Ph.D. Dissertation of JINLAN PIAO

The Effect and Limitation of Drug Diffusion by a Novel Pressurized Intraperitoneal Aerosol Chemotherapy in an Ex Vivo Model Mimicking the Human Abdominal Cavity

새로운 가압 복강 내 에어로졸 화학 요법에 의한 인간 복강을 모방 한 **Ex Vivo** 모델에서의 약물 확산의 효과와 한계

August 2021

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Seoul National University
Obstetrics and Gynecology

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Submitting a Ph.D. Dissertation of Public Administration

August 2021

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Confirming the Ph.D. Dissertation written by
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August 2021

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Abstract

Background: Pressurized intra-peritoneal aerosol chemotherapy (PIPAC) has been introduced as a novel technique of intraperitoneal chemotherapy for the treatment of peritoneal metastasis (PM) caused by advanced or recurrent solid tumors. PIPAC has been implemented in clinical medicine mainly in Europe. But the PIPAC machine has not been imported to South Korea and is not affordable in market. So, before conducting this part of ex vivo experiment, we collaborated with medical biomechanics and design laboratory in Seoul National University redesigned and reconstructed the well-established prototype and this project implemented to investigate the pattern of tissue penetration according to different nozzle position and to find the best position resulting in best drug delivery.

Material and Methods: Fresh postmortem peritoneum tissues were cut into 8 identical pieces and fixed at different spatial places A-H considering the asymmetrical abdominal cavity in 2cm-,4cm- and 8cm- ex vivo models. Ex vivo experiment performed using a novel prototype, that sprayed about 30-μm droplets at flow rate of 30ml/min under the pressure of 7 bars. Methylene blue staining aera on six faces in the three ex vivo models were observed with naked eyes. The penetration depth was evaluated with the depth of concentrated diffusion (DCD) and the depth of maximal diffusion (DMD) of doxorubicin using confocal laser scanning microscopy after the application of 4',6-diamidino-2-phenylindole to the tissue specimens. The counted highest number of DCD and DMD was performed to compare the score of three ex vivo models.

Results: In terms of the distribution, the 4cm- and 8cm-ex vivo models showed more stained faces than the 2cm-ex vivo model, the closer nozzle to the bottom of the model, the more unevenly distributed methylene blue was observed. With different nozzle positions, the best tissue penetration found at different tissue sample and the pattern of doxorubicin penetration in the 8 sample tissues change accordingly. After we counted the number of the highest DCD and DMD values and found that 4cm-ex vivo model showed the highest score of 5 (62.5%).

Conclusion: Drug delivery into the peritoneum tissue during PIPAC differs according to different nozzle position and the optional nozzle position for best drug

delivery should be determined in consideration of the three-dimensional structure of the abdominal cavity. Further in vivo study is needed to determine optional nozzle position and rotated nozzle can be expected to improve diffusion and penetration of aerosol during PIPAC.

Keywords: Pressurized intraperitoneal chemotherapy, Peritoneal metastasis, Ex vivo, Nozzle

Student Number: 2017-39829

Chapter 1. Ex vivo Experiment of Pressurized Intraperitoneal Aerosol Chemotherapy: A Review

1.1 Introduction

1.1.1 Ovarian cancer

Ovarian cancer is well known as rank fifth in cancer deaths among women, rank first for deaths among female patients with reproductive system [1]. According to Korea Central Cancer Registry, there are about 2702 new cases of ovarian cancer patients, death cases of 1149 and prevalence cases of 21,013 in South Korea 2017 [2]. It is estimated about 6.8 age adjusted crude incidence rate per 100,000 and 10.5 crude incidence rate per 100,000 in South Korea in 2017 [2]. Moreover, there are about 2.25 million new cases of ovarian cancer patients diagnosed worldwide and about 1.54 million cases of death every year [3]. Due to subtle or absent symptoms, shortage of screening methods and emergence of chemoresistance and lack of effect treatment ovarian cancer patients diagnosed at advanced stages. So, development of effective treatment methods of ovarian cancer patients especially those with advanced stage have been a very headache problem to gynecological doctors.

1.1.2 Peritoneal metastasis

Peritoneum is a large serous organ with both histologically epithelial and mesenchymal features. As peritoneum is a dynamic organ has important role in keeping movements of intraabdominal organs and maintaining a balance of organs within the abdominal cavity [4]. The peritoneum not only effects in inflammation causes fibrotic adhesion formation after surgery or infection but also highly effects in tumor metastases of many types of malignancies [5]. Peritoneal metastasis (PM) is a phenomenon of cancer cells' deposition within the parietal and visceral

peritoneum. PM often is the last condition that causing difficulty in treatment because of the numerous tumor depositions throughout the abdominal cavity [4, 6]. According to embryology, the embryonic germ cells and the celomic epithelial cells of the peritoneum and the germinal epithelial cells of the ovaries may transform into malignant cells, finally develop into PM [7, 8]. Mostly PM results from intracavitary dissemination of tumor cells that may arise from a variety of different primary sites such as gastrointestinal, urinary system, gynecologic or mesothelioma and pseudomyxoma et al [9]. Researchers consider PM not only as a part of the process of cancer but also considered as a 'peritoneum' disease and the therapeutic method of PM has undergone a lot of changes [9].

Due to the large surface area and complex three-dimensional structure of the peritoneal cavity as well as poor vascularization of the peritoneum, PM with a lot of tumor deposits throughout the abdominal cavity very difficult to treat [10]. So, researchers suggest that PM should be considered clinically the same as gynecologic cancers such as ovarian and fallopian tube cancers. The median survival of women who diagnosed with recurrent ovarian cancer with PM is especially poor ranging between 4 and 15 months [9]. The poor prognosis and progression of gynecological malignancies is often associated with dissemination and growth of tumor cells in the peritoneal cavity. Patients with ovarian cancer have been shown to eventually die from complications of locoregional tumoral widespread and development of this condition finally into PM [9].

1.2 Diagnosis of Peritoneal Metastasis

Preoperative PM diagnosis is very difficult. Techniques like computed tomography-scan (CT) and magnetic resonance imaging (MRI) are used in diagnosing and implemented before planning cytoreduction surgery and preventing needless laparotomy in patients with unresectable disease. However, the imaging techniques have low sensitivity for small volume disease especially early stage of PM. The gold standard of PM is the direct peritoneal visualization with laparotomy or laparoscopy [9].

1.3 Treatment of Peritoneal Metastasis

1.3.1 Intravenous chemotherapy (IV)

The initial standard treatment for ovarian cancer patients is intravenous chemotherapy (IV) combined with cytoreductive surgery [11]. It is reported about 80% of the ovarian cancer patients respond to IV chemotherapy, but more than 70% found recur within 5 years after treatment and develop drug resistance [12]. Safety checklist is very important in IV chemotherapy of ovarian cancer patients with PM, such as renal and liver functioning test and patients should keep hospital stay for several days and renal and liver complications should be recorded for 30 days [13]. We can infer that the toxic side effects caused by high concentration within circulatory system is very severe in IV chemotherapy. In contrast to high concentration within circulatory system, the chemotherapy agent concentration within the tumor tissue is relatively low [14].

