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Original Article

Efficacy of Hyperthermic Pressurized Intraperitoneal Aerosol Chemotherapy in an In Vitro Model Using a Human Gastric Cancer AGS Cell Line and an Abdominal Cavity Model

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ABSTRACT

Purpose: Peritoneal carcinomatosis (PC) presents a major challenge in the treatment of late-stage, solid tumors, with traditional therapies limited by poor drug penetration. We evaluated a novel hyperthermic pressurized intraperitoneal aerosol chemotherapy (HPIPAC) system using a human abdominal cavity model for its efficacy against AGS gastric cancer cells.
Materials and Methods: A model simulating the human abdominal cavity and AGS gastric cancer cell line cultured dishes were used to assess the efficacy of the HPIPAC system. Cell viability was measured to evaluate the impact of HPIPAC under 6 different conditions: heat alone, PIPAC with paclitaxel (PTX), PTX alone, normal saline (NS) alone, heat with NS, and HPIPAC with PTX.
Results: Results showed a significant reduction in cell viability with HPIPAC combined with PTX, indicating enhanced cytotoxic effects. Immediately after treatment, the average cell viability was 66.6%, which decreased to 49.2% after 48 hours and to a further 19.6% after 120 hours of incubation, demonstrating the sustained efficacy of the treatment. In contrast, control groups exhibited a recovery in cell viability; heat alone showed cell viability increasing from 90.8% to 94.4%, PIPAC with PTX from 82.7% to 89.7%, PTX only from 73.3% to 74.8%, NS only from 90.9% to 98.3%, and heat with NS from 74.4% to 84.7%.
Conclusions: The HPIPAC system with PTX exhibits a promising approach in the treatment of PC in gastric cancer, significantly reducing cell viability. Despite certain limitations, this study highlights the system's potential to enhance treatment outcomes. Future efforts should focus on refining HPIPAC and validating its effectiveness in clinical settings.

Keywords: Peritoneal carcinomatosis; Chemotherapy; Hyperthermia; Gastric neoplasms; Cell line

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

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Author Contributions
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INTRODUCTION

Metastasis to the peritoneum often occurs ab initio in late-stage or upon recurrence of cancer, appearing in 60%–70% of individuals with malignant solid tumors such as ovarian, gastric, and colorectal cancers [1-3]. Managing peritoneal carcinomatosis (PC) remains a significant hurdle even in modern oncology care. Depending on the type of cancer, many patients display extensive, inoperable conditions with a dismal prognosis, sometimes leading to obstructive symptoms. For gastric cancer accompanied by PC, the Gastric Cancer Guidelines of Korea and Japan recommend palliative systemic chemotherapy and optimal supportive care as the preferred treatment [4,5]. Despite recent advancements in our understanding of disease biology and therapeutic innovations, patients still have a dismal prognosis [6]. The management of PC is challenging due to limited treatment options and poor drug penetration in the peritoneal cavity, necessitating innovative therapeutic strategies. Adding a locoregional treatment approach such as intraperitoneal (IP) chemotherapy can be beneficial. Since its introduction, IP has emerged as an effective approach [7]. Pressurized intraperitoneal aerosol chemotherapy (PIPAC) has emerged as a promising approach to enhance the distribution and penetration of chemotherapeutic agents within the peritoneal cavity [8]. The aerosolized form of chemotherapy is believed to increase the therapeutic index, especially in hard-to-reach tumor nodules. Additionally, hyperthermia has long been explored for its potential in enhancing the effects of chemotherapy by increasing drug penetration and directly inducing cytotoxicity in cancer cells [9]. Before this study, we developed an IP drug delivery system called hyperthermic pressurized intraperitoneal aerosol chemotherapy (HPIPAC), which integrated the advantages of both PIPAC and hyperthermic intraperitoneal chemotherapy (HIPEC) and proved its safety using a large animal model [10]. While small animal PC models are available for experimentation, no large animal models that meet the requirements of our study, specifically the need for an abdominal cavity as large as that of a human to simulate laparoscopic conditions accurately, are available. This highlights a critical need for representative models that can bridge the gap between in vitro cell line studies and in vivo animal or human studies. We developed an innovative silicon-based abdominal cavity model for this study, designed to mimic the human peritoneal environment. Using this model, our objective was to assess the efficacy of heat combined with elevated intra-abdominal pressure and chemotherapeutic agent using our HPIPAC system on the AGS cell line under various conditions.

