Am J Cancer Res 2025;15(3):1213-1223
www.ajcr.us /ISSN:2156-6976/ajcr0162228

Original Article

Safety of BromAc® with Mitomycin C during hyperthermic intraperitoneal chemotherapy (HIPEC): a preclinical study

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Received November 24, 2024; Accepted February 5, 2025; Epub March 15, 2025; Published March 30, 2025

Abstract: Peritoneal cancer patients are often treated with hyperthermic intraperitoneal chemotherapy (HIPEC). BromAc®, a mixture of bromelain and acetylcysteine, has demonstrated anticancer properties with chemotherapeutic agents. Although bromelain and acetylcysteine have anti-inflammatory, anti-coagulant and wound healing properties, their effect with Mitomycin C is unknown in HIPEC. Hence, we investigated their safety using a rat model. Sixteen Wistar rats were divided into 4 groups (N=4). Controls received saline, whilst the others received BromAc®, Mitomycin C (MMC) or BromAc®+MMC. Three doses were given at 30-minute intervals. Animal weights were monitored for 7 days before euthanasia. Peritoneal fluid and blood samples were collected for pharmacokinetic analysis. Colon anastomosis healing was evaluated with burst pressure and collagen density assessment. Internal organ histology and coagulation factor X were performed in plasma with an enzyme-linked immune assay. All rats were healthy, with similar weight fluctuation patterns, although the MMC-treated rats, with or without BromAc®, showed higher weight loss during the first 4 days. Whilst the burst pressure was similar in all groups, the BromAc® group showed a slightly higher value. Collagen densities were similar in all groups. The results showed that the histology of vital organs of the treated and controls were similar. BromAc® concentration in peritoneal fluids increased over 90 min with a higher increase when given with MMC. BromAc® or the combination did not affect coagulation Factor X. In conclusion, general well-being, wound healing, organ histology, pharmacokinetics and coagulation factor evaluations indicated that BromAc® with or without MMC was safe during HIPEC.

Keywords: Acetylcysteine, BromAc®, bromelain, colon anastomosis, HIPEC, MMC

Introduction

The majority of Peritoneal carcinomatoses (PC) are mucinous and primarily originate from gastrointestinal or gynecological sources. The most effective treatment for PC in selected patients involves cytoreductive surgery (CRS) combined with hyperthermic intraperitoneal chemotherapy (HIPEC) [1]. During HIPEC, heated cytotoxic drugs are delivered intraperitoneally to treat malignant cells [2]. Hyperthermia, often performed at 42°C, can enhance the effects of certain chemotherapy agents. Mitomycin C (MMC) is a chemotherapeutic agent most frequently used for HIPEC and is efficient and safe for the treatment of peritoneal tumor metastasis of colorectal origin [3]. HIPEC procedure usually includes the resection of part of the intestine, with anastomosis being performed after the completion of HIPEC.

Mucins are made of a protein backbone abundant in serine and threonine, connected to a variety of O-linked oligosaccharide side chains [4]. Bromelain (Brom) and acetylcysteine (Ac) are two mucolytic agents demonstrated interference with the growth of malignant cells, on their own [5, 6]. Combining bromelain with acetylcysteine (BromAc®) enhanced these effects and has proven to be highly effective against gastrointestinal (GI) cancer cells [7] and organoids [8]. Additionally, it has demonstrated efficacy as a mucolytic for pseudomyxoma peritonei (PMP) mucin [9, 10]. Hence, PC patients may benefit from BromAc® and HIPEC co-treatment.

https://doi.org/10.62347/HQJH9945

Colon anastomotic leak is a major complication following CRS-HIPEC [11] and is associated with a high morbidity and mortality rate, which could escalate if the anastomosis is performed under conditions that may impact wound healing [11-13]. Although, patients with PMP have been treated with BromAc®; it has not been investigated in patients with colon anastomosis [14, 15]. Our earlier study of BromAc®, using IP administration, on colon anastomosis rat model showed no adverse effect on the healing process [16]. However, the study involved intraperitoneal injection delivery, unlike the current study that involves HIPEC alone and in combination with MMC chemotherapy. Based on the characteristics of BromAc® mentioned earlier, adding chemotherapy and hyperthermia may have a significant effect on wound healing, leading to a compromise in the anastomosis. Hence, we tested the effects of intraperitoneal BromAc® with MMC on the healing process of colon anastomosis using a rat model of HIPEC.

