MICRONUTRIENT-ENHANCED HYPERTHERMIC INTRAPERITONEAL CHEMOTHERAPY FOR TREATMENT OF PERITONEAL METASTASIS: A NOVEL EXPERIMENTAL DESIGN

by

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Abstract

Introduction: Peritoneal carcinomatosis is an end stage sequela occurring in 10% of patients with colorectal cancer. Palliative approaches have evolved over the past several decades and the role for surgical cytoreduction with hyperthermic intraperitoneal chemotherapy (HIPEC) has proven efficacy in several studies. Optimization of HIPEC therapy includes the addition of adjuncts to the carrier solution of intraperitoneal chemotherapy to improve tumor cell killing. In this study the addition of vitamin C, selenium, and quercetin ("micronutrient combination") to mitomycin C is evaluated in-vitro, and a novel murine model of HIPEC is developed using a hyperthermic chemotherapy infuser device designed de novo and printed on a 3D resin printer.

Methods: HCT-116 cells were grown in culture and divided into treatment groups including: control, micronutrient combination, mitomycin C, and mitomycin C + micronutrient combination. Groups were cultured up to 72 hours after treatment and then subjected to MTT assay, crystal violet assay, trypan blue synergy assay, clonogenicity assay, cell cycle assessment by flow cytometry with propidium iodide, and western blotting for cleaved caspase-3. The infuser device was designed in a CAD environment, printed on a 3D resin printer, and underwent fluid temperature stability analysis and flow experiments by infusing methylene blue into live mice followed by necropsy and analysis of dyeing patterns.

Results: MCC treated cells proliferated at 32.7%, and tumor cells treated with MCC + MNC carrier solution proliferated at 27.3%. Normothermic MCC and the MNC alone
caused a 26.8% and 33.3% reduction in cell survival, and MCC delivered to cells in the micronutrient combination solution decreased cell survival by 53.2%. 95.3% and 99% of cells treated with MCC or MNC alone demonstrated viability, and 85% of cells treated with MCC + MNC demonstrated short term viability, suggesting synergy. HCT-116 clonogenicity is disrupted by MCC and MNC individually, and nonexistent in the MCC + MNC treatment group. Cleaved caspase-3 mediated apoptosis is upregulated by MCC, and by MNC to a lesser extent. Flow cytometry apoptosis demonstrates increased S-phase cell cycle arrest in the MCC + MNC sample. The mouse infuser HIPEC apparatus demonstrated an thorough distribution of blue dye in predictable regions of the abdomen with an acceptable range of hyperthermic regulation.
Introduction

Peritoneal carcinomatosis develops in about 10% of patients diagnosed with colorectal cancer\(^1\). At the time of diagnosis, 8-15% of cases of colorectal cancers involve the peritoneal surface, and in 25% of patients with recurrent disease, peritoneal carcinomatosis is the isolated site of metastatic disease\(^2\)\(^-\)\(^5\). Hyperthermic intraperitoneal chemotherapy (HIPEC) is now considered the standard of care for certain peritoneal surface malignancies. Multiple consensus statements from Oncologic specialists worldwide support the use of HIPEC in patients with peritoneal metastases\(^6\). The survival advantage of cytoreductive surgery (CRS) with HIPEC in patients with peritoneal carcinomatosis from colorectal cancer exceeds 31 months, and has been confirmed in multiple studies\(^7\)\(^-\)\(^8\). However, the methods employed in administering HIPEC vary significantly across institutions, and there is no standardized therapy. While multiple protocols are advocated, there is a scarcity of data that demonstrates the efficacy of one protocol over another. This is, in part, due to the many variables of HIPEC left up to the digression of the surgical oncologist. An emerging trend to standardize HIPEC regimens for different tumors is challenged by the expectation that HIPEC therapy can be further improved.