1.3.2 Intraperitoneal chemotherapy (IP)

Since peritoneum is the primary site of metastatic disease in ovarian cancer, the most common way of metastasis is intraperitoneal spread, a lot of clinical trials have targeted intraperitoneal metastatic cancer cells in ovarian cancer with advanced stage to find effective treatment methods. Such as targeted immunotherapy insufflating intraperitoneal antibodies directly into peritoneum cavity [9]. To further exert therapeutic effect, the anticancer agents must effectively penetrate target tissue and should reach all cancer cells as much as possible [15]. Intraperitoneal chemotherapy (IP) for treating solid tumor with peritoneal metastasis developed in 1950s using nitrogen mustard intraperitoneally for treating malignant ascites [16]. The concept IP chemotherapy was developed in 1970s that high volume of chemotherapeutic drugs demonstrated directly into the peritoneal cavity to make a ‘abdominal bath’ filled with chemotherapy agents [17].
IP chemotherapy eradicate residual peritoneal disease by increasing drug concentration within the peritoneal cavity and reduce chemotherapy drug flow

directly into circulatory system, inducing high concentration of drugs within peritoneal cavity and reduce systemic cytotoxic effects of standard intravenous chemotherapy. It is also known that the peritoneal-plasma barrier limits the reabsorption of drugs from the peritoneal cavity into the circulation. Infusing chemotherapeutic drugs directly into the abdominal cavity improves the local toxicity and reduces the systemic toxicity obviously [18, 19]. It is reported that chemotherapeutic agents directly contact with the intraperitoneal cancer cells and the metastatic nodules, inducing higher bioactivity with longer half-life in the peritoneal tumor tissues than does the agents within systemic chemotherapy, demonstrating an superior effect of intraperitoneal chemotherapy [19]. According to recent large clinical trials by Gynecologic Oncology Group study, GOG 172, the phase II/III large randomized clinical trials, the median overall survival rate was improved by about 17 months in patients who received IPC after IV chemotherapy than patients treated with IV alone [20]. Armstrong et al also did a clinical trial with ovarian cancer patients of stage III, after randomly treated patients with IV chemotherapy and IP chemotherapy the median duration of overall survival and progression free survival in IP chemotherapy group (65.6 months) better than IV chemotherapy group (49.7 months), but the quality of life of patients treated with IP chemotherapy was worse than IV chemotherapy group patients [11], suggesting higher toxicity in patients who treated with IP than IV chemotherapy. This may be caused by high dose of chemotherapy agents within peritoneal cavity, the capillary uptake of chemotherapy agents from peritoneal surfaces to capillary is incomplete and longer but lower systemic exposure than that of IV chemotherapy.

To exert best penetrating of chemotherapy drugs into the target tissue, target tissue should be reached by chemotherapy agents as much as possible with possible drug concentration and exposure period of time. The poor vascularization of peritoneum tissue and increased interstitial fluid pressure during PM causing therapeutic limitations of IP [21]. Poor vascular distribution in the peritoneum tissue causing decreased uptake of chemotherapy agents into the target within peritoneum, the vascular distribution also different according to peritoneum geometry, some necrotic zones in peritoneum leading to poor blood supply and poor systemic uptake of chemotherapy agents [22]. Moreover, study found interstitial fluid pressure increased in solid tumors than normal tissue, the altered interstitial fluid

pressure of tumor tissue also causing poor uptake of chemotherapy agents into the tumor tissue [23].

So, physical interventions are needed to increase drug uptake such as increasing intraperitoneal pressure, converting chemotherapy agents into ‘gas-like’ aerosols and generating hyperthermia et al [21].

1.3.3 Hyperthermic intraperitoneal chemotherapy

Hyperthermic Intraperitoneal Chemotherapy (HIPEC) was conducted with hyperthermic condition during surgery with opened abdomen, the purpose of the HIPEC is to eliminate potential micro-metastasis nodules that cytoreductive surgery alone cannot remove by impairing DNA repair with hyperthermia [24-26]. HIPEC directly induce liquid with chemotherapeutic drugs into the peritoneal cavity intraoperatively, the perfusing period of HIPEC took about 90 minutes. It took about 120 minutes total to perform one HIPEC procedure. From the moment reached the temperature (42℃-44℃), the duration of the chemotherapeutic drugs perfusion into the abdomen cavity was exactly 30 minutes. Since the high cost of HIPEC functioning in the operating room and the tumor oxygenation increases mainly during the first 30 minutes, the timer HIPEC procedure conduct mainly fixed at 30 minutes [27].

A large-scale clinical trial has been implemented by van Driel et al found patients conducted with HIPEC in addition with interval cytoreductive surgery could improve outcomes of stage III ovarian cancer patients than the patients treated with CRS and systemic chemotherapy [25]. After follow-up about 4.7 years, the median overall survival of surgery group was 33.9 months and the median overall survival of CRS with HIPEC was 45.7 months with similar rate of adverse events happened in both groups [25]. As we can see, HIPEC combined with adequate CRS can significantly improve prognosis of patients with PM.

But the hyperthermia and direct infusion of chemotherapy agents into the abdominal cavity can lead to acute systemic side effects [28]. The side effects also caused by large operative wound during operation, catheter-related complications

and so on. HIPEC is also known as become effective after complete tumor resection [25].

1.3.4 Pressurized intraperitoneal aerosol chemotherapy

Recently, pressurized intraperitoneal aerosol chemotherapy (PIPAC) as a palliative therapy has been introduced to treat patients with PM mainly in Europe that overcomes defects of IPC and HIPEC with aerosolized liquid solutions and high pressure within the peritoneal cavity of patients [29]. PIPAC was developed under the theory of increased pressure and aerosolized chemotherapy agents increase drug delivery.

According to pharmacokinetics, the boosted pressure within the peritoneal cavity increases the uptake of drug agents into the tumor tissue [30]. Esquis et al found that increased peritoneal pressure strengthen the penetration of cisplatin in peritoneal tumor tissue of rat model after treated with higher intraperitoneal pressure [31]. Fancy et al also implemented in vivo experiment with two groups of swine models each treated with HIPEC and HIPEC with high pressure (18mHg), found swine model treated with high pressure resulted in enhanced diffusion of chemotherapy agent both in visceral and parietal peritoneum [32]. Similar results also found at clinical trials by Kusamura et al, patients who treated with HIPEC with high abdominal pressure found increased drug concentration in the peritoneum tissue than patients with HIPEC without high abdominal pressure [33] . Improved drug delivery into the peritoneal cavity also could be reached by aerosolized chemotherapy agents using an aerosol-creating device that produces highly concentrated drug particles in a form of “therapeutic chemo-aerosol” within the peritoneal cavity[21]. More homogeneous and intense delivery methylene blue was observed after PIPAC in a large animal model than animal model treated with conventional lavage [34]. According to pharmacological study PIPAC found better tissue concentration per chemotherapy drug dose apply than systemic chemotherapy and IP chemotherapy [35]. From the published data we can infer that PIPAC have far better homogeneity and penetration of chemotherapy agents than IP chemotherapy.

According to the published data, PIPAC may also increase quality of life by postpone the end-stage disease scenarios [29]. PIPAC also can be used repeatedly and allow repeated peritoneal tissue sampling to further assessment. As we can see, PIPAC not only easier to repeat because of its minimally invasive operation, but also have better pharmacokinetic advantages with higher tumor tissue concentrations and less systemic toxicity [36]. But both in vivo and ex vivo experiment data about tissue penetration found PIPAC induce specific high concentration at place such as tissue located below the nozzle, and in ex vivo experiment found even changes the nozzle position still found highest concentration at the point below the nozzle [37, 38], drug gradient caused from top to the bottom, from center to the periphery. The homogeneous distribution and penetration of chemotherapy agents within the peritoneal cavity is still not achieved.