Seven days post-surgery, we measured the burst pressure at the site of anastomosis after colon surgery to determine differences compared to control animals, which were only exposed to saline. Further, we examined the accumulation of collagen and colon to assess wound healing. The concentration of Factor X in plasma was assessed to investigate the effects on coagulation. The concentration of the various agents was also determined at different time points (to 90 min) in the intraperitoneal fluid during HIPEC and up to 7 days in plasma post-HIPEC to assess the pharmacokinetics of the drug. Further, owing to the absorption of BromAc® systemically, we examined the tissue histology of the different internal organs to determine any adverse effects.

Materials and methods

Drug preparation

BromAc® was manufactured by Mucpharm Pty Ltd., AUS as a sterile solution. A pharmaceutical-grade MMC was used in the study (AUST R 243552, Omegapharm, AUS). The drug diluent was 0.9% NaCl solution. The drug used in the study was prepared as one batch and stored at -30°C until use.

HIPEC study ethics and design

Sixteen Wistar rats (Ozgene ARC, WA, AU) weighing an average of 250 g were divided into four groups (N=4); both sexes equally. The "resource equation approach" has been used to determine the sample size [17]. Groups were treated with HIPEC and either 0.9% saline (control), BromAc® [Brom (0.03 mg/mL) + AC (3 mg/mL)], MMC (0.02 mg/mL) or BromAc®+MMC was applied during HIPEC treatment. Drug doses were selected according to levels tested in prior studies [18-20]. A 250 mL perfusate was circulated over the 90 min treatment period. At any single time point, approximately 25 mL of perfusate would be in the peritoneal cavity.

Before surgery, rats were anesthetized with isoflurane. The surgical site was shaved and disinfected with chlorhexidine, and a midline laparotomy was performed. A sterile plastic drape was sutured to the abdominal wall to prevent fluid leaks. The abdomen was left open. The saline perfusate was pre-warmed in a water bath and driven by a roller pump. The intraperitoneal temperature was maintained between 40.5-41.5°C. Temperature was monitored using digital 2 Way K-Type temperature thermometer equipped with K-Type thermocouples placed intraperitoneal (upper and lower abdomen) and in the perfusate. Drugs were added to the perfusate in three divided doses at 30 min intervals (i.e. 0, 30 and 60 min). Intraperitoneal perfusate samples were collected before the addition of the subsequent dose to evaluate drug concentration. After 90 min of open perfusion, the perfusate was drained. Then, the cecum was identified, and a 1.5 cm transverse incision was made. A colon anastomosis was then repaired in longitudinal continuous fashion using 5/0 PDS inverting suture technique. Then, a seromuscular interrupted 5/0 PDS suture was used over the repair. Then, the abdomen is closed in two layers.

Rats were monitored daily for health parameters and signs of intra-abdominal infection. On Day 7 post-HIPEC, rats were euthanized by inhalation of carbon dioxide. Day 7 is considered clinically as an important checkpoint for assessing healing and identifying potential complication. Then, the midline laparotomy was re-opened. Signs of inflammation, anastomotic leak, abscesses, or adhesions were explored. Then, tissue specimens were collected for histopathological evaluation.

Quantitative analysis of coagulation factor X

Rat Factor X levels have been measured in plasma using ELISA Kit (#IRTF10KT, Innovative Research, MI, US).

Anastomotic burst pressure

A 10 cm segment of the bowl that includes the anastomosis site was ligated proximally, and a catheter was inserted distally, with fixation using a 3/0 silk tie to prevent leak. The catheter was connected to a syringe and sphygmomanometer via a stopcock. The syringe was used to insufflate the bowl gradually with air until a sudden loss of pressure occurred. This pressure was recorded as Anastomotic Burst Pressure (ABP).

Histological evaluation

Formalin-fixed, paraffin-embedded sections of colon anastomosis and other organs were H&E-stained. Fibrosis was assessed based on trichrome-stained sections. All tissues/slides were examined by an accredited independent contracted veterinary histopathologist for the absence or presence of abnormal histopathological features. The pathologist was masked to group treatments but was familiar with background information.

Quantitative analysis of bromelain, MMC and acetylcysteine

A fluorescence assay was used to measure Bromelain enzymatic activity in peritoneal and plasma samples collected during the HIPEC procedure. Samples were filtered using Amicon® Ultra-2 10 kD filters at 1500 rpm for 15 min at 4°C, and the supernatant was analyzed for Protease Activity. Fluorescence intensity was recorded using a VANTAstar® microplate reader. Meanwhile, MMC and Acetylcysteine concentrations in peritoneal fluids were measured via High-Performance Liquid Chromatography (HPLC). After thawing and centrifuging the samples, the supernatant was injected into a Shimadzu HPLC system for detection at 351 nm and 214 nm, respectively.