MATERIALS AND METHODS

HPIPAC system

In our previous study, we demonstrated the safety of the HPIPAC system using a large animal model. **Fig. 1** illustrates the HPIPAC controller unit and **Fig. 2** presents a schematic diagram detailing the operation of the system [10]. Three 12 mm trocars were placed on the abdomen. The afferent CO_2 tube wound with heat generating coil was inserted into a trocar, and the efferent tube was inserted into another trocar. The heat module in the HPIPAC controller circulates hyperthermic CO_2 in a closed-loop circuit and creates a gas-based dry IP hyperthermia. A 12 mm balloon trocar (Kii Balloon Blunt-Tip Access System, Rancho Santa Margarita, CA, USA) was placed at the 6'o clock hole, and 2 additional 12 mm balloon trocars were placed through the 3'o clock and 9'o clock holes on the silicone roof of the model. The temperature probe was inserted through a separate small hole on the silicone roof just above the liver. The nozzle was inserted through the 6'o clock trocar, an afferent CO_2 tube wound with a heat-generating coil was inserted into the 9'o clock trocar, and the efferent tube was

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Fig. 1. Hyperthermic pressurized intraperitoneal aerosol chemotherapy system controller [10].

inserted into the 3'o clock trocar. Heated CO_2 was insufflated and circulated in a closed circuit until the intra-abdominal temperature reached 42°C. A newly developed 10-mm dual-flow nozzle, which uses IP CO_2 gas to generate and disperse the drug aerosol, was used for drug nebulization.

Abdominal cavity model construction

The model depicted in **Fig. 3A** was specifically designed and custom-built to simulate the human abdominal cavity both in structure and material composition, offering a close representation for experimentation (Medical IP, Seoul, Korea). The overall size of the abdominal cavity model was 375×280×300 mm. It was constructed using acrylic plates, ensuring a sturdy base. The inner surface of the model was lined with a skin-colored silicone layer, which provided a realistic tactile feel mimicking the inner surface of the human

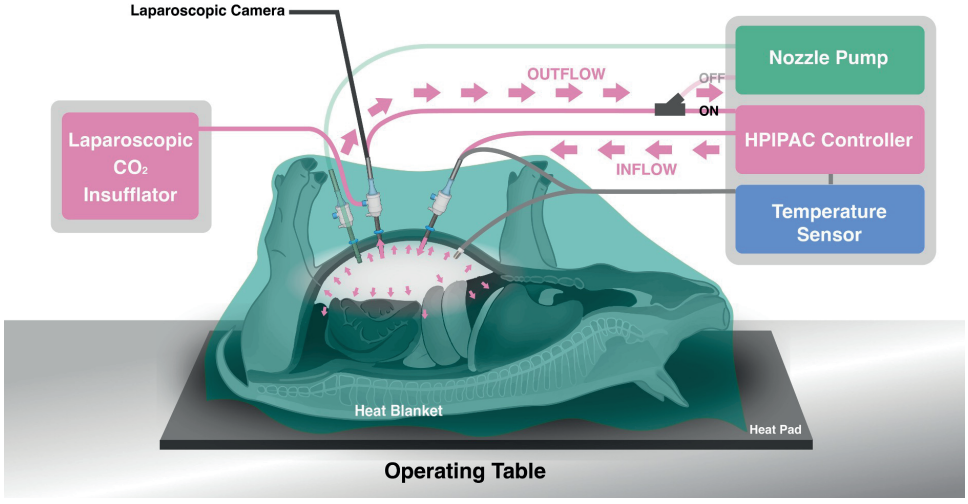

Fig. 2. Mechanism of the hyperthermic pressurized intraperitoneal aerosol chemotherapy system [10].

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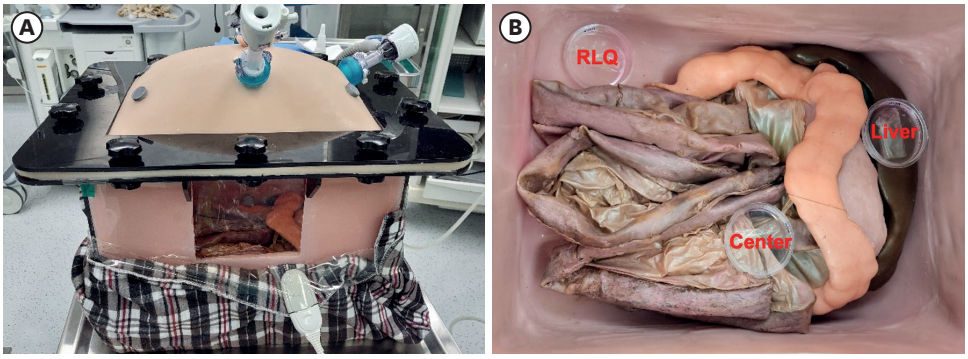