1.4 Methodological Aspects of PIPAC

First of all, CO_2 insufflated to enlarged the abdominal cavity and keep 12mmHg within the pneumoperitoneum, two different trocars (5 and 12 mm) are inserted into the abdominal cavity in an operating room with laminar airflow to keep the operators' safety. 5mm trocar used for measuring temperature, 12mm trocar used for insufflating CO_2 and aerosolized liquid. The peritoneal carcinomatosis index (PCI) is calculated according to anatomic structures and lesion size score based on the tumor size and distribution. 13 abdominopelvic regions of the anatomic structures such as central abdominal wall, epigastrium, right lower and upper abdominal wall, left lower and upper abdominal wall, left and right flank, upper and lower jejunum, upper and lower ileum calculated with the lesion size score from 0 to 3. The score 0 to 3 each means no visible carcinomatosis, isolated tumor nodules, multiple tumor nodules and confluent lesions. Counts the PCI score by adding up lesion size scores [39]. The peritoneal tissue biopsies are taken for histologic confirmation of malignancy during the first procedure and for further confirmation of tumor regression during the following steps [40, 41]. Ascites and mucus volumes are also documented. Then a nebulizer with trocar is connected to a high-pressure injector line inserted into the abdomen.

This dosage of chemotherapy agent is based on published data. The chemotherapy drugs are set at a flow rate of 30ml/min with an upper limit pressure of 200psi. The procedure is controlled via remote controller to keep operators' safe., the therapeutic capnoperitoneum keep for 30min at a temperature of 37℃. Then the chemotherapy aerosol is pumped out via a closed line through two sequential microparticle filters into the air-waste system of the hospital. Figure 1 shows a cartoon of the PIPAC procedure. Time duration is also need to consider delay-weeks of anti-cancer chemotherapy drugs, usually PIPAC performed every 6-8 weeks.

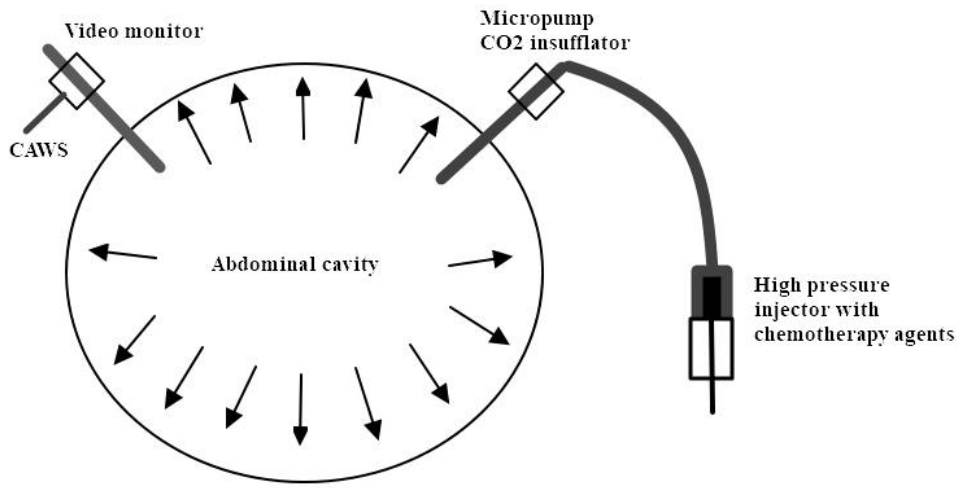

Figure. 1 Procedure of PIPAC. During the procedure, keep 12mmHg of abdominal cavity with CO_2, pressurized chemotherapy aerosols sprayed into the peritoneal cavity via micropump. Keep tightly closed abdominal cavity for 30 min. After 30 min, the aerosols with agents are released through a Closed Aerosol Waste System (CAWS) safely into the external environment. The entire procedure implemented via remote controlled system and performed in an operating room.

1.5 The Microinjection Pump

Microinjection pump (MIP) consists of a high-pressure injector, a high-pressure injector connecting line and a nozzle. Chemotherapy drug was filled in a sterile plastic syringe and connected with injector head of the high-pressure injector line

and then connected to the nozzle connecting port via a high-pressure line [37]. As a single-fluid nozzle, Figure 2 gives a three-dimensional view of the microinjection pump (MIP) nozzle head developed by Daniel Gohler et al [42]. It consisted of shaft (A), concentric bar (B), threaded sleeve (C), needle sleeve (D), free-moving nozzle needle (E), the face plate (F) and the nozzle cap (G). The liquid agent supplied through annular gap between (A) and (B), whereas (B) located inside the (A). The liquid then reaches into the (C), (D) and the (G) that connects (A) and (B). As the E is located in the D, liquid passes into D, E and F subsequently. Afterward, the liquid sprayed through the F and reaches outside the MIP as an aerosol [42].

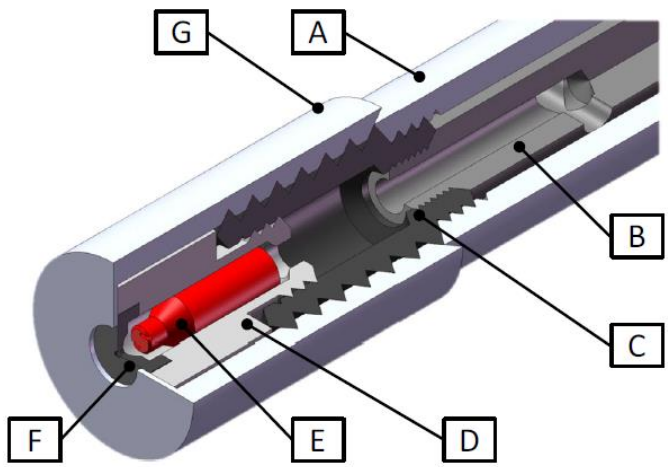

Figure. 2 A sectional view of 3D nozzle model developed by Daniel Gohler et al [42]. (A) shaft (B) concentric bar (C) threaded sleeve (D) needle sleeve (E) free-moving nozzle needle (F) face plate (G) the nozzle cap.

1.6 Ex Vivo Experiment of PIPAC

To further evaluate the delivery of chemotherapy agents, the ex vivo experiment of PIPAC has been implemented to evaluate the optional MIP position for maximizing aerosol delivery in the ex vivo model. It is a laparoscopy-like ex vivo experiment with fresh swine peritoneum tissue and ex vivo model that mimicking human abdominal cavity to investigate the penetration and distribution of aerosolized chemotherapy agents during PIPAC.

1.6.1 Ex vivo model of PIPAC

The experiments of PIPAC implemented in an ex vivo model simulating human abdominal cavity with fresh tissue samples. A lot of ex vivo models has been previously described in published articles, such as EVA bag with blue ink [43], ex vivo bovine urinary bladder model [44] and ex vivo model with plastic box. The ex vivo PIPAC model with plastic box was the most commonly used model, it is cheap, easy to imitate and large enough to mimic the human abdominal cavity [37, 44] (Figure 3). To maintaining the constant temperature of 36˚C, the model was situated in a water bath during the entire PIPAC procedure (Figure 3) [37].

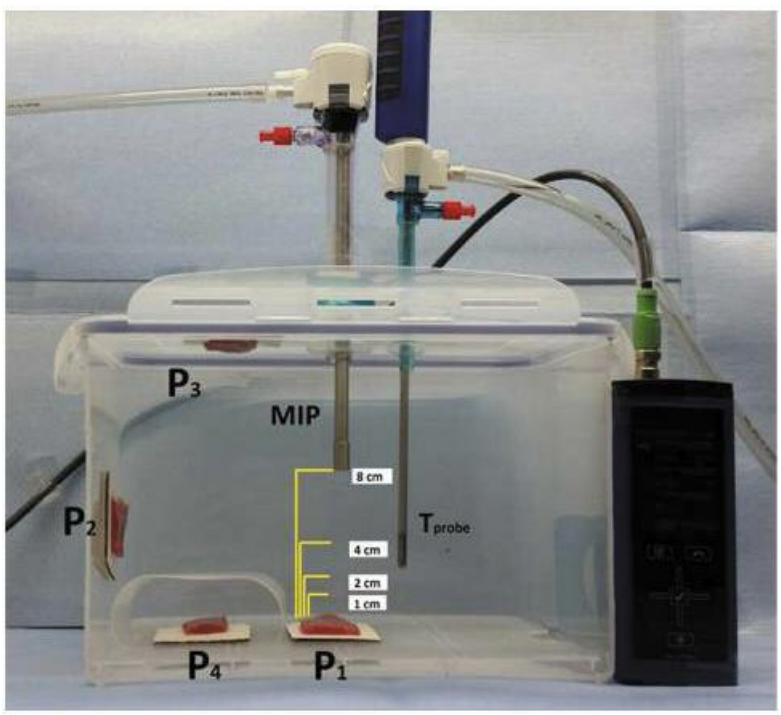

Figure 3. Laparoscopy-like ex vivo model of PIPAC by Khosrawipour V et al [37]. Nozzle placed at the center of the top cover of the model. Distance between the nozzle to the bottom at 1cm, 2cm, 4cm, 8cm. Swine tissues placed at P1: opposite the spray jet. P2: Side faces of ex vivo model. P3: Cover of the ex vivo model. P4: Bottom covered with tunnel.