For plasma Acetylcysteine quantification, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used with protein precipitation (PPT) via acetonitrile. The supernatant containing Acetylcysteine was separated after centrifugation and analyzed with a reversed-phase C18 column. The mass spectrometer operated in positive ion mode with multiple reaction monitoring (MRM). MMC concentration in plasma was also measured using HPLC. After sample thawing and dilution, acetonitrile was added for precipitation, followed by centrifugation. The supernatant was injected into the HPLC system for MMC detection at 351 nm.

Statistical analysis

Data were analyzed using GraphPad Prism version 10.0 (GraphPad Software Inc., San Diego, CA, USA). One-way ANOVA and Repeated Measures Two-way ANOVA (RM-ANOVA) were used to determine the significance difference between groups. Tukey's and Dunnett's multiple comparison tests were used after significant one-way ANOVA and RM-ANOVA, respectively to compare the means of the control and treated groups. Differences were considered statistically not significant when $P>0.05$ (n.s); significant when $P\leq0.05$ (*), very sigroups.t. $P=0.001$ to 0.01 (***), and extremely significant when $P=0.0001$ to 0.001 (***).

Results

During examination of the abdomen of control and drug-treated rats there were no signs of abscess, anastomosis leaks or signs of infection including presence of purulent drainage. There was only mild omental adhesion to the anastomoses observed in all rats.

RM-ANOVA showed a significant effect of both time and treatment on body weight, $P<0.0001$ and 0.0025, respectively. There was weight loss in the control and all treated groups in the first 3 days (**Figure 1A**); however, the difference in body weight between the control and treated groups was not significant.

Following this, there was a weight gain from day 4 to 7 except with the MMC-treated group. The body weight of the control group did not differ significantly from the BromAc® alone group. The BromAc® and MMC treated group showed significant weight loss when compared with control on day 5 only ($P=0.027$). On days 5, 6, and 7, there was a significant decrease in body weight of MMC alone treated group when compared with control ($P=0.0203$, 0.0291, and 0.0185, respectively). General health and well-being parameters monitored during the study showed the same patterns between control and treated animals.

Factor X levels were measured to investigate the effects of drug treatment on coagulation.

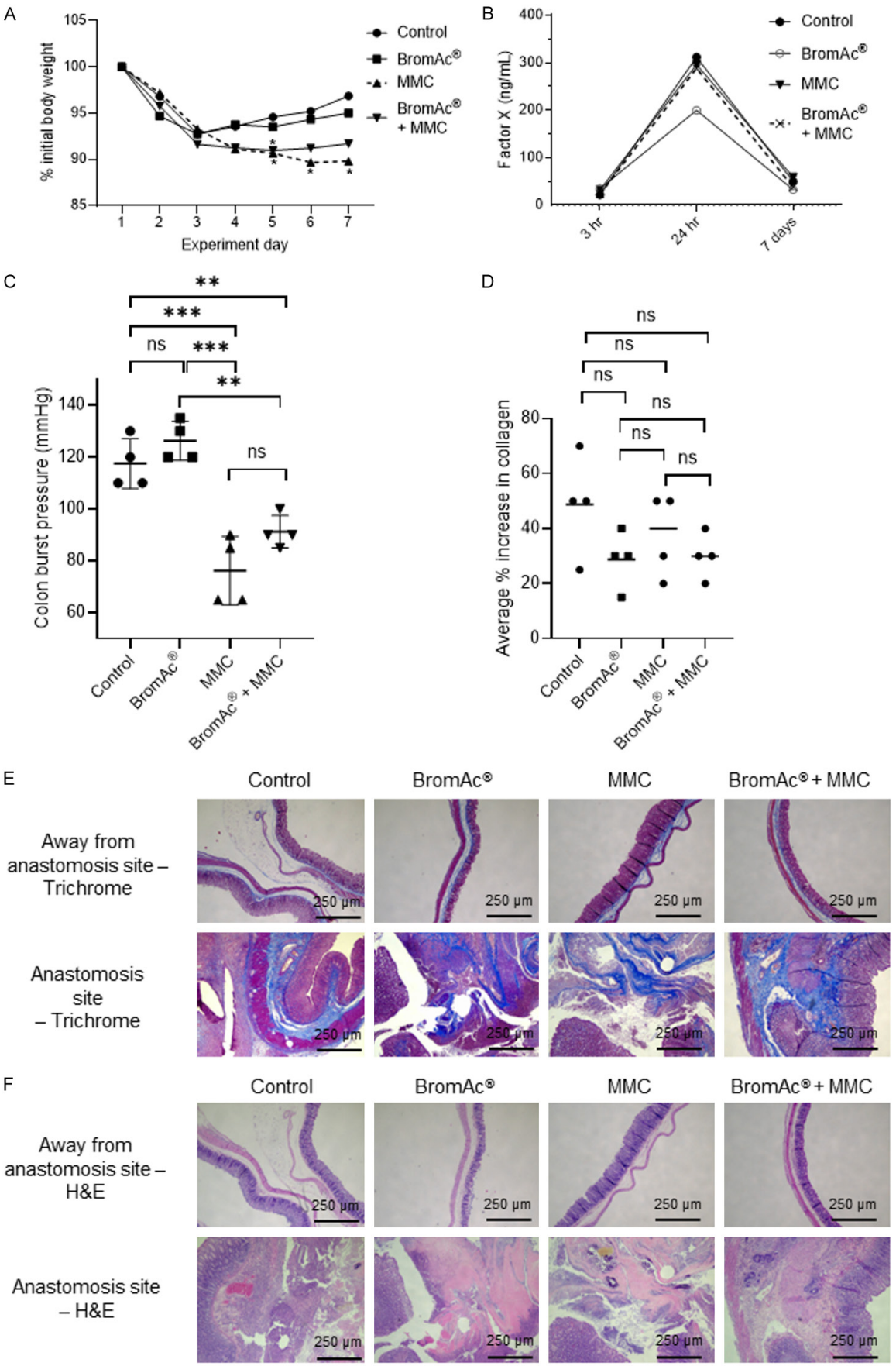
A
% initial body weight
Experiment day
Control
BromAc®
MMC
BromAc® + MMC
B
Factor X (ng/mL)
3 hr
24 hr
7 days
C
Colon burst pressure (mmHg)
ns
D
Average % increase in collagen
E
Away from anastomosis site – Trichrome
Anastomosis site – Trichrome
F
Away from anastomosis site – H&E
Anastomosis site – H&E
250 µm