Fig. 3. (A) Abdominal cavity model, (B) placement of AGS cell line culture dishes.
RLQ = right lower quadrant.

abdominal cavity. The roof of the model was crafted to resemble the abdominal wall. It comprised a central portion made of skin-colored silicone. This central silicone segment was surrounded by acrylic plates, ensuring structural integrity. The roof was fastened securely to the body of the model using screws, guaranteeing an airtight seal essential for conducting experiments under laparoscopic conditions. This airtight environment was crucial as our experiment required such conditions to prevent aerosolized paclitaxel (PTX) from escaping the model, and for ensuring the safety of the operator. This necessity distinguishes our model from other enclosed space models, which do not offer the required safety and functionality for our experiment. Nine strategically positioned holes were crafted into the model to facilitate the insertion of trocars. These holes were sealed using plastic buttons to prevent potential air leakage when the trocars were not inserted. The model featured a comprehensive representation of intra-abdominal organs, except for retroperitoneal structures. Each organ was meticulously crafted to mirror its human counterpart in both shape and texture. The liver and colon were constructed using plastic, whereas the stomach and peritoneum were made with silicone. Small bowel and its mesentery were crafted from latex, providing a flexible and life-like feel. The lining of the retroperitoneal organs was also constructed using silicone. During the experiment, the lower part of the model was surrounded by a heating blanket to mimic the body temperature of the human abdominal cavity.

Cell culture and cell viability assay

For the experiment, we used human gastric cancer AGS cells (gastric adenocarcinoma, KCLB 21739; Korean Cell Line Bank, Seoul, Korea). There were other cell-lines that were available such as ascites derived SNU5, SNU16, YCC2, and NCC20, which are suspension cells, meaning they float and grow within the medium. Considering our future research, we used attached cells like the AGS cell-line to assess the effect of drug penetration into the peritoneum. The AGS cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin–streptomycin (Gibco; Thermo Fisher Scientific, Waltham, MA, USA). Cells were maintained in a humidified incubator with 5% CO_2 at 37°C. 5×10^5 cells were plated in a 60 mm cell culture dish (TPP, Trasadingen, Switzerland) and incubated for 1 day. The cell dishes were then transferred into the in vitro abdominal cavity model (**Fig. 3B**). Control cells for all experiments were cultured without treatment in a humidified incubator with 5% CO_2 at 37°C.

Cell viability was measured by a water soluble tetrazolium salt (WST) cytotoxicity assay with the EZ-Cytox solution (Dogen; Daeillab, Seoul, Korea).The remaining medium volume in the 60 mm dish was measured after the experiment and then the EZ-Cytox solution was added to each culture dish according to the manufacturer's protocol. The protocol specifies that the

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solution must react in the medium for 2 hours, with a maximum allowable time of 4 hours, before measuring cell viability. This necessitated a washing process following the viability measurement. Afterwards, cells were incubated in a humidified incubator with 5% CO_2 at 37°C for 2 hours. Next, the optical density was measured at 450 nm in a multi-detection microplate reader (Molecular Devices, San Jose, CA, USA). Cell viabilities were estimated as relative values compared to the untreated controls. Upon completing the cell viability assessment, the cells underwent 2 washes with PBS, followed by the addition of 3 mL of culture medium, before continuing with the culture. Furthermore, we evaluated cell viability at 48 and/or 120 hours of culture.

In vitro experiments

The culture dishes were placed at 3 different depth levels (**Fig. 3B**). The lowest part in the cavity model was at the right lower quadrant (RLQ) space where the retroperitoneal lining was exposed. The middle depth level was on the small bowel mesentery (center) and the highest level in the cavity model was above the liver.

To assess the efficacy of the HPIPAC system in this experiment, a chemotherapeutic agent, PTX (Samyang Biopharm, Seongnam, Korea), was used. Other chemotherapeutic agents used for IP include oxaliplatin. Oxaliplatin, in comparison to PTX, demonstrates a synergistic effect with hyperthermia by enhancing tissue penetration through increased interstitial permeability. However, regarding gastric cancer, no studies involve IP administration of oxaliplatin alone. Therefore, PTX was the preferred choice for our experiment, as it already possesses preclinical data regarding the initial dosage [11]. Normal saline (NS) served as a control in comparison to PTX. The experiment was structured around 6 distinct conditions, with the first 5 acting as control groups in relation to the 6th, the experimental group, HPIPAC with PTX treatment. Each condition was repeated at least thrice. The six experimental conditions and procedures were as follows:

1. HPIPAC on its own, without either NS or PTX (heat only). Hyperthermia was induced at 42°C in a pressurized environment and the temperature maintained for 1 hour without the administration of NS or PTX, thereby mimicking the conditions of gas-based HIPEC.
2. PIPAC combined with 50 mL of 100 μM PTX (conventional PIPAC procedure). PTX was nebulized and then kept for 30 minutes in a pressurized environment to simulate the standard PIPAC procedure.
3. Sole aerosolization of 50 mL of NS, without heat (NS aerosolization only). NS was nebulized without the subsequent 30-minute wait to establish a basic sham control group.
4. Aerosolization of 50 mL of 100 μM PTX without heat (PTX aerosolization only). PTX was nebulized without the subsequent 30-minute wait to evaluate the time effect in PIPAC.
5. HPIPAC system with 50 mL of NS (heat with NS). Hyperthermia at 42°C was initially induced. Subsequently, NS was nebulized after which hyperthermia was continued for an additional hour to investigate the effect of PTX in an experimental condition.
6. HPIPAC combined with 50 mL of 100 μM PTX (HPIPAC with PTX). Hyperthermia was first induced at 42°C. Following that, PTX was nebulized and hyperthermia was then resumed for an additional hour. This protocol simulated the actual HPIPAC procedure.

Cell viability was assessed immediately after each experiment (0 hours) and after 48 hours of incubation (48 hours) for the control groups (conditions 1–5). For the group treated with HPIPAC using PTX, an additional incubation period of 120 hours was conducted.

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Regarding the dose of PTX, the advised phase I dose for PTX in PIPAC was established at 15 mg/m^2 in an earlier large-animal study conducted by Tan et al. [12]. In this study, the body surface area of a 58 kg pig was 1.05 m^2. The PTX dose translated to 15.75 mg, which became 122.99 μM when diluted in 150 mL of NS, the typical amount used in PIPAC aerosolization. Based on these findings, we adjusted the PTX dose for our study to 100 μM.

RESULTS

Control group

The specific cell viability assay results for all the experiments, described in **Table 1** and **Fig. 4**, show an accurate representation of the change in average cell viability in the 6 conditions. The average cell viability changes of each condition, from immediately after the experiment to after 48 hours of incubation, regardless of the location is as follows; heat only 90.8% → 94.4%, PIPAC with PTX 82.7% → 89.7%, PTX only 73.3% → 74.8%, NS only 90.9% → 98.3%, heat with NS 74.4% → 84.7%. All the cell viability assays from the control group showed the recovery of AGS cells after 48 hours of incubation.

HPIPAC with PTX

In **Table 1**, the detailed changes in cell viability for the HPIPAC with PTX condition are documented, spanning from immediately after the experiment to 48 and 120 hours of incubation. **Fig. 5** presents these changes graphically, illustrating a clear decline in cell viability from an initial average of 66.6% immediately after the experiment, to 49.2% at 48 hours, and further down to 19.6% after 120 hours of incubation. Microscopic images in **Fig. 6** also depict this trend, showing a persistent and even amplified decrease in cell viability across all culture dishes throughout the extended 120-hour incubation period.

DISCUSSION

We have introduced and proven the feasibility and safety of our HPIPAC system in another study involving a large animal model. In the experiment, 3 pigs were able to tolerate the survival model after circulation of hyperthermic CO_2 (40°C–42°C) [10]. The absence of an animal model for PC in gastric cancer necessitated the development of a 3-dimensional (3D) printed silicon-based abdominal cavity model that replicated the human peritoneal environment to evaluate IP therapies. We developed this peritoneal cavity model to conduct in vitro cytotoxicity tests using the AGS cell line.

Table 1. Cell viability assay results showing percentages of viable AGS cell-line in each dish

Experiment number	Location of dish	Heat only (%)		PIPAC + PTX (%)		PTX only (%)		NS only (%)		Heat + NS (%)		HPIPAC + PTX (%)		
		0 hours	48 hours	0 hours	48 hours	0 hours	48 hours	0 hours	48 hours	0 hours	48 hours	0 hours	48 hours	120 hours
Experiment 1	Center	100	95	83	93	77	81	100	100	69	92	67	51	23
	Liver	93	93	82	92	65	72	96	100	85	92	65	42	15
	RLQ	83	90	81	82	67	65	90	100	79	88	67	45	23
Experiment 2	Center	93	100	85	91	79	79	92	100	76	82	69	59	21
	Liver	91	95	84	92	75	71	95	95	65	86	65	41	20
	RLQ	83	98	78	89	69	69	91	98	75	83	69	49	15
Experiment 3	Center	98	98	88	92	81	83	100	100	83	91	71	55	18
	Liver	95	100	83	90	76	78	73	100	66	76	57	53	18
	RLQ	81	81	80	86	71	75	81	92	72	72	69	47	23