1.6.2 Position to detect drug penetration depth

The swine peritoneum tissue samples each measuring 3cm*3cm*0.5cm were placed at different space positions of the ex vivo model. Khosrawipour V et al selected 4 different positions within the ex vivo model: (P1) located at the bottom of the model opposite the MIP, (P2) on the side walls of the ex vivo model, (P3) on the cover of the ex vivo model and (P4) on the bottom of the ex vivo model with a bilaterally opened plastic cover to mimic anatomic barriers within the abdomen (Figure 3) [37].

1.6.3 Nozzle position

Khosrawipour V et al selected the distance between MIP and bottom of the ex vivo model 1 cm, 2 cm, 4 cm and 8 cm [37].

1.7 Previous Studies of Ex Vivo Experiment of PIPAC

1.7.1 Effect of MIP position on drug delivery in the ex vivo model of PIPAC

Khosrawipour V et al found the mean depth of doxorubicin penetration was different according to different distance between MIP and bottom of the ex vivo model. Study found the drug penetration significantly higher in peritoneum tissue directly exposed below MIP than tissue located at other places. A closer positioning of the MIP toward target peritoneum tissue found highest penetration of drug within the samples. Even though changing MIP positions cannot achieve homogeneous penetration of doxorubicin among all target tissues, found still highest at tissue located below the MIP [37].

1.7.2 Effect of internal pressure on the drug delivery in the ex vivo model of PIPAC

During the ex vivo experiment by Khosrawipour V et al, fixed all conditions such as distance of between MIP and the bottom of the ex vivo model and doxorubicin dosage and changing the internal pressure (0mmHg, 12mmHg, 20mmHg) of the ex vivo model with constant CO_2 gas flow. The changes of internal pressure did not observe any drug penetration of sample tissues placed at under the spray jet, wall, top and bottom [37]. Suggesting changes of internal pressure do not have significant impact on the tissue samples of the ex vivo model.

1.7.3 Effect of drug dosage on drug delivery in the ex vivo model of PIPAC

Khosrawipour V et al fixed all conditions such as internal pressure, distance between MIP and bottom of ex vivo model except drug dosage found with different doxorubicin concentration the doxorubicin penetration into the peritoneum tissue different obviously. The penetration of doxorubicin found increased in all target tissues along with increased doxorubicin concentration. With different drug concentration 1.5mg/50ml, 3mg/50ml, 4.5mg/50ml, 6mg/50ml and 9 mg/50ml, different drug penetration198±38μm, 336±34μm, 401±39μm, 454±60μm and 585±22μm were measured respectively. As we can see, the drug concentration may be one of the important factors effect tissue drug penetration significantly [37].

1.7.4 Exploring drug distribution in the ex vivo model

The drug distribution pattern was explored by Daniel et al in an ex vivo model. Unlike the ex vivo model established with plastic box Daniel et al used a fresh bovine urinary bladder (2-3L) as a model with a balloon trocar inserted through an incision at the bladder neck, sprayed methylene blue under the pressure of

15mmHg at room temperature maintained for 15min, after the entire procedure opened the urinary bladder found homogeneous staining substance. Daniel et al thought oval like organ more likely simulate human abdominal cavity along with minimal cost and easy to use [44]. Apart from urinary bladder ex vivo model, EVA bag also used as ex vivo model explored distribution pattern by Leen et al [43].

1.7.5 Effect of irradiation on the tissue penetration depth in the ex vivo model of PIPAC

Khosrawipour V et al compared the impact of single fractional and bi-fractional irradiation on the depth of doxorubicin penetration within the peritoneum tissue in ex vivo experiment of PIPAC with plastic box. Research found both single fractional and bi-fractional irradiation did not significantly change doxorubicin penetration into the normal peritoneum tissue. With higher single fractional irradiation lower penetration depth observed in normal peritoneum tissue, but with higher fractional irradiation did not found significant changes of penetration depth within the normal peritoneum tissue. Still further studies are needed to further confirm the impact of irradiation on the tumor cells in ex vivo experiment of PIPAC[45, 46].

1.7.5 Exploring particle stability and structure during the ex vivo experiment of PIPAC

Not only drug penetration or spatial distribution pattern, but the behavior of the drug aerosol particles and structure during PIPAC was also studied by Khosrawipour et al in a standard ex vivo model with 5mm trocar inserted center of the top cover of the model, swine peritoneum sample attached at the side faces of the model. The differences in behavior of drug particles when implementing PIPAC and the changes in the peritoneum structure were investigated to study how the drug particles interact with peritoneum in this study with electron microscopy.

During the PIPAC procedure, doxycycline that used in this experiment created a 200nm nanofilm height on the peritoneal surface creating a therapeutic capnoperitoneum regardless of the size of initial particle hitting. But the diameters of nanofilm differ according to initial diameter of aerosol particles that hitting peritoneum spot, wider than the diameter of original initial aerosol particles. When the aerosol particles coated with liposome, the particles will adhere with the peritoneal surface creating a nanofilm. According to this article the effect of pressurized intraperitoneal aerosol on the peritoneum is similar to liquid film distribution than to that of a gas. Suggesting nebulizer should be improved to producing more gas-like aerosols on the peritoneum when implementing PIPAC procedure [47].

1.7.6 Research about particle application range during ex vivo experiment of PIPAC

In addition to changes of physical characteristics of ex vivo model and effect of particle structure during the laparoscopic-like ex vivo experiment of PIPAC, studies also investigated whether the therapeutic nano or microparticles could be applicated to be delivered via PIPAC or not. The experiment was performed with standard ex vivo model established with plastic box. During this ex vivo PIPAC procedure, human serum, bacteria cultures and macrophage cells are aerosolized into the ex vivo model of PIPAC and analyzed the human serum composition, measured viability of bacteria and macrophage cells after the PIPAC implementation. Within aerosol formation, the concentration and viability of bacteria did not significantly change, but macrophage cells showed structural disintegration. So, PIPAC can be feasible to deliver complex particles, however, larger and more complex particles of structures might be changed during PIPAC procedure [48].

1.8 Discussion

Ovarian cancer very well known as diagnosed at late stage (III, IV) and also well-known as have poor prognosis despite IV chemotherapy, targeted or immune-oncologic therapy [11]. Approximately 60-70% of ovarian cancer patients developed into stage III-IV disease [49]. PIPAC as a novel treatment method, locoregionally administrate chemotherapeutic drugs directly into the abdominal target tissue and overcome the limitations of IPC such as lower systemic toxicity with aerosolized nano-therapeutic liquid solutions [29]. Creating aerosols via nozzle is the most important part in PIPAC. Since the PIPAC prototype is not available in South Korea, creating a PIPAC prototype with novel nozzle that creating appropriate aerosol droplet diameter, spray diameter and spray velocity comparable to the nozzle prototype used in Europe is main problem to solve.