Several studies have demonstrated improved efficacy by using non-standard strategies during both in-vitro and in-vivo experiments. Lehman, et al., for example, demonstrated that the addition of diethyldithiocarbamate to the HIPEC carrier solution induced oxidative stress and improves survival in a murine colon cancer model of
HIPEC\textsuperscript{9}. The drug carrier solution is, in general, an attractive medium to optimize the efficacy of treatment because of its intimate relationship with both the cytotoxic drug and the tumor microenvironment. The primary mode of drug delivery in HIPEC is through local drug diffusion into the tumor, with systemic absorption and recirculation a distant second\textsuperscript{10}. The extent to which the carrier solution prolongs and enhances exposure of the drug to the tumor cells has an impact on anti-neoplastic potential\textsuperscript{11}. The prospect of a carrier solution that could act synergistically with the chemotherapeutic drug motivated our work with a micronutrient-enhanced carrier solution.

A review of recent literature of compounds that have antineoplastic activity and protective effects on normal cells identified three specific naturally occurring micronutrients that are investigated in this study. Vitamin C has been found to exhibit cytotoxic effects selectively on tumor cells and is also well known to protect normal cells through its antioxidant properties\textsuperscript{12-15}. In vivo studies demonstrate antineoplastic activity at high concentrations that may be achieved locally with intraperitoneal therapy. Quercetin is a plant-derived flavonoid with antioxidant properties that has been investigated for its strong anti-cancer effects in colorectal tumors\textsuperscript{16,17}. In-vitro data suggests that quercetin promotes apoptosis in cancer cells specifically by inducing reactive oxygen species (ROS) accumulation\textsuperscript{18}. Thirdly, selenium is an essential micronutrient that is toxic in high doses, but plays an important role as a cofactor for antioxidant enzymes. It has been investigated as a potential supplement in cancer prevention and treatment. Selenium has been shown in-vitro to induce cell death in cancer cells, and it has also demonstrated anti-metastatic properties by inhibiting cell
invasion and angiogenesis\textsuperscript{19-22}. The first goal of this investigation is to determine whether the addition of vitamin C, quercetin, and selenium to an isotonic carrier solution for mitomycin C increases the efficacy of the chemotherapeutic drug in vitro.

The second arm of this investigation is to optimize a murine model of HIPEC for testing the micronutrient-enhanced carrier solution. The standard murine models prevalent in the literature include an open model and a closed model. The open model is also termed the “Coliseum” technique, and uses a laparotomy incision with the skin and abdominal wall suspended anteriorly in order to create an accessible peritoneal cavity for the instillation of HIPEC solution. In this model, the mouse is placed on an orbital shaker and a warming pad during therapy. Although effective at distributing HIPEC solution throughout the abdomen, this model lacks hyperthermia since the solution is instilled at the beginning and quickly normalizes to the mouse peritoneal temperature (which is hypothermic due exposure to the open air of the laboratory. Additionally, since the HIPEC solution is instilled but not cycled or flowing from a solution reservoir, the similarity to clinical conditions in humans is limited. The closed method described in the literature uses a peristaltic pump and warm water bath for the drug solution, and inflow and outflow catheters inserted intraperitoneally. Although this model accomplishes continuous flow and a hyperthermic bath for the HIPEC solution, temperature is monitored within the mouse abdomen, which is not the same as the instillation temperature. An additional pitfall of this method is that uniform flow may be compromised by the variable placement of the catheters in the abdomen, as well as the tight space within the peritoneal cavity. The second goal of this investigation is to
optimize a murine model of HIPEC that overcomes the limitations of available techniques.

**Methods and Materials**

All experimental protocols were in accordance with institutional oversight committee requirements.

**Chemicals**

Mitomycin C (MCC) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Vitamin C, quercetin, selenium, and McCoy’s 5A cell culture medium were purchased from Sigma-Aldrich (St. Louis, MO). Four treatment solutions were prepared, including: 1) negative control consisting of only McCoy’s 5A cell culture medium; 2) Micronutrient combination (MNC) prepared with 1.25mM of vitamin C, 150µM of quercetin, and 20µM of sodium selenite in McCoy’s 5A; 3) MCC (5µg/ml) in McCoy’s 5A; 4) MCC (5µg/ml) and MNC in McCoy’s 5A. Treatment solutions were filter sterilized (0.22µm) and formulated prior to use.