PIPAC = pressurized intraperitoneal aerosol chemotherapy; PTX = paclitaxel; NS = normal saline; HPIPAC = hyperthermic pressurized intraperitoneal aerosol chemotherapy; RLQ = right lower quadrant.

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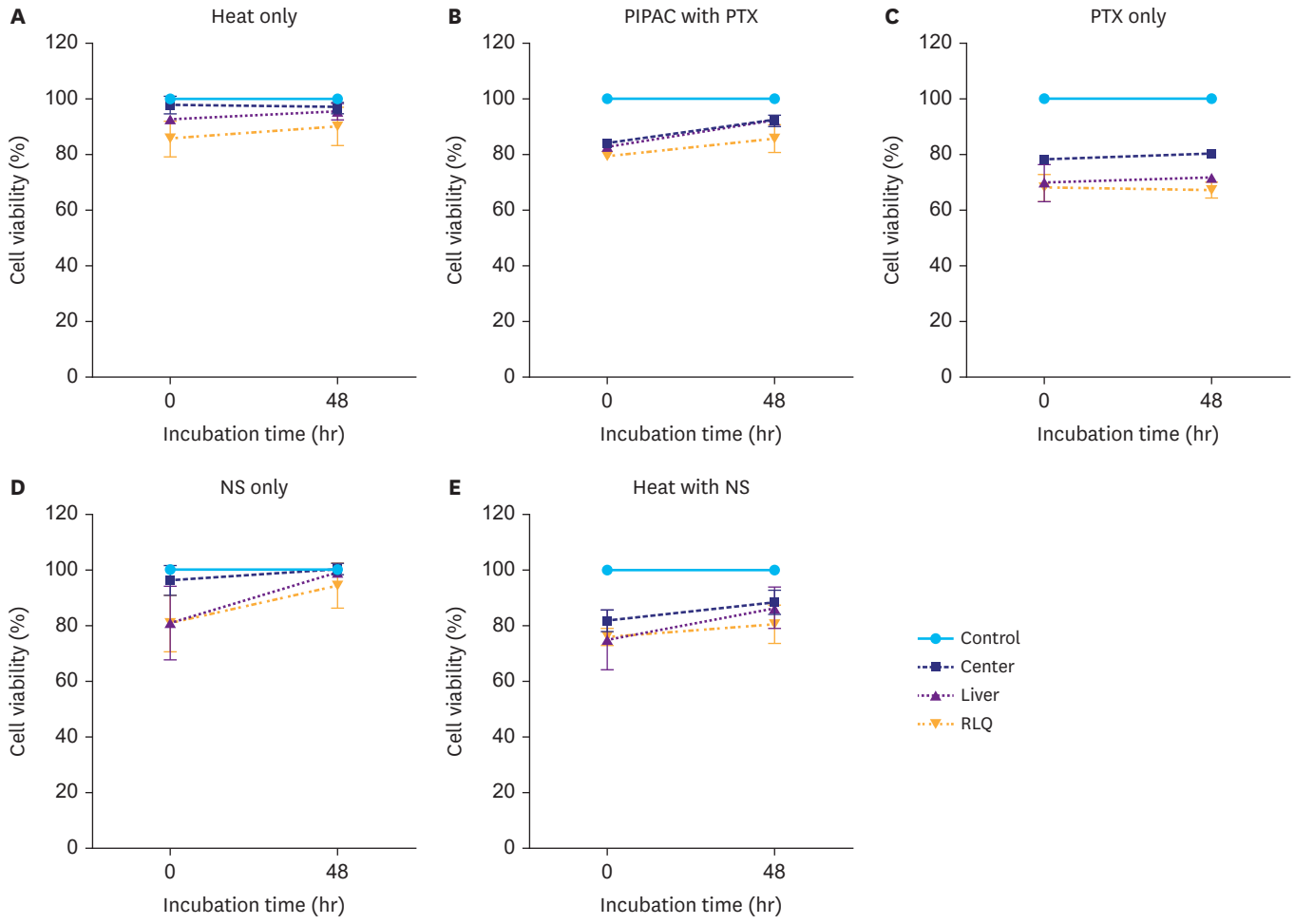

Fig. 4. Cytotoxic effect on the control group. (A) Heat only, (B) PIPAC with PTX, (C) PTX aerosolization only, (D) NS aerosolization only, and (E) Heat with NS. Cell viability (%) measured immediately after the experiment and 48 hours after incubation.
PIPAC = pressurized intraperitoneal aerosol chemotherapy; PTX = paclitaxel; NS = normal saline; RLQ = right lower quadrant.