A lot of ex vivo experiments of PIPAC have been implemented to investigate how environmental parameter (pressure, temperature, irradiation et al) change effect drug penetration depth, distribution, tumor cell apoptosis and to the aerosol particle structure and application et al. Most of the ex vivo experiments have been performed with a human abdominal cavity mimicking plastic box with trocar inserted into the top cover, the distribution within ex vivo model was explored with bovine urinary bladder model and EVA bag with methylene blue, considering the plastic box walls could not absorb methylene blue and condensate after aerosolization, this could affect the result of distribution within the ex vivo model. The distribution pattern of methylene blue by PIPAC mainly conducted with in vivo swine model [38].

The ex vivo model designed by Khosrawipour et al also has some defects. The drug distribution and penetration depth within the abdominal cavity affected by three-dimensional abdominal structure when implementing laparoscopy-like PIPAC and when conducing laparoscopy, the trocar inserted not at the center of the abdominal cavity, most importantly according to the published data discussed only 4 the target tissues located different positions in this ex vivo model.

Moreover, A lot of clinical experiments confirmed that PIPAC better at homogeneous and penetration of chemotherapy agents than conventional IP chemotherapy and HIPEC [30, 33, 35]. But PIPAC still has some limitations, according to the research about PIPAC we can infer that it causing uneven diffusion within abdominal cavity, in the in vivo swine model found highest

penetration at small intestine and in the ex vivo model highest penetration found at tissue located below the nozzle. Moreover, in ex vivo experiment changing the distance between nozzle and bottom of the model still found highest penetration depth at location below the nozzle with the similar pattern observed in target tissues in ex vivo model. So, this problem still needed to be solved by researchers. And optional MIP position should be further discussed to reach best drug delivery in PIPAC.

1.9 Conclusion

Due to the limitations of the market situation in South Korea and limitations in current ex vivo experiments of PIPAC, more research is needed to further discuss drug delivery of PIPAC with new nozzle to find optional position to result in best drug delivery and how the pattern of drug delivery changes according to different nozzle positions.

Chapter 2. Evaluation of Drug Delivery into the Peritoneum Based on Nozzle Position during Pressurized Intraperitoneal Aerosol Chemotherapy in an ex vivo Model

2.1 Study Background

Approximately 60-70% of ovarian cancer patients with stage III-IV developed into peritoneal metastasis[49]. The standard therapy of PM is systemic chemotherapy with an initial cytoreductive surgery, it is considered as the standard treatment worldwide [50]. The late-stage patients with PM development of the clinical interventions such as targeted immuno-chemotherapy have moderate effect on the patients' survival. The mortality of ovarian cancer patients who developed peritoneal metastasis is about 35% [51]. Thus, there is an urgent need to develop therapies that could more efficiently treat malignancies with peritoneal spread.

Recently, several alternative therapeutic approaches have been discovered for improving management of PM. Such as IP chemotherapy found have potential to improve prognosis of patients by reaching high drug concentration within peritoneal cavity. After IPC local drug concentration increased a lot with the longer half-life of the drugs observed in the peritoneal cavity. Due to the peritoneum-plasma barrier IPC keep anticancer drugs in the peritoneal cavity with high concentrations, so it can achieve high response rates in patients compared with IV chemotherapy [21]. Even though keeping high concentrations within abdominal cavity causing high toxicity than IV chemotherapy during 4-6 weeks after IPC implementation, but the quality of life and survival rate 1 year after IPC much better than IV chemotherapy [11].

A procedure known as HIPEC is a mostly widely known post-CRS procedure that is a locoregional chemotherapy treatment, heated to increase the penetration of anticancer drugs and cytotoxicity on the tumor cells. HIPEC need mature surgeons to perform and the results of this procedure depend on residual tumor mass after CRS [52].

The abdomen initially filled with a solution (Dialysis or Ringer's solution), then the carrier solution pass through the HIPEC machine, heated to about temperature of

42℃, then the chemotherapeutic agent is added to the solution and start the procedure. The temperature of abdominal cavity is measured every minute, recorded and documented. The HIPEC procedure continued about 30-120 min, the carrier solution is drained along with the chemotherapeutic agents, lavage the abdominal cavity with 8-10L Ringer' solution [28].

Furthermore, due to the carrier solution is initially heated to a temperature between 40-43℃, after administered for 30-120 min, the abdominal cavity has to be lavage out and the patient is bound to be monitored in the ICU (Intensive Care Unit). HIPEC increases penetration depth of the chemotherapeutic agent into tissue, could direct treatment of free intraperitoneal tumor cells, reach relative homogeneous distribution of chemotherapeutic agent than IV chemotherapy. HIPEC also have some disadvantages. Firstly, during the procedure cytotoxicity of the chemotherapeutic drugs elevated due to the temperature maintain above 41℃, but heat shock proteins up-regulated because of the high temperature, that may be able to induce some thermal tolerance of the patients. Secondly, because of the platinum and mitomycin C and other factors contained in chemotherapeutic drugs the cytotoxic effect of chemotherapeutic agents obviously increased, so the HIPEC lack of repeatability than PIPAC. And in an experimental study found only 10-15% of chemotherapeutic drugs could be detected compared with peritoneal perfusates in patients[53]. In addition to this, the price of the HIPEC is too expensive to perform for most of the patients [5, 28, 54].

Thereby safety and effective of HIPEC is still controversial for curative treatment. Recently, PIPAC implemented as a palliative treatment for treating PM with solid tumor especially ovarian cancer. PIPAC is an innovative IPC approach that increase the effect of chemotherapy by taking advantage of the physical properties of aerosol and high internal pressure, overcomes limitations of IPC and HIPEC. PIPAC is reported to be safe and well tolerated, with relatively little post-operative hepatic and renal toxicities [21].

In addition, the way PIPAC sprays chemotherapeutic agents in a form of aerosol resulting in higher local drug bioavailability and a better therapeutic index. Aerosols consist of two phases: a liquid phase (droplets) and a gaseous phase (gas-like). The gaseous phase of aerosols distributed more homogeneously within the abdominal cavity, remaining equal drug concentration within the entire abdominal

cavity [55]. PIPAC offers the advantage of increased intraperitoneal cavity pressure of 12mmHg that counteracting the intra-tumoral pressure increasing the drug penetration of the tumor tissue.

Unlike IPC and HIPEC, PIPAC creates artificially generated pressure gradient between internal and external abdominal cavity and when the aerosols are sprayed within the pressurized abdominal cavity, enhancing the diffusion of liquids and substances through the peritoneum. Moreover, in contrast to conventional IPC and HIPEC, it can be applied repeatedly. In in vivo studies that performed with doxorubicin found it only penetrates 10-30μm after HIPEC [56], whereas penetration depth of doxorubicin in peritoneal tissue found to be about 469μm in ex vivo experiments of PIPAC [37] and penetrates about 349μm in in vivo studies with swine model [38].

Even though drug penetration after PIPAC far deeper, according to the published in vivo and ex vivo data found highest penetration of doxorubicin observed at tissue opposite to nebulizer [37, 38]. In ex vivo experitment found apart from the position below the nozzle the penetration of doxorubicin found significantly lower at other positions, even changes of nozzle position the highest penetration found still at position below the nozzle. Also foun with different nozzle position, the penetration of target tissue changes accordingly [37]. What's more, the exsiting ex vivo model did not considered asymmertrical three-dimensional human abdominal cavity very much.

More importantly, the PIPAC prototype mainly used in Europe, have not imported to South Korea yet. Before implementing this experiments, we collaborated with Seoul National University biomedical engineering developed a novel prototype.

