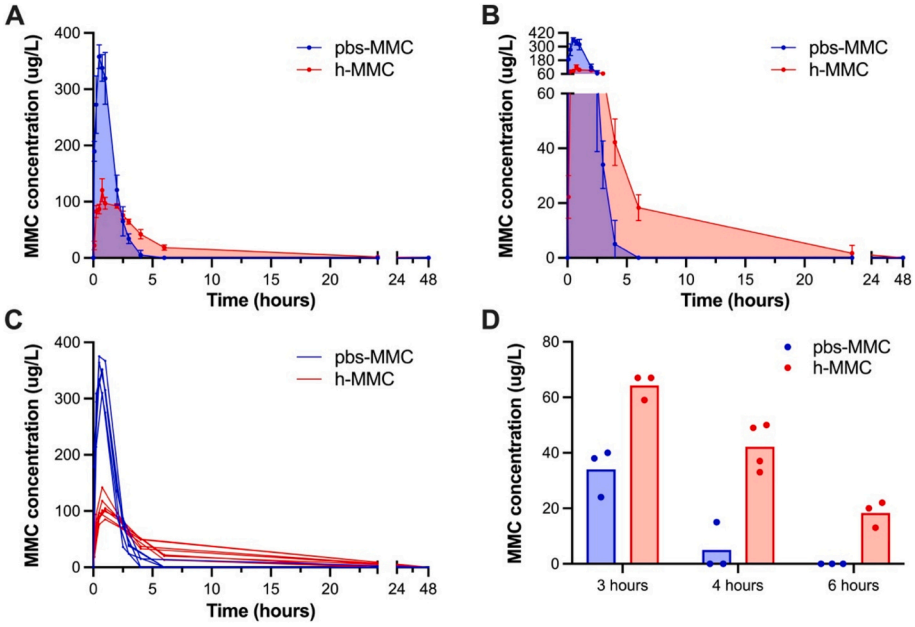

Fig. 1. MMC concentration-time curve in plasma for h-MMC (n = 7) and pbs-MMC group ($n = 6$). (A) Full Y-axis representation of MMC concentration over time. (B) Partial Y-axis displayed for better visualization of differences between groups. (C) Concentration over time curve for each individual animal. (D) Bar chart of the 3-, 4-, and 6-h time points, where the most relevant differences are observed.

Table 2
Plasma and hematological parameters of the animals in the experiment. Values are displayed as mean (SD). Means were compared with a two-way analysis of variance (ANOVA) with post-hoc Tukey's multiple comparison test.

	h-MMC 24(*n = 4)*	h-MMC 48 h(*n = 4)*	pbs-MMC 24 h(*n = 3)*	pbs-MMC 48 h(*n = 3)*	Reference values*
Liver function and damage					
AST (U/L)	114 ± 16	77.3 ± 9.9	109 ± 15	91 ± 15.9	74–134
ALT (U/L)	38 ± 4.1	23 ± 1.4	52 ± 4.9	33 ± 4.0	18–45
ALP (U/L)	98.5 ± 15.9	60.3 ± 5.4	152 ± 7.5	120 ± 6.7	62–230
GGT (U/L)	<3	<3	<3	<3	0–3
Albumin (g/dL)	1.03 ± 0.6	0.79 ± 0.08	1.38 ± 0.044	1.40 ± 0.07	3.4–4.8 1.57[b]
Kidney function					
eGFR (mL/min/ 1.73m^2)	89.3 ± 1.4	87.6 ± 2.3	>90	87.2 ± 0	
Inflammation					
CRP	<1	<1	<1	<1	<1
Haptoglobin (mg/ dL)	21 ± 2	34 ± 3	18 ± 6	23 ± 1	10–35
Electrolytes					
Sodium (mmol/L)	139 ± 0.8	140 ± 0.95	140 ± 0.58	143 ± 1	138–147
Potassium (mmol/ L)	4.85 ± 0.22	4.6 ± 0.33	5.11 ± 0.52	4.61 ± 0.62	4.31–5.55
Bone marrow functioning					
Hemoglobin (g/ dL)	9.7 ± 1.1	9.3 ± 1.4	12.2 ± 0.1	12.7 ± 0.6	13.7–17.6 14.6[b]
Platelets (10^3/L)	584 ± 62[a]	501 ± 65[a]	770 ± 19.7	458 ± 406[a]	638–1177
White Blood Cells (10^3/μL)	6.6 ± 1.6	4.9 ± 0.95	4.9 ± 0.15	4.2 ± 1.6	1.96–8.25