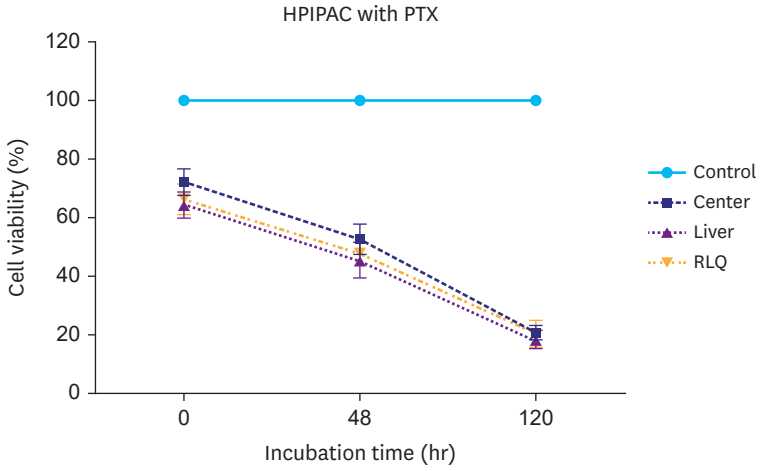

Fig. 5. Cytotoxic effect on HPIPAC with PTX. Cell viability (%) measured immediately after the experiment and 48 hours after incubation with an additional 120 hours of incubation.
HPIPAC = hyperthermic pressurized intraperitoneal aerosol chemotherapy; PTX = paclitaxel; RLQ = right lower quadrant.

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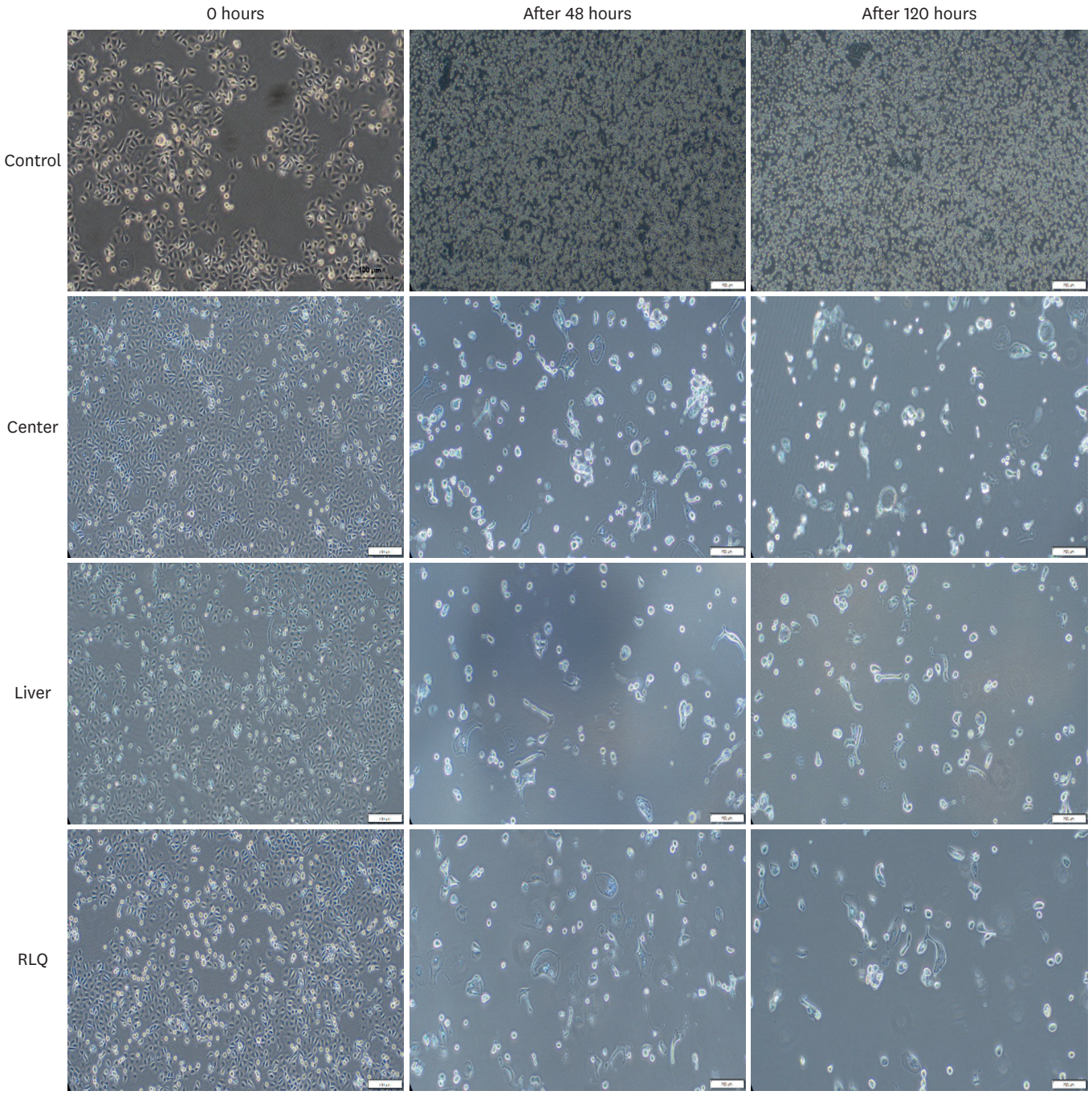