2.2 Purpose of Research

Homogeneity delivery of drug into the pritoneum cavity is still not ideal. The three-demensional structure of human abdominal cavity, adhesion and tumor spread effect drug delivery with PIPAC. In this experiment we developed a newly designed ex vivo model simulating human abdominal cativy as much as possible to

further evaluate how the drug penetration changes according to different nozzle positions. And further evaluate the best nozzle position resulting in best drug delivery into the ex vivo model.

2.3 Materials and Method

2.3.1 Novel PIPAC system developed in Seoul National University

Before implementing this experiment, we collaborated with Institute of Medical and Biological Engineering, Medical Research Center, Seoul National University College of Medicine developed novel PIPAC prototype, the chemotherapy agents delivered under the pressure of 100psi (6.89 bar) with a constant flow rate of 30ml/min and generates aerosols sized 25μm at a velocity of 60km/h (Figure 4) [57]. Doxorubicin (doxorubicin hydrochloride, purchased from Sigma-Aldrich) and methylene blue (purchased from Sigma-Aldrich) filled in a sterile syringe (50ml) and connected to the high-pressure injector and then connected to the high-pressure line and to the connecting port of the nozzle, with the help of high pressure the doxorubicin and methylene blue delivered into the ex vivo model of PIPAC as gas-like aerosol. The diameter of prayed region by this prototype was 18.5±1.2cm, and the penetration depth ranged from 360 to 520μm, that were comparable to the values using the microinjector from previous studies (Capnopen® ; 133 Capnomed, Villingendorf, Germany) [37, 57].

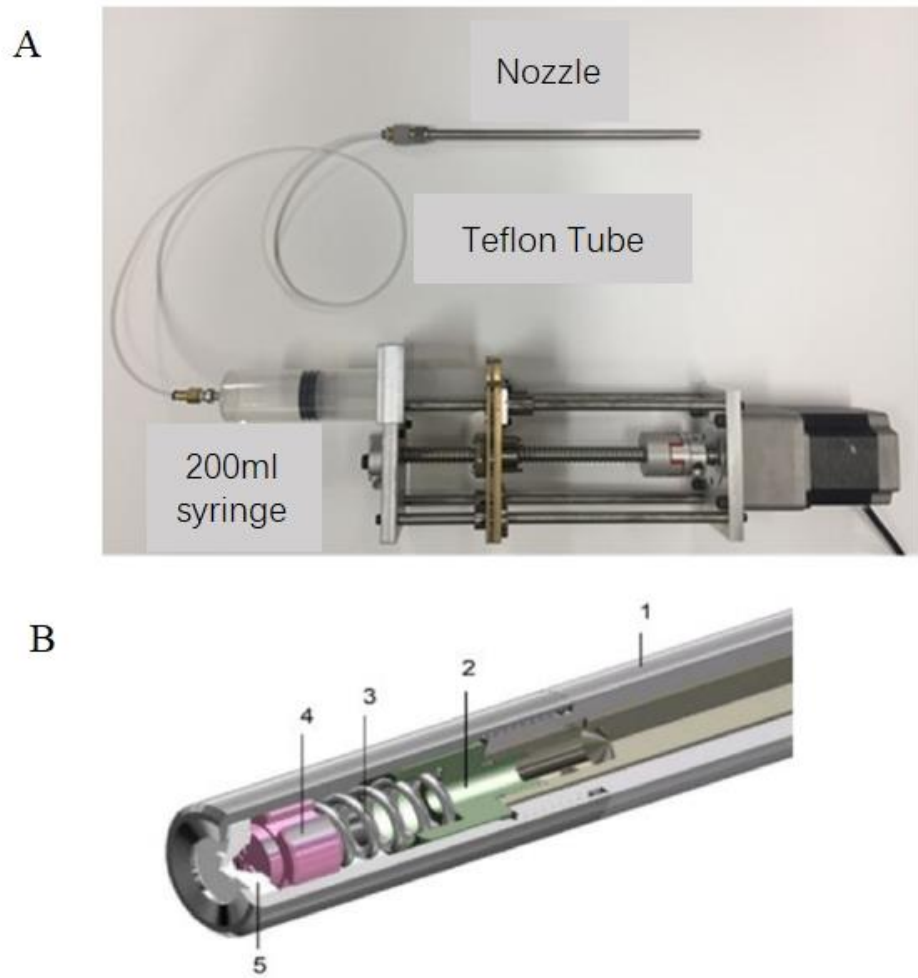

Figure 4. (A) A novel Seoul National University(SNU) PIPAC system with a 200ml syringe and nozzle developed by Lee et al [57] (B) Three-dimensional image of nozzle head developed by SNU. 1: Sleeve 2: Compartment 3: Pushing spring 4: Nozzle groove

2.3.2 Novel ex vivo model of PIPAC

Figure 5 is the newly designed simulated image of ex vivo model of PIPAC. It is mainly consisted of 21cm*15cm*16cm sealable plastic box that mimicking abdominal cavity and two 12mm and 5mm trocars (purchased from Dalim Group) one for delivering chemotherapy agents (12mm) and the other for suction pump (5mm). For obtaining the fresh tissue peritoneal specimens, this study was

approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital in advance (No. 18-0051-S1A0). Fresh postmortem swine peritoneum tissues each measuring 3cm*3cm*0.5cm were attached at 8 different spatial positions (A-H) of the box. The spatial positions of each tissue were determined considering the asymmetrical three-dimensional structure of abdominal cavity. The plastic box situated in a water bath maintain a constant temperature of 37℃ during the PIPAC procedure.

In the novel ex vivo model, the nozzle placed top cover of the plastic box, left 1/3 (7cm) away from the side faces of the box and 1/3 (5cm) away from the front face of the box. The suction pump also placed top cover of the model beside nozzle. Peritoneum tissue A placed right below the nozzle. Tissue B placed at the left side face of the box, 1/3 (5cm) away from the front face of the box, 1/2 (8cm) away from the top cover of the box. Tissue C placed on the top cover of the plastic box and between the left side face and the nozzle (3.5cm). Tissue D also placed top cover of the plastic box and right 1/3 (7cm) away from the right face, 1/3 (5cm) away from the front wall of the box. Tissue E placed at the right-face wall of the box, 1/2 (8cm) away from the top cover of the box, 1/3 (5cm) away from the front face of the box. Tissue F placed right below the tissue D with a tunnel-like covered over the tissue. Tissue G placed at the anterior face of the box, 1/3 (7cm) away from the left face of the box, 1/2 (8cm) away from the top of the box. Tissue H placed at the posterior face of the box, 1/3 (7cm) away from the left face of the box, 1/2 (8cm) away from the top of the box. As we can see, the tissue samples are located at as many spatial positions as possible.