* References values from Wistar rats.
[a] Indicates $p < 0.0001$ compared to pbs-MMC 24 h.
[b] Indicates mean value obtained from two control WAG/Rij animals from previous (unpublished) experiments.

in kidney tissue. Histological findings in small intestine are also shown in Fig. 2. Apoptosis in crypts was seen after 24 h in both groups (Fig. 2B, red arrows). After 48 h in the h-MMC group, progressive apoptosis (red arrows), but also inflammation (Fig. 2B, black arrows) and regeneration (Fig. 2B, blue arrows) was seen in duodenal epithelium. After 48 h in the pbs-MMC group, also regeneration was seen. During autopsy of the four animals in the h-MMC group after 48 h, macroscopic lesions were noticed in the urine bladder and seminal vesicle during visual inspection. Additional histologic analysis showed edema, dilated blood vessels, and active hemorrhage. The seminal vesicle was edematous as well in these animals. These macroscopic changes on urine bladder and seminal vesicle tissue were absent in all other groups. Histological abnormalities in urine bladder and seminal vesicle can be found in the Supplementary Files.

4. Discussion

In this study on healthy rats, we evaluated in vivo pharmacokinetics and toxicity of a novel MMC-loaded UPy-PEG supramolecular hydrogel formulation (h-MMC) used as drug delivery system (DDS) for extended chemotherapy within the peritoneal cavity, compared to MMC dissolved in PBS (pbs-MMC). Our results suggest that the bioavailability of MMC in the peritoneal cavity was prolonged when administered via the supramolecular hydrogel. Specifically, in the group receiving h-MMC, MMC was detectable in plasma samples up to 24 h post-administration whereas in the pbs-MMC group this was limited to 4 h post-administration. There were no statistically significant differences in biochemical parameters indicating acute-to-subacute adverse effects on liver- or kidney function, although a statistically significant lower number of platelet count was found in the h-MMC group after 24 and 48 h and pbs-MMC group after 48 h, compared to pbs-MMC after 24 h. Furthermore, hemoglobin values were lower than reference values for all experimental groups. No signs of short-term adverse effects on kidney tissue were found, whereas in liver tissue histological analysis revealed the presence of vacuolated macrophages in animals that had hydrogel administered. Furthermore, in duodenal tissue signs of apoptosis were seen in all animals.

Our primary objective was to assess the clearance rate of MMC from the peritoneal cavity, as determined by plasma MMC concentrations over time, to evaluate the bioavailability and systemic exposure of MMC administered as h-MMC compared to pbs-MMC. The concentration over time curve of pbs-MMC exhibited a clear peak, corresponding to the short half-life time of 25 min, which is supported by data of other pharmacokinetic experiments with MMC in rats [22]. In contrast, the h-

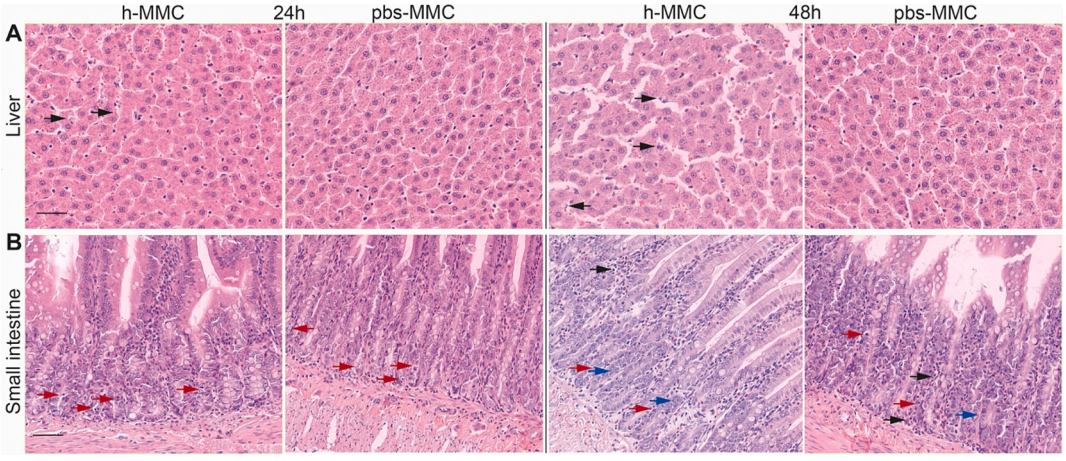

Fig. 2. Hematoxylin & Eosin (H&E) staining of liver tissue (A) and small intestine tissue (B) in all experimental groups. In panel A, black arrows in indicate vacuolated (residence) macrophages, only observed in animals that had h-MMC administered. In panel B, red arrows indicate apoptotic cells, black arrows indicate inflammatory cells, and blue arrows indicate regenerative cells. The scale bar indicates 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Main histological findings of intraperitoneal MMC exposure in both experimental groups.