Fig. 6. Representative microscopic image of the changes in cell viability of the AGS cell line at each location, from immediately after the experiment to 48- and 120-hour postincubation, in HPIPAC with PTX experiment.
HPIPAC = hyperthermic pressurized intraperitoneal aerosol chemotherapy; PTX = paclitaxel; RLQ = right lower quadrant.

While treatments with heat only, PIPAC with PTX, PTX only, NS only, and heat with NS demonstrated regrowth of AGS cells following incubation, the combined application of PTX with our HPIPAC system resulted in a significant reduction in cell viability and an increase in cell death over time. The unexpected dip in cell viability observed in the NS only experiment could be attributed to alterations in the culture medium properties caused by aerosolized NS, which, in turn, impacts the cells. Additionally, optimal cell growth conditions typically

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involve humidification with 5% CO_2 at 37°C in the incubator; however, aerosolization with NS alone, even without heat, can lead to reduced cell viability due to the increased pressure in the model from 100% CO_2, a situation that applies to all experiments conducted. The observed increase in cell viability after 48 hours in the PTX containing PIPAC with PTX and PTX only groups can be attributed to the specific method used, and qualities of the EZ-Cytox solution used for measuring cell viability. The solution's protocol necessitates a washing step after the 2-hour evaluation period due to its stipulation that the maximum reaction time is 4 hours. Following PTX aerosolization and the immediate addition of the EZ-Cytox solution for viability assessment, any residual solution and PTX are removed from the cells, which are then incubated for 48 hours without the presence of PTX. This procedural detail means that during the 48-hour incubation, the cells are not under the influence of PTX, allowing cells unaffected by the initial exposure to proliferate. This washing step, mandated by the solution's limitations, effectively creates a PTX-free environment, facilitating cell recovery and proliferation. Furthermore, due to the solution's effects, damaged cells retaining NADH-dehydrogenase activity could still appear alive when assessed immediately after the experiment. This could explain the minimal (less than 5%) decrease in cell viability seen after 48 hours of incubation in certain cases: specifically, the center dish of "Exp 1" with Heat only, and the RLQ and Liver dishes of "Exp 1" and "Exp 2" treated with PTX.