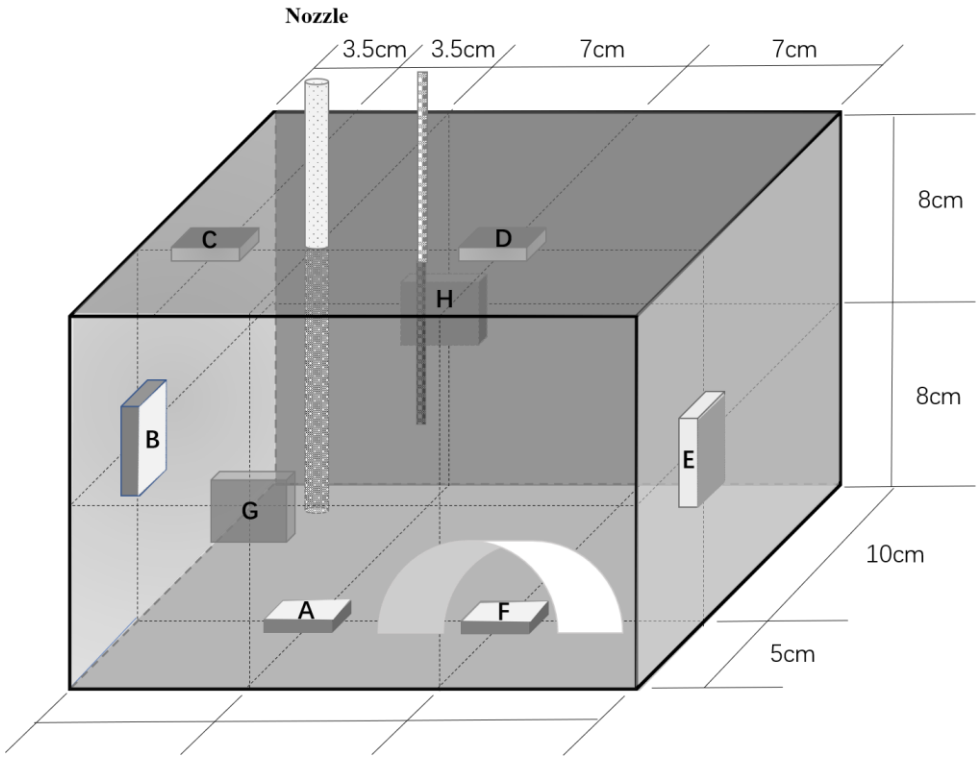

Figure 5. Simulated image of 21cm*15cm*16cm ex vivo PIPAC model. Tissue samples attached from A-H as Image show. A-H: peritoneum tissues. Barrier: tunnel-like cover over tissue F.

2.3.3 Procedure of ex vivo experiment of PIPAC

Postmortem Fresh swine peritoneum tissue samples were cut from the swine model. The 8 swine fresh peritoneum tissue samples attached at the positions (A-H) as Figure 5 show. We tightly sealed the ex vivo model and the capnoperitoneum of 12mmHg was maintained with CO_2 gas during this experiment with SNU laparoscopic system (KARL STORZ Endoscopy 136 Korea CO., Ltd., Korea).

The published articles used doxorubicin to evaluate the penetration depth of the peritoneum tissue samples, so in this experiment we also used doxorubicin to evaluate the penetration depth of peritoneum tissue samples. In this experiment, doxorubicin 4.9mg diluted in 70ml 0.9% NaCl at room temperature (23°C) and (consider the doxorubicin left at connecting line between syringe and the nozzle is about 20ml) sprayed at a flow rate of 30ml/min. After aerosolization, the tissue

samples were exposed within aerosolized chemotherapy agents for another 30 minutes. After 30 minutes the doxorubicin pumped out with suction line to an air-waste system equipped with a glass microfiber filter impregnated with a carbon layer (Laparo Clear Smoke Filtration Kit, pore size 0.027 um, diameter 50 nm, GVS Inc., Italy) in the operating room. Repeat the procedure 3 times changing the distance between nozzle and the bottom (2cm, 4cm, 8cm). The experiments were repeated three times for each ex vivo model.

The spatial distribution of chemotherapy agents in the novel ex vivo model of PIPAC, evaluated with 1.5% methylene blue solution (240 ml) diluted with 120ml NaCl 0.9% instead of doxorubicin, fill the sterilized syringe with 70ml diluted methylene blue and aerosolized into the ex vivo model at a flow rate of 30ml/min. Also repeat 3 times changing the distance between nozzle and the bottom of the box (2cm, 4cm, 8cm). Observe the pattern of spatial distribution and drug penetration depth according to different distance between nozzle and the bottom.

2.3.4 The distance between nozzle and the bottom of the model

The nozzle was positioned 2cm, 4cm and 8cm away from the bottom of the ex vivo model.

2.3.5 Detection of doxorubicin penetration depth using confocal fluorescence microscopy

To evaluate the penetration depth of doxorubicin, all tissue samples were rinsed with 0.9% sterile NaCl solution and then immediately frozen in liquid nitrogen. Prepare 24 cryosections (3*3*0.5cm) with a thickness of 7μm from three different aeras of each specimen and applied with 1.5μg/ml 4,6-diamidino-2-phenylindole (DAPI) (purchased from Sigma-Aldrich Inc., Korea) to stain nuclei. The penetration depth of doxorubicin was monitored using confocal laser scanning microscope (Leica TCS SP8) and compared between the three ex vivo models. In

this study we evaluated the penetration depth of doxorubicin by the depth of concentration diffusion (DCD) and the depth of maximized diffusion (DMD) as follows. The DCD measured the distance between the luminal surface and the positive staining of the most accumulated surface. And all the data reported in micrometers. The DMD was the distance between the luminal surface and the visualized innermost depth for doxorubicin accumulation.

2.3.6 Distribution analysis

To investigate the drug distribution, we used 1% methylene blue sprayed into the ex vivo model observed with naked eyes and compared the stained areas on the six faces in the three ex vivo models.

2.3.7 Statistical analysis

Statistical analyses were performed using SPSS version 22 software (IBM Corp., Armonk, 166 NY, USA), and the data were analyzed by the Kruskal-Wallis test and Mann-Whitney U test 167 with Bonferroni correction. In this study, a significant p-value was defined as $p < 0.05$.

2.4 Results

2.4.1 Microscopic confocal laser analysis of DCD and DMD at various nozzle positions according to the distance between nozzle and the bottom

Microscopic confocal laser analysis of sample tissues show the highest DMD of the sample B, C, D, F, G found to be when the distance between nozzle and bottom is 2cm (2cm-ex vivo model), increase with increased distance between nozzle and the bottom of the model. The highest DMD of the sample A, H found at 2cm-ex vivo model and lowest at 8cm-ex vivo model, decrease with increased distance between nozzle and the bottom of the model. Microscopic confocal laser analysis shows the highest DCD of the sample C, D, F found to be at 8cm-ex vivo model and lowest at 2cm-ex vivo model. The highest DCD of the sample E, G, H found to be at 4cm-ex vivo model and lowest at 8cm-ex vivo model. DCD of the sample A found no big difference among the 2cm-, 4cm- and 8cm-ex vivo model (Figure 6).

2.4.2 Comparison of DCD at various nozzle positions according to the distance between the nozzle and the bottom

Figure 7 and Table 1 show the DCD comparison of tissue samples (A-H) according to the different nozzle positions. As we can see, sample C, D, F, G show highest DCD at 8cm-ex vivo model (C: 245.3 ± 12.1µm, D: 114.4 ± 9.8µm, F: 265.5 ± 6.1µm, G: 258.8 ± 5.4µm), lowest DCD at 2cm-ex vivo model (C:125.4 ± 4.5µm, D: 94.4 ± 5.2µm, F: 174.8 ± 5.3µm, G: 111.2 ± 5.9µm). Sample A, E, H show highest DCD at 2cm-ex vivo model (A: 268.7 ± 23.5µm, E: 84.7 ± 1.8µm, H: 257.9 ± 6.8µm), lowest DCD at 8cm-ex vivo model (A: 256.4 ± 3.1µm, E: 57.6 ± 1.1µm, H: 92.8 ± 3.4 µm). Sample B shows no big difference between 2cm-ex vivo model (133.9 ± 4.5 µm), 4cm-ex vivo model (131.3 ± 3.8 µm) and 8cm-ex vivo model (133.2 ± 3.5 µm) nozzle position and have no statistical significance. All

data were analyzed using Kruskal-Wallis test with post-hoc Mann-Whitney U test including the Bonferroni correction.