**Cells**

Colorectal cancer cells (HCT116) were acquired from American Type Culture Collection (Manassas, VA). Cells were plated at 30,000 cells/75 mm dish and maintained in McCoy’s 5A with 10% fetal bovine serum and 1% penicillin/streptomycin. They were
cultured at 37°C in 5%CO₂ and 95% humidity, and passaged when cells reached 75% confluence in T-75 flasks.

**Mitomycin C and micronutrient concentration determinations**

HCT116 tumor cells were plated in 96 well dishes and treated with ascending concentrations of MCC in McCoy’s 5A from 0 to 20µ/ml for 90 minutes at 37°C. Treatment solutions were then removed, plates rinsed, and cells were returned to the incubator in sterile media. At 72 hours MTT analysis (see Proliferation assay below) was performed to determine an IC₅₀ curve for MCC on HCT-116 cells. The same procedure was performed for the MNC using static concentrations of the micronutrients and descending concentrations of the stock micronutrient formulation in McCoy’s 5A medium.

**Proliferation assay**

HCT116 tumor cells were plated in 96 well dishes at a concentration of 1000 cells/well and cultured overnight. Two treatment arms were then prepared for parallel experiments at 37°C and 41.5°C. Cells were treated with either 1) negative control, 2) MNC medium, 3) MCC (5µg/ml) in medium, or 4) MCC + MNC in medium, and incubated at 37°C and 41.5°C for 90 minutes. After treatment, cell plates were rinsed and placed in sterile media in standard cell culture conditions. At 72 hours, 20µl of 5mg/ml thiazolyl blue tetrazolium bromide (MTT) was added to all cells-containing wells and a column of empty wells. The plate was then incubated at 37°C for 3.5 hours. Media was then
removed and 150µl of MTT solvent (4mM HCl, 0.1% Nondet P-40 in isopropanol) was added. Plates were agitated for 15 minutes on an orbital shaker, then absorbance was read at 590nm with a photospectrometer.

**Growth assay**

Tumor cells were plated in 6 well dishes and allowed to incubate overnight. Each well was then treated with one of the four treatment groups noted above. At 72 hours, media was removed and cells were fixed with 1% paraformaldehyde in PBS then stained with 0.5% crystal violet solution. Cells were rinsed multiple times and optical intensity was measured with a photospectrometer.

**Viability assay**

In a multi-well dish, tumor cells were plated and incubated overnight. IC\textsubscript{25} formulations of both MCC and the MNC treatment solutions were prepared and substituted for the IC\textsubscript{50} formulations used in the other experiments. At 72 hours the cells were extracted from the wells and suspended in a balanced salt solution in labeled microtubes. 0.4% trypan blue was added to each microtube. Cells were counted with a hemacytometer after 5 minutes to determine cell viability as the percentage of unstained cells for each treatment group.

**Clonogenicity assay**

About 1000 cells were seeded into four 100mm dishes and treated at 18 hours, before sufficient time had elapsed for colony formation. At 96 hours post-treatment, cells were
stained with methylene blue, photographed, and counted with the aid of a microscope and ImageJ software (Wayne Rasband, NIS, USA). Colony formation in treatment groups was compared with negative control for quantity and histological characteristics.

**Western blotting**

Cells were cultured in 100mm dishes and treated with either micronutrient combination or 5µg/ml MCC when confluence had reached about 50%. At 48 and 72 hours post-treatment, lysates were prepared. A protein determination was performed on the respective lysates and they were electrophoresed across a gel and transferred to a nitrocellulose membrane. Cleaved caspase-3 antibodies were used to determine apoptotic activation.

**Cell cycle with propidium iodide (Flow cytometry)**

HCT-116 cells were grown in culture and plated in 100mm dishes at 5000 cells/dish. At 24 hours, the cells were treated with one of the four different treatment groups. 24 hours later, the cells were harvested and resuspended in 70% ethanol. RNAase and propidium iodide were added according to sample volume the cell solutions were incubated at 37°C for 30 minutes. Samples were then analyzed for cell cycle characteristics using the Becton Dickinson FACSCanto II flow cytometer.