Figure 1. (A) Percentage changes in body weights of rats monitored post-HIPEC. (B) Levels of coagulation Factor X in plasma at different time intervals. (C) Potency of colon anastomotic burst pressure 7 days post-HIPEC. (D) Average percentage increase in collagen present in the colon-anastomosis sites. (E) H&E-stained colon sections. (F) Trichrome-stained colon sections. Final magnification in (E and F), ×50; scale bar =250 μm.

RM-ANOVA revealed a significant effect of time but not treatment on Factor X levels (P=0.0001 and 0.5629, respectively) (**Figure 1B**). There was a significant increase in Factor X levels at 24-hr in control and drug-treated groups compared to their own 3-hr levels (P=0.0058, 0.0012, 0.0651, and 0.0636, respectively).

Post-euthanasia, burst perforations were located at or next to the anastomosis sites in all rats. One-way ANOVA revealed a significant effect of at least one treatment on burst pressure (P<0.0001). There were no significant differences in the burst pressure between the control and the BromAc®-treated groups (P= 0.5779), while the MMC and BromAc®+MMC groups decreased significantly (P=0.0003 and 0.0096, respectively) (**Figure 1C**). Interestingly, treatment with MMC alone or in combination with BromAc® decreased burst pressure significantly when compared with the BromAc® alone-treated group (P<0.0001 and 0.0011, respectively). There was no significant difference in burst pressure between MMC and BromAc®+MMC groups (P=0.1690).

The fibrosis at the sites of colon anastomosis was interpreted based on trichrome-stained (collagen density) sections (**Figure 1D**, **1E**). One-way ANOVA showed no statistical difference in collagen density between the groups (P=0.1953).

Inflammatory changes (lamina propria, submucosal, muscle layers, and serosal surfaces), the loss of structures/necrosis (e.g., epithelial cells, crypts), and vascular congestion, both at a point away from the site of the anastomosis and the site of the anastomosis on H&E-stained sections were evaluated. No difference between the control and the other treated groups 7 days post-surgery (**Figure 1F**).

Histopathological evaluations showed no abnormalities in the heart, lung, or kidney sections of the control and treated groups (**Figure 2**; **Table 1**). Similar findings were observed upon comparison of sections from the liver, pancreas, and spleen with only ±1/4 difference between groups. Sections from the small intestine showed hyperplastic Gut-Associated Lymphoid Tissue (GALT) in control groups (3/4). BromAc® alone and MMC alone control groups showed a decrease in this observation (only 2/4). However, co-treatment with BromAc® and MMC normal GALT appearance with complete resolution of the hyperplastic phenomena (0/4). Hence, these results indicated the safety profile of the intraperitoneal BromAc® administered with HIPEC on the examined body organs over the study period.