	h-MMC 24h(*n* = 4)	h-MMC 48 h(*n* = 4)	pbs-MMC 24 h(n = 3)	pbs-MMC 48 h(*n* = 3)
Liver	Vacuolated kupffer cells	Vacuolated kupffer cells	None	None
Kidney	None	None	None	None
Small intestine	Apoptosis	Progressive apoptosis, inflammation, regeneration	Apoptosis	Regeneration

MMC group showed a plateau-shaped curve. This curve demonstrates a slow and sustained release with decreased peak exposure in the h-MMC group. The Cmax was three times higher in the pbs-MMC group compared to the h-MMC group. The AUC h-MMC/pbs-MMC ratio of 87 % suggested that the overall (systemic) bioavailability was similar between both groups.

The prolonged detection of plasma MMC in the h-MMC group demonstrate that the supramolecular hydrogel effectively extended the intraperitoneal residence time of MMC. This can be attributed to hydrophobic interactions between MMC and the hydrophobic compartments of the supramolecular UPy-PEG network, which retain MMC within the hydrogel structure. Notably, this retention effect is more pronounced for compounds with greater hydrophobicity, as demonstrated in an experiment by Bakker et al. [19]. Upon intraperitoneal administration, the UPy-PEG hydrogel undergoes gelation, forming a localized drug depot that enables sustained MMC release into the peritoneal cavity, from where it is gradually absorbed into the blood stream. This mechanism modulates peritoneal clearance and extends systemic MMC presence without altering its intrinsic stability or metabolism.

The extended residence time is expected to have an effect on survival, due to the increased tumor cell apoptosis. Most side-effects of MMC are dose-dependent [23,24] and are associated with systemic peak drug conditions [25]. Therefore, the potential advantage of h-MMC over pbs-MMC lies in its ability to mitigate these side effects by maintaining lower systemic drug levels while still ensuring local delivery to the peritoneal cavity.

In a previous experiment, we demonstrated improved overall survival of h-MMC in a validated PM-CRC rat model by treating the animals with an identical hydrogel formulation as used in the current experiment [14]. The overall survival rate at 120 days following h-MMC administration was 78 %, significantly higher than the 38 % survival rate observed with pbs-MMC. The pharmacokinetic data from the current experiment suggest that the extended intraperitoneal residence time of MMC with h-MMC may explain this improved survival outcome. This extended residence time maintains a local concentration gradient, promoting gradual drug diffusion into the tumor, which enhances tumor penetration. This slow and steady drug diffusion is crucial for effective delivery to the target cells [8,26,27], leading to increased apoptosis. Consequently, this mechanism of action most likely explains the therapeutic benefit of h-MMC found in our previous study.

In this experiment, we have found a Cmax of 120 μg/L and 358 μg/L in the h-MMC and pbs-MMC group, respectively. This has proven to be a clinically relevant dose in rats as we found improved survival rates in rats treated with h-MMC compared to pbs-MMC with a similar dose of 10 mg/m^2. An experimental study comparing clearance rate of MMC in rats and humans conclude that the rat appears to be a suitable model for MMC disposition in humans [22]. Clinical studies investigating the pharmacokinetics of intraperitoneal MMC after HIPEC report that the Cmax varies between 110 and 500 μg/L at a cumulative dose of 35 mg/m^2 MMC [10], indicating a comparable dose range to what we found in our study. Most colon cancer cell lines undergo apoptosis at a drug concentration of around 100 μg/L in vitro [10], although plasma levels will never reach this therapeutic level due to severe side effects. Due to the plasma-peritoneal barrier, high intraperitoneal concentrations can be accomplished, whilst systemic drug concentrations remain low. The concentration of MMC in intraperitoneal perfusate has been reported to be 10 to 29 times higher than in plasma [10]. This means that the local intraperitoneal concentration in the h-MMC group might be between 1200 μg/L and 3480 μg/L, whereas this is between 3580 μg/L and 10,400 μg/L in the pbs-MMC group. These data suggest that the intraperitoneal concentration is high enough, also in the h-MMC group, to cause apoptosis of the tumor cells, which is also in line with the therapeutic effect that was demonstrated in the PM study [14].