*Computer assisted design and 3D printing*
Using Autodesk 123D Design (Autodesk, Inc) and an Objet350 Connex 3D resin printer (Stratasys Ltd.) and a resolution of 0.01mm with FullCure 720 Model Resin, an infuser device (hereafter termed “Mouse Infuser,” Fig. 1) was designed and printed.

![Mouse Infuser design in AutoDesk 123D Design](image)

**Fig. 1: MouseInfuser design in AutoDesk 123D Design**

*Methylene blue solution flow analysis*

A murine HIPEC apparatus was set up using a peristaltic pump, a warm water bath, vacuum containers for the infusion solution, small caliber tubing, and a heat lamp. Six mice were matched for body weight and underwent 30-60 minutes of HIPEC with either the MouseInfuser or the cannula technique, and infused with methylene blue. After infusion, mice were sacrificed and necropsy was performed. Distribution of HIPEC solution was scored based on a 5-point assessment (Fig.2) of blueness of the internal
abdominal walls and viscera. This was based on the completeness and intensity of blue dyeing throughout the viscera. A secondary endpoint was the number of times the circuit required troubleshooting for flow disturbances.

<table>
<thead>
<tr>
<th>Region</th>
<th>Liver</th>
<th>Small bowel</th>
<th>Large bowel</th>
<th>Abdominal wall</th>
<th>Diaphragm</th>
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<tbody>
<tr>
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<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

Fig. 2: Methylene blue mouse necropsy score

**HIPEC infusion temperature analysis**

During infusion with warmed PBS, temperatures were serially recorded in the warm water bath, within a test tube to which the MouseInfuser was installed, and from within the inflow catheter of the MouseInfuser. The heat lamp distance to test tube was also recorded and adjusted to optimize the apparatus set-up to minimizing the difference in temperature between the warm water bath and the test tube into which the solution was unfused.

**Statistics**

Comparative data were analyzed with a student’s t test, and the threshold was a p value < 0.05.
Results

*Micronutrient combination inhibits tumor cell proliferation on its own, and enhances the effect of Mitomycin C*

MTT assay was performed at 72 hours after treatment of HCT116 cells for 90 minutes at 37°C. Results are quantified as a percentage of proliferating cells compared to the normalized negative control. The proliferation of rapidly dividing HCT-116 cells is reported corresponding to mitochondrial activity. Cells treated with MNC alone were 57.6% of the normalized negative control. MCC treated cells proliferated at 32.7%, and tumor cells treated with MCC + MNC carrier solution proliferated at 27.3%. Effects were enhanced when the cells were treated for the same duration at 41.5°C. Tumor cells treated with MCC in hyperthermic micronutrient combination carrier solution proliferated 6.9% less than their normothermic counterparts.
HCT116 tumor cell membrane integrity is compromised by the addition of micronutrient combination to Mitomycin C

HCT116 tumor cell survival was quantified by the percentage of cells that remained adherent to the culture dishes at 72 hours post-treatment compared to the negative control. Lysed cells detached from the culture dishes and were subsequently not stained by crystal violet. Adherent cells were lysed with acetic acid and optical intensity was quantified by photospectrometry. Results are reported in terms of cell survival. While normothermic MCC and the MNC alone caused a 26.8% and 33.3% reduction in cell survival, MCC delivered to cells in the micronutrient combination solution decreased cell
survival by 53.2%. Treatment in hyperthermic conditions resulted in an average 11.5% decrease in cell survival across all treatment groups.