Bromelain, Acetylcysteine and MMC were measured in peritoneal fluids during HIPEC to investigate the bioavailability of the drugs in the peritoneal cavity. RM-ANOVA showed significant effect of both time and treatment on bromelain concentration, P=0.0001 and 0.0035, respectively. There was an increase in the bromelain concentration over time in the peritoneal perfusate in BromAc® and BromAc®+MMC groups (**Figure 3A**). At 30 min, there was no significant difference in bromelain concentration between BromAc® and BromAc®+MMC treated groups and control (P=0.4558 and 0.4400, respectively). At 60 min, the increase in the bromelain concentration in BromAc® and BromAc®+MMC groups was significant compared to control (P=0.0196 and 0.0140, respectively). At 90 min, the increase in bromelain concentration was not significant in BromAc® (P=0.0878). Co-administration of MMC and BromAc® resulted in a significant amount of bromelain in the peritoneal fluid (P=0.0391).

RM-ANOVA analysis showed significant effect of both time and treatment on Acetylcysteine concentration in peritoneal fluids, P=0.0002 and P<0.0001, respectively (**Figure 3B**). At 30 min, there was no significant difference in Acetylcysteine concentration between BromAc® and BromAc®+MMC treated groups when compared to control (P=0.0551 and 0.0079, respectively). The increase in the Acetylcysteine concentration in BromAc® and BromAc®+MMC groups was significant at 60 min (P=0.0385 and 0.0034, respectively) and at 90 min (P=0.0126 and 0.0039, respectively).

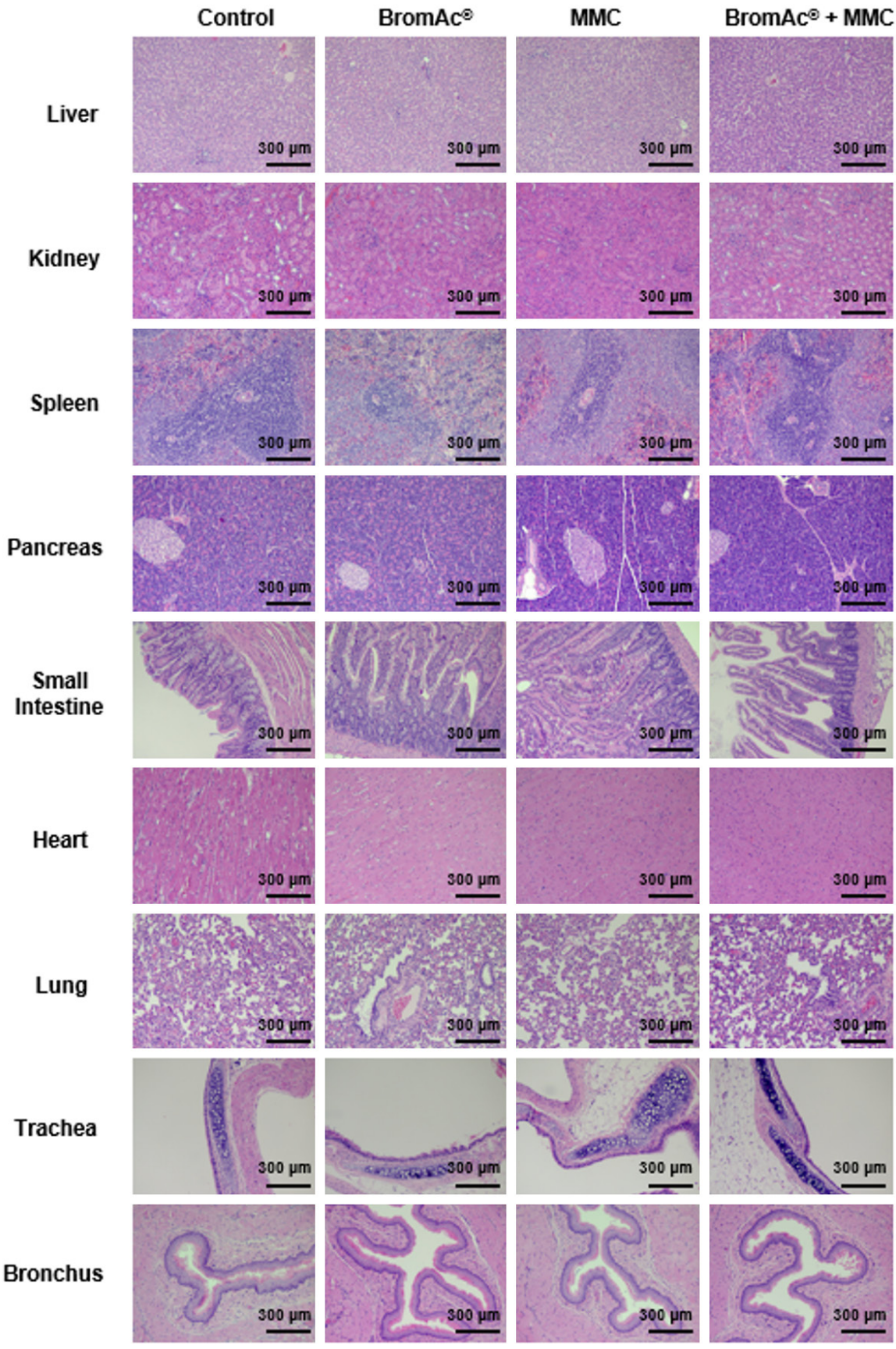
Control
BromAc®
MMC
BromAc® + MMC
Liver
Kidney
Spleen
Pancreas
Small Intestine
Heart
Lung
Trachea
Bronchus
300 μm