The current study focuses on evaluating possible acute adverse effects resulting from prolonged intraperitoneal exposure to MMC. We examined organs involved in MMC metabolism, including liver and kidney, as well as organs with high cell turnover rates susceptible to chemotherapy effects, such as the intestine and bone marrow. Importantly, no signs of microscopic or systemic liver- or kidney damage were observed after 24 and 48 h in either group, indicating no acute toxicity in these organs. The vacuolated macrophages found in the liver tissue in the h-MMC group were comparable with those found in our previous experiment. These vacuolated macrophages were attributed to the degradation of PEG-based materials, as reported in other studies, and were not linked to any findings of toxicity or adverse effects in our experiments [18]. Apoptosis in the duodenal tissue was noted, a common finding after systemic chemotherapy exposure [28]. This effect was consistent across both groups, showing no significant differences. Furthermore, no significant differences in white blood cell count were observed between groups. We were not expecting to find signs of myelosuppression in this experiment, as it typically occurs about a week after intraperitoneal exposure with HIPEC [29]. Remarkably, a statistically significant reduction in mean platelet count was found in animals administered h-MMC and pbs-MMC after 48 h compared to those given pbs-MMC after 24 h. The clinical relevance of this finding is uncertain, as a temporarily lower platelet count does not necessarily result in adverse effects. The lower platelet count in the pbs-MMC group might be the direct consequence of MMC exposure, although this effect then occurs earlier than expected. Previous experiments by our research group found lower platelet counts at 14- and 28-days post-administration of unloaded hydrogel compared to control animals [18], suggesting that it might be a side effect of hydrogel metabolism. In the h-MMC 48 h group, the lower platelet count can also be explained by the active hemorrhage observed in the urinary bladder. Furthermore, the mean hemoglobin levels were lower than reference values, though not statistically significantly different between the groups. The direct relation between intraperitoneal MMC exposure and bladder abnormalities has not been studied before. However, it can be hypothesized that metabolites formed during drug and/or hydrogel breakdown may lead to bladder hemorrhage. Alternatively, local urological damage may occur by prolonged exposure to the drug or its metabolites as a result of slower uptake from the peritoneal cavity to the systemic compartment. Unfortunately, we did not collect urinary bladder and seminal vesicle tissue in the other animals, as these were no pre-defined target organs. By the time the abnormalities in the h-MMC group were noted, the other animals had already been euthanized.

This study has three limitations. First, we focused exclusively on acute adverse effects of MMC exposure, comparing MMC dissolved in PBS with hydrogel-loaded MMC administered intraperitoneally. Longer-term adverse effects, including potential cumulative toxicities, need to be investigated in future studies to provide a more comprehensive understanding of the safety profile of the treatment. Second, we could only

evaluate the MMC concentrations in plasma. Our analytical LC-ESI-MS method was not optimized to measure MMC that is extracted or remains in the UPy-PEG gel in peritoneal fluid. Unfortunately, there is currently no reliable method available to quantify MMC concentration when it is loaded in the hydrogel. Future studies are developed to carry out a tumor uptake study in rodents to determine the primary pharmacology of the h-MMC. This study, combined with the therapeutic efficacy study and the current study, provides the data to fully understand the pharmacokinetics and pharmacodynamics of MMC released from the hydrogel. Third, this study did not account for potential gender-related pharmacokinetic differences, as only male animals were used. Future studies should include both male and female animals to better assess potential gender-related variations in pharmacokinetics.

In summary, the results of this experiment demonstrate that h-MMC offers a prolonged presence in plasma compared to pbs-MMC, indicating extended intraperitoneal residence time without peak systemic exposure. The (systemic) AUC remained highly comparable between both groups. While liver and kidney function remain unaffected, a statistically significant lower platelet count was observed after exposure to h-MMC and pbs-MMC after 48 h. Furthermore, histological analysis demonstrated the presence of vacuolated macrophages in the liver tissue in the hydrogel group. These findings suggest that h-MMC has the potential to enhance localized chemotherapy efficacy while reducing systemic toxicity, making it a promising approach for improving intraperitoneal drug delivery.

CRediT authorship contribution statement

Anne G.W.E. Wintjens: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Peter-Paul K.H. Fransen:** Writing – review & editing, Methodology, Investigation, Funding acquisition, Conceptualization. **Kaatje Lenaerts:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Hong Liu:** Investigation, Conceptualization. **Geert C. van Almen:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Marion J. Gijbels:** Writing – review & editing, Methodology, Conceptualization. **Ben J.A. Janssen:** Writing – review & editing, Resources, Methodology, Conceptualization. **Ignace H.J.T. de Hingh:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Patricia Y.W. Dankers:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Kurt Van Der Speeten:** Writing – review & editing, Methodology, Conceptualization. **Nicole D. Bouvy:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Wouter Marchal:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2025.113763.

Data availability

Data will be made available on request.

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