*HCT116 tumor cell mortality is disproportionally decreased by mitomycin C in the micronutrient combination carrier solution, which suggests synergy.*

Treated and control tumor cells were exposed to trypan blue after being treated with 25% inhibitory concentrations of MCC and MNC, as opposed to the IC_{50} formulations used in
the other experiments. Nonviable cells preferentially take up trypan blue, while the membranes of healthy cells resist the dye. While 95.3% and 99% of cells treated with MCC or MNC alone demonstrated viability, only 85% of cells treated with MCC + MNC demonstrated short term viability. This represents a 9.3% decrease in mortality over the expected value if the effects of MCC and MNC were merely additive, which suggest synergy.
**HCT116 tumor cell clonogenicity is disrupted by MCC and MNC individually, and nonexistent in the MCC + MNC treatment group**

The aggregation of individual cells and the adherence of dividing cells in colonies manifests as solid metastatic lesions in peritoneal carcinomatosis. The population doubling time for HCT-116 colorectal cancer is about 21 hours, and this corresponds to the appearance of grossly visible colonies in cell culture monolayers. By sparsely plating cells and treating them by 18 hours, we examined the effects of our treatments of cells after adherence but prior to significant colony formation. We found that colonization was interrupted completely in the MCC + MNC group, with zero colony formation. Only a few ill-appearing colonies formed in the MCC group, while > 100 formed in the MNC group. The negative control group formed > 500 colonies.

*Mechanistic studies indicate that cleaved caspase-3 mediated apoptosis is upregulated by MCC, and by MNC to a lesser extent.*

Western blotting was performed at 48 and 72 hours after treatment and an antibody for cleaved caspase-3 was used. There was no obvious cleaved caspase-3 expression in the negative control, while at 48 hours both MCC and MNC demonstrated apoptosis activation. At 72 hours, cleaved caspase-3 expression was increased in both in MCC and MNC treated cells. This indicates that both MCC and MNC induce cell death through cleaved caspase-3 mediated apoptotic pathways.
Flow cytometry apoptosis demonstrates increased S-phase cell cycle arrest in the MCC + MNC sample.

Apoptosis in the control groups occurred predominantly in the G1 phase (34.6%) and in G2 phase (22.1%) to a lesser extent. The MCN samples showed a similar distribution, with cell cycle arrest in G1 (31.9%) and G2 (34.1%). The MCC samples primarily arrested in G2 (58.5%), then S-Phase (36.6%), and to a lesser extent in G1 (4.9%). In the combination MCC + MNC samples, most cells underwent apoptosis during S-Phase (72.4%), followed by G2 (23.5%) and G1 (4.1%)

The MouseInfuser HIPEC apparatus demonstrated a superior distribution of blue dye in predictable regions of the abdomen to the cannula technique.

Six mice matched for weight underwent HIPEC with methylene blue. Three mice from the MouseInfuser group (Fig. 3), and three mice from the cannula technique (Fig. 4) were sacrificed and underwent necropsy and scored according to the grading system above (Fig. 1). Mice in the MouseInfuser group averaged a score of 14/18, with the most points earned uniformly throughout the small bowel and completely along the abdominal wall.
Mice in the cannula group averaged a score of 10.6/18, with points earned variably through the abdomen, and the greatest intensity primarily between the inflow and outflow catheters.

Fig. 3: MouseInfuser installed in anesthetized mouse.
Troubleshooting was unnecessary in the MouseInfuser technique.

The MouseInfuser technique required no adjustments during infusion experiments in any of the mice. Catheters in the cannula technique needed to be adjusted 2.6 times on average due to outflow obstruction. Notably, two mice in the cannula technique arrested late in the experiment due to abdominal distention secondary to obstructed outflow, compared to one mouse in the MouseInfuser group, which arrested for unknown reasons.

A constant temperature was achievable using the MouseInfuser, with a minimal difference between the infusion point and warm water bath temperature.

The temperature probe installed directly with the MouseInfuser allowed for a real time assessment of inflow temperatures. With the warm water bath at 42.0°C and the heat
lamp at a distance of 35.5cm from the infusion point, the inflow temperature of the solution was 40.1°C, with a goal temperature between 40.0 - 40.5°C (Fig. 5).