Figure 2. Representative microscopic histological images of different internal organs stained with H&E. Final magnification, ×100; scale bar =300 μm.

Table 1. Histopathological features scores in the HIPEC *in vivo* model

Histopathological Findings	Control	BromAc®	MMC	BromAc®+MMC
Heart, lung, kidney	0/4	0/4	0/4	0/4
Liver				
Focal capsular reaction with fibroplasia, mesothelial cells	0/4	1/4	0/4	1/4
Pancreas				
Fibroplasia, and/or inflammatory cells	1/4	1/4	1/4	2/4
Spleen				
Capsular reaction with fibroplasia, inflammatory cells, and/or neovascularisation	2/4	1/4	1/4	1/4
Small Intestine				
Hyperplastic GALT	3/4	2/4	2/4	0/4
Mesenteric lymph nodes				
Moderate to severe reactive lymphoid hyperplasia	2/4	3/4	2/4	2/4
Colon, away from the surgical site - Lamina propria				
Lymphocytic or inflammatory cell infiltration	3/4	3/4	3/4	2/4
Fibroplasia	3/4	3/4	3/4	2/4
Submucosal oedema	0/4	2/4	1/4	0/4
Mesothelial hypertrophy	0/4	1/4	0/4	0/4
Neovascularisation	1/4	0/4	0/4	0/4
Colon, surgical site - Lamina propria				
Diffuse colitis, or mixed inflammation	4/4	4/4	4/4	4/4
Multifocal necrosis	4/4	4/4	4/4	4/4
Foreign body pyogranuloma	4/4	2/4	3/4	3/4
Oedema	2/4	1/4	3/4	3/4

RM-ANOVA showed significant effect of both time and the treatment on MMC concentration in peritoneal fluids, $P<0.0001$ for both (**Figure 3C**). There was a significant increase in all MMC concentration measurements overtime in both MMC alone and BromAc®+MMC treated groups when compared to control at 30 min ($P>0.0009$ and 0.0072, respectively), at 60 min ($P=0.0028$ and 0.0015, respectively), and at 90 min ($P=0.0054$ and 0.0011, respectively).

To investigate further the pharmacokinetics of the therapy, the drug levels were measured in plasma post-HIPEC. RM-ANOVA showed significant effect of both time and the treatment on Bromelain concentration in plasma, $P=0.0001$ and 0.0035, respectively (**Figure 3D**). At 3-hr, there was a significant increase in bromelain concentration in BromAc®+MMC ($P=0.0097$) but not in BromAc® ($P=0.1539$) treated groups when compared to the control. At 24 days, there was no significant difference between the BromAc® and BromAc®+MMC treated groups when compared to control ($P=0.2866$ and 0.3516, respectively). The plasma levels of acetylcysteine (**Figure 3E**) and MMC (**Figure 3F**) were under the detection limits 3-, 24-hr, and 7-days post-HIPEC.

Discussion

The present study on rats showed an initial drop in body weights of all animals after the surgical interventions, indicating that the nutritional intake was reduced in all treatment groups, suggesting trauma, pain, and discomfort in the animals. The loss of body weight was a general indication of well-being after surgical intervention and treatment in all the animals. Noticeably, the loss of body weight in the BromAc®-treated group was eventually reversed from Day 4-7. The loss of body weight continued after day 4 in the MMC group and the combination group of MMC and BromAc®. Weight gain in the MMC group on the seventh day was absent compared to all the other groups further