HIPEC has previously been established as an effective approach in augmenting the penetration of chemotherapy drugs, with hyperthermia itself exerting direct cytotoxic effects on malignant cells [9]. Few studies have highlighted the role of hyperthermia in initiating apoptosis, thereby impeding tumor growth [13,14]. A notable investigation from China detailed the molecular processes through which hyperthermia induces apoptosis in AGS cells, unequivocally illustrating hyperthermia's critical contribution to anticancer effects on AGS cells in a temperature- and time-dependent manner [15]. Gill et al. [16] suggested that hyperthermia not only prompts apoptosis but also leads to protein denaturation, hinders deoxyribonucleic acid repair, and facilitates increased drug concentration within tumor nodules. When cancer cells are in contact with PTX, cell cycle arrest at the G2/M phase is induced, cell division is disrupted, and cell death results [17]. PTX, hyperthermia and increased pressure inside the model affects both the cells and the culture medium during the experiment. The HPIPAC with PTX experiment, displaying a sustained reduction in cell viability across 120 hours, illustrated the synergistic impact on cell damage characterized by extended apoptosis and the hyperthermia-induced enhancement of drug accumulation, coupled with the inhibition of cell proliferation by PTX. Our results showed similar results as other studies. Kokura et al. [14] and Zhou et al. [15] presented significant findings in the arena of hyperthermia and its effects on gastric cancer cells. Kokura et al. [14] established that solitary hyperthermia exhibited substantial cytotoxicity at 43°C on MNK45 gastric cancer cells. Building on this, Zhou et al. [15] articulated persuasive evidence pointing to the conspicuous suppression of tumors in AGS gastric cancer cells under the influence of hyperthermic CO_2 (42°C–44°C) coupled with a pneumoperitoneum (15 mmHg) sustained over a span of 2–4 hours. They asserted that this strategy yielded a cytotoxicity that was markedly superior to what hyperthermia alone could achieve [15].

Several studies on colorectal cancer in vitro experiments with human colorectal cancer cell-lines are available. The HCT8 cell line, a popular choice in preclinical research, has been extensively characterized, primarily due to its resistance and propensity to exhibit high metastatic rates in animal models, thereby offering a simulation that closely mirrors the clinical scenarios observed in patients with PC [18-20]. However, limited data on in vitro

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experiments with the AGS cell line specific to this type of abdominal cavity model and no large animal gastric cancer PC models are available. The abdominal model crafted in this study did not only to mimic the human abdominal cavity but also minimized the potential errors inherent in conducting experiments of this nature using a model. We strategically placed cell dishes at three locations (center, liver, RLQ) within the cavity, each at a different depth (approximately 5 cm difference), to represent the irregular terrain of the abdominal cavity, hence aiming for more precise results. As shown in **Table 1**, the cell viability values for the center, liver, and RLQ differ across each experiment. While these values may not be identical in every instance, the placement of the nozzle could have been slightly different in each experiment, and also may have resulted from differences in circulating CO_2 turbulence, which varies with each experiment. Our previous study on the development of the HPIPAC system established that the maximum intra-abdominal temperature was set to range from 40°C to 42°C [10]. Changes in air temperature may lead to alterations in turbulence, potentially causing the observed differences in aerosol distribution between temperatures of 40°C and 42°C.

Khosrawipour et al. [21] conducted an in vitro PIPAC experiment utilizing the HCT8 cell line. In this study, cells were cultivated on a permeable membrane in contact with an underlying medium, directly exposed to atmospheric conditions [21]. In contrast to this, we employed AGS cell line cultures submerged within the medium, a methodology that might be regarded as inaccurate based on previous research, given that the cells are not directly subjected to the influences of hyperthermia and pressure [21]. We factored in the biological context in which cancer cells are not situated on the surface but are shielded by a semi-permeable barrier known as the peritoneum. Taking this into consideration, we chose to maintain the medium within the cultivated cell environment.

Our study is subject to several limitations. Although we endeavored to replicate the abdominal cavity, our simulation does not perfectly mirror a live abdominal environment. Given the absence of a suitable large animal model of PC in gastric cancer, constructing an artificial abdominal cavity was deemed our best option. Furthermore, the placement of culture dishes within this cavity does not fully emulate the conditions of actual peritoneal seeding nodules, despite the use of the AGS cell line. The lack of an animal model for PC with an abdominal cavity comparable in size to humans necessitated our reliance on placing living AGS cell cultures in dishes within the model. Efforts were made to strategically position these dishes to approximate the entirety of the abdominal cavity. Lastly, the HPIPAC system developed in our prior research required further enhancements [10]. The system's ability to raise and sustain temperature, along with the circulation of heated CO_2 and aerosolization of medication, demanded more sophisticated refinements to minimize error, as cell viability is sensitive to even minor temperature fluctuations.

Our study underscores the potential of the HPIPAC system and a 3D printed silicon-based model to simulate the human peritoneal environment for evaluating IP therapies in gastric cancer treatment. Despite demonstrating the feasibility and safety of hyperthermic CO_2 circulation and its synergistic effect with chemotherapeutic agents, particularly PTX, we acknowledge limitations such as the inability to perfectly replicate the live abdominal cavity and the absence of a large animal model for PC in gastric cancer. These findings highlight the necessity for further refinement of the HPIPAC system to enhance its precision and effectiveness. Future research should focus on overcoming these challenges to better mimic human conditions, thereby improving the therapeutic outcomes for patients with PC.

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