Fig. 5: Temperature assessment
Discussion

Efforts to optimize intraperitoneal chemotherapy by modifying the carrier solution are three-fold. Researchers have used conventional carriers and attempted to demonstrate an advantage of isotonic, hypotonic, hypertonic, or high molecular weight (HMW) isotonic solutions. To date, most institutions use isotonic carriers because no significant advantage of non-isotonic or HMW solutions is apparent, and fluid shifting, as well as post-operative bleeding, is a concern\textsuperscript{23-25}. Investigators have also challenged the use of conventionally formulated chemotherapeutics used in IV administration, citing disadvantages such as rapid clearance from the peritoneal cavity, lymphatic absorption, and a lack of tumor selectivity\textsuperscript{10,26}. Innovations in IP therapy drug carriers have been discussed, and include the development of nanoparticles, microspheres, liposomes, and micelles, among others\textsuperscript{27-30}. The third method of optimizing drug delivery, including the published work cited above, is the addition of compounds to conventional carrier solutions to increase drug efficacy. Here we present the first data showing that the addition of naturally occurring micronutrients to an isotonic carrier solution improves tumor cell killing.

Micronutrient-enhancement of carrier solutions for HIPEC is a compelling method to explore to optimize therapy. The micronutrients we tested have a good safety profile. Although in high doses selenium and quercetin are known to be toxic, the concentrations of these micronutrients for HIPEC carrier solutions is extremely dilute at
20µM and 150µM. In vivo studies are underway to confirm low systemic absorption and local healthy tissue tolerance.

While further mechanistic studies are needed, we hypothesize that the effect of MCN demonstrated here on colorectal cancer cells involves the paradoxical anti-oxidant and reactive oxygen species inducing properties of these compounds. This may further explain why these micronutrients are selectively cytotoxic for tumor cells, and cytoprotective in normal tissue. This study demonstrates that MNC is cytotoxic to colorectal cancer cells on its own, but to a far lesser degree that MCC, as expected. In proliferation studies, tumor cells treated with MNC alone propagated 25% more than cells treated with MCC, which corresponds with diminished cleaved caspase-3 activity in the MCN cell group. The combination MCC + MNC therapy was particularly devastating on the sparsely plated cells in the colonization assay, which demonstrated an absence of colony formation. Cell colonies in peritoneal carcinomatosis come in the form of both already established metastatic lesions and microscopic peritoneal implants. Following adequate cytoreduction, IP chemotherapy then works to eradicate residual tumor cells. This data suggests that MCC + MNC carrier solution may increase residual tumor cell lysis. Flow cytometry unexpectedly demonstrated that cell cycle arrest occurred predominantly in S-phase in the MCC + MNC treatment group. This suggests an alternate pathway to apoptosis is involved, and further investigation into the role of p53 and Chk1 activation by this drug and carrier solution combination is warranted.

The penetration of HIPEC drug into residual lesions remains a challenge, since studies have shown that the maximum depth with conventional drug carriers is 3mm\textsuperscript{31}.  

25
Our data is based on a monolayer of cultured cells, and animal studies are necessary to investigate whether a MNC-enhanced carrier solution can improve tissue penetration and tumor volume reduction.

The MouseInfuser device demonstrated a more predictable and complete distribution of infused intraperitoneal solutions into the abdomen than existing methods, with the advantage of using a temperature-controlled closed circuit. The 1.9°C drop in temperature from the warm water bath to the infusion point makes this apparatus an acceptable means of testing hyperthermic infusion of different pharmacologic agents, since extreme temperatures that adversely affect drugs pharmacokinetics are not necessary. Additionally, temperature is measured from within the MouseInfuser device, instead of within the mouse abdomen, where the fluid temperature is influenced by mouse intraabdominal temperature. This has the advantage of more accurate infusion temperatures, and avoids the need for elevated warm water bath temperatures.

Hyperthermia is well documented to increase the penetration of mitomycin C, and is know to be synergistic with certain chemotherapeutic drugs\textsuperscript{32,33}. Here we present data that suggests a micronutrient-enhanced carrier solution can also have a synergistic effect with hyperthermic chemotherapy. This can lead to the development of optimized carrier solutions for different chemotherapeutics and tumor types that may increase HIPEC efficacy and help eradicate residual tumor cells after cytoreductive surgery. The novel murine model described here will facilitate testing the vitamin C, selenite, and quercetin micronutrient-enhanced carrier solution in-vivo, and promises utility in many other murine HIPEC experiment
References