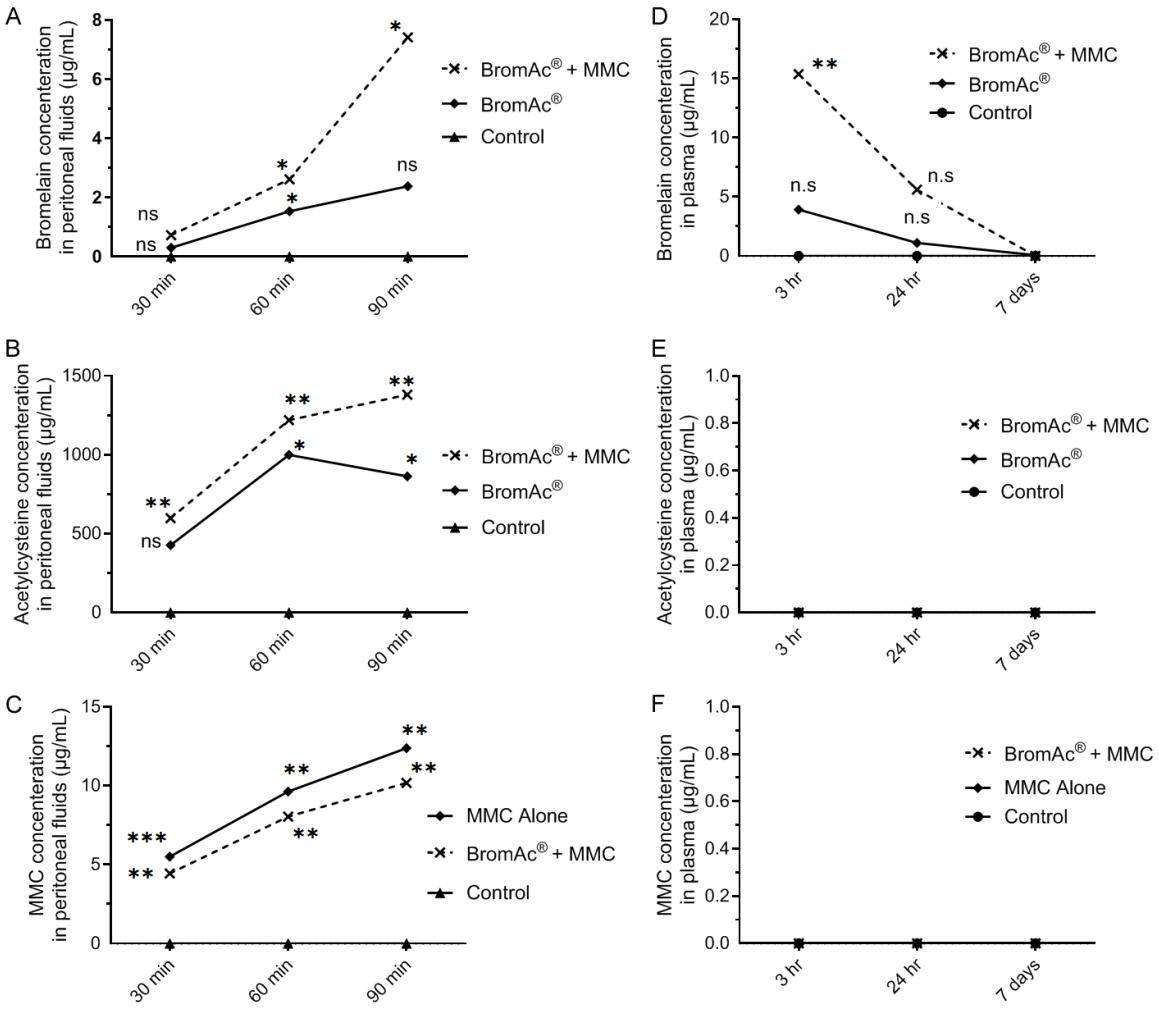

Figure 3. Graphs show the concentration levels of the drug agents in peritoneal perfusates and plasma. Graphs (A) Bromelain, (B) Acetylcysteine, and (C) MMC show drug levels in peritoneal perfusate during HIPEC. Graphs (D) Bromelain, (E) Acetylcysteine, and (F) MMC show drug levels in plasma.

indicating much higher trauma and systemic toxicity, potentially due to the presence of MMC which is known to induce systemic organ toxicity [21].

Bromelain, Acetylcysteine and MMC have anticoagulant properties. Hence, it was vital to investigate the effect of drug treatment on coagulation to assess the safety of the drug. Factor X is a crucial component of the coagulation cascade. It plays a critical role in both the intrinsic and extrinsic pathways. Hence, regardless of the pathway initiating it, Factor X is activated leading to the formation of a fibrin clot. The results showed that the type of drug treatment had no effect on coagulation at 3-hr, 24-hr, or 7-days post-treatment, demonstrating the safety of the drug treatments. There was an increase in the coagulation Factor X in all groups including control at 24-hr, indicating that this increase is related to the HIPEC surgery, not the drug treatment.

Importantly, no anastomosis leakage was observed post-euthanasia in either the control or the treated groups. Previous studies using rat models reported very low incidences in control rats, ranging from 0 to 5% (0-1 out of 20 animals) [22, 23]. While another study showed that bromelain can prevent intra-abdominal adhesion [24], mild post-operative intra-abdominal adhesions were observed in all control and drug-treated groups in our study. Although there were no significant differences in burst pressure between the control and the BromAc®-treated groups post-surgery, the MMC and

BromAc®+MMC groups showed a significant decrease in burst pressure compared with the control group.

The individual components of BromAc®, bromelain and acetylcysteine, are known to promote healing [25, 26]. This effect might have been present but undetectable due to the limited sample size. Additionally, the presence of MMC may have interfered with the healing process, as MMC has been shown negative effect on anastomotic strength during HIPEC in rats [27, 28].

Collagen is a fibrous protein crucial for wound healing, strengthens tissues at the wound site, and prevents tears under pressure. Our study found similar collagen deposition levels at day 7 in all groups (control, BromAc®, MMC, and BromAc®+MMC). This suggests that neither BromAc® alone nor its combination with MMC significantly affects collagen synthesis, a vital process for wound repair.

Systemic absorption and distribution of the tested drugs (BromAc® and MMC) were anticipated as the drug was administered intraperitoneally during the HIPEC procedure in this study. Therefore, histological analysis of various vital organs, including lungs, heart, kidneys, spleen, and pancreas, was performed to assess the potential pharmacological and biochemical effects of BromAc®. Hyperplastic GALT was observed in both control and BromAc®-, as well as MMC-treated rats. This observation could be related to an underlying condition such as inflammation caused by colon anastomosis surgery. In rats co-treated with BromAc® and MMC, hyperplastic GALT disappeared, potentially suggesting an anti-inflammatory effect of the drug combination. No other significant histological abnormalities were observed in either the saline or the treated groups across these organs. This finding suggests that, at the administered concentration, BromAc® did not induce microstructural alterations in vital organs, further supporting its systemic safety profile within the parameters of this protocol.

The pharmacokinetics of the drug agents has been investigated. Analysis of the intraperitoneal perfusate indicated that there was an increase of bromelain over the 90-min HIPEC due to three equal doses delivered at 0, 30 and 60 min. This also shows that bromelain did not get absorbed instantly by the organs and the peritoneal membrane, probably due to its large size [29]. The concentration of bromelain was much higher when delivered with MMC as compared to delivery without MMC. This observation is most probably due to competitive absorption between MMC and bromelain and this should be further investigated. There was also an increase of Acetylcysteine concentration overtime in the peritoneal perfusate in BromAc® and again this may be due to acetylcysteine being hydrophilic and although its molecular weight is only 163 Daltons, lipophilic molecules get absorbed more readily through the blood vessels [29]. Data showed an increase in MMC concentration over time in the peritoneal perfusate in MMC and BromAc®+MMC groups. However, the increase was much higher in the MMC group compared to BromAc®+MMC group indicating enhancement of absorption of MMC by BromAc®. The results showed that bromelain was observed in the blood up to 24 hr post-HIPEC. Upon oral delivery, Bromelain is absorbed by the body through the intestines. It stays effective in the body for about 6 to 9 hr [30]. In addition, the results showed that both Acetylcysteine and MMC disappeared readily in the blood. Previous data showed that both agents has a short half-life of 5.6 hr [31] and 8.2-51.8 min [32], respectively in the blood after systemic administration.

Although the present study indicates that the use of BromAc® alone or in combination with MMC is safe during HIPEC procedures in healthy rats, there are limitations to transferring these findings to patients. Patients with peritoneal cancers are often immunocompromised and have other comorbidities, which may cause the healing process to vary depending on their health, age, nutritional status, and other conditions. Further pre-clinical studies are planned to investigate the safety of BromAc® administration with other chemotherapeutics under both hyperthermic and normothermic conditions.

Acknowledgements

We would like to thank Lorna Rasmussen from Cerberus Sciences, Melbourne, for helping with the histopathological evaluation. This research was funded by Mucpharm Pty Ltd., Australia.

Disclosure of conflict of interest

Mucpharm Pty Ltd. solely owns or has sublicensed the intellectual property surrounding

BromAc®. D.L.M. and S.V. are shareholders of Mucpharm Pty Ltd. A.H.M., M.B., M.K.R., K.P., A.S., F.O., S.B., and J.A., are employees of Mucpharm Pty Ltd.

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