From Figure 9 we can see the variation tendency of DCD according to different nozzle positions and the bottom at a glance. From this line chart we can see DCD tested highest at tissue C,D and G in the 4cm- and 8cm- ex vivo model, tissue F tested highest at 8cm-ex vivo model, the DCD tissue A and B have no big difference according to the different nozzle positions. Tissue D and E also found almost the same DCD between different nozzle positions. Other tissue samples found different DCD according to the different nozzle positions.

2.4.3 Comparison of DMD at various nozzle positions according to distance between the nozzle and the bottom

Figure 8 and Table 2 show the DMD of 8 tissue samples (A-H) at different nozzle positions. Sample B, C, D, F, G show highest DMD at 8cm-ex vivo model (B: 404.6 ± 2.9µm, C: 527.7 ± 10.3µm, D: 188.7 ± 7.8µm, F: 518.9 ± 16.5µm, G: 537.2 ± 8.7µm) and lowest at 2cm-ex vivo model (B: 218.7 ± 7.4µm, C: 184.6 ± 6.8µm, D: 143.4 ± 3.4µm, F: 418.2 ± 15.6µm, G: 322.5 ± 8.7µm). Sample A, E, H found doxorubicin DMD highest at 2cm-ex vivo model (A:517.5 ± 6µm, E: 162.7 ± 1.3µm, H: 528.1 ± 16.3µm) and lowest at 8cm-ex vivo model (A: 329.1 ± 8.9µm, E:122.8 ± 5.1µm, H: 214.1 ± 9.9µm). All data were analyzed using Kruskal-Wallis test with post-hoc Mann-Whitney U test including the Bonferroni correction.

Figure 10 show DMD tested highest at tissue D, F and G in the 4cm- and 8cm- ex vivo model, tissue E and H tested highest at 2cm- and 4cm-ex vivo model, tissue D and E found no big fluctuation among 2cm-, 4cm- and 8cm-ex vivo models. Other tissue samples found different DMD between different nozzle positions.

To compare different nozzle positions between three ex vivo models, counted the number of tested values of DCD and DMD that showed highest among three models (Table 3). When the nozzle position located at 4cm showed the highest

value of 5 in DCD and DMD, which means it maximize the aerosol delivery to about 62.5% of the ex vivo model area.

2.4.4 Spatial distribution pattern of methylene blue in the novel ex vivo model of PIPAC

This experiment analyzed spatial distribution pattern with methylene blue aerosolized in the ex vivo model. When we compared the stained areas on the six faces between the three ex vivo models, at the 2cm-ex vivo model the methylene blue mainly stained at the bottom of the model, seldom distributed on the top of the model and side faces of the model. At the 4cm-ex vivo model the side face stain of methylene blue better than at 2cm-ex vivo model, but still rarely stained on the top cover of the ex vivo model. At the 8cm-ex vivo model, the methylene blue stained on the top cover of box is far better than 4cm nozzle position and 2cm-ex vivo model. When the distance between nozzle and the bottom is 8cm the methylene blue better distributed on the side faces than 4cm-ex vivo model and 2cm-ex vivo model (Figure 11).

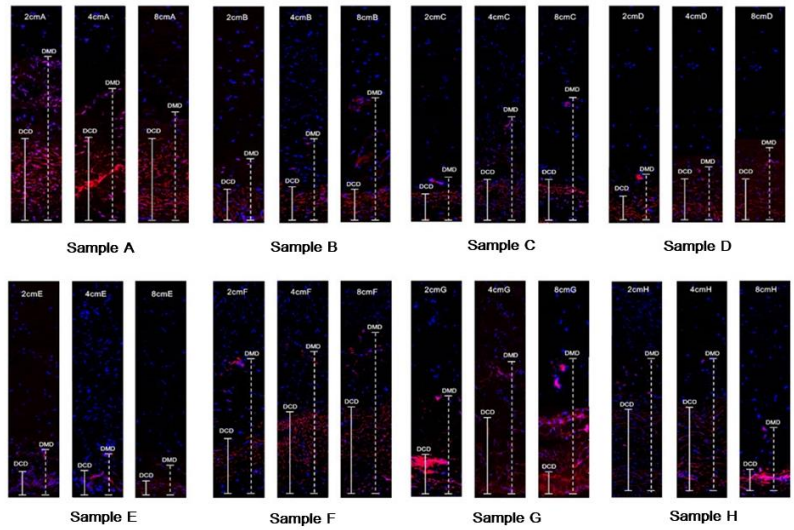

Figure 6. Microscopic confocal laser analysis of the concentrated penetration depth (DCD) and maximized penetration depth (DMD) of doxorubicin in fresh peritoneal tissue samples (A-H) each with different distance between nozzle and bottom (2cm, 4cm, 8cm). Doxorubicin concentration (3mg/50ml). Nuclei (blue) were stained with 40,6-diamidino-2-phenylindole (DAPI), DAPI concentration (1.5µg/ml).

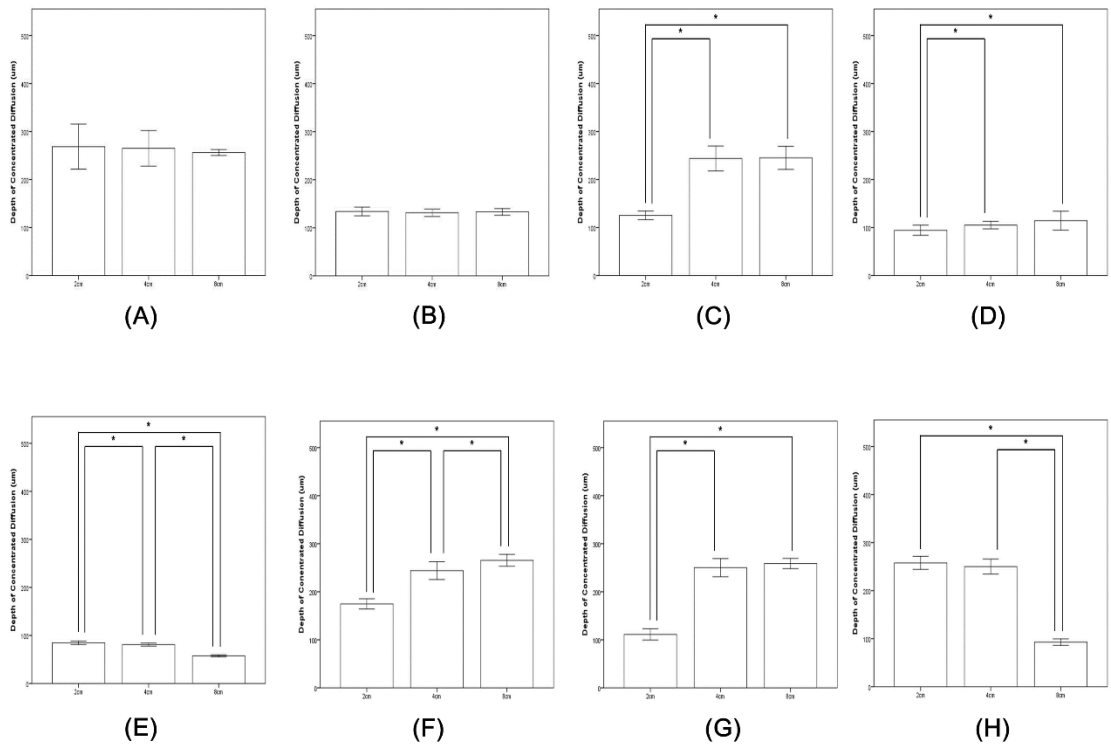

Figure 7. Penetration depth of concentrated diffusion (DCD) of doxorubicin in tissue samples (A-H) according to different distance between nozzle and bottom (2cm. 4cm. 8cm). *$p<0.05$ **$p<0.01$ ***$p<0.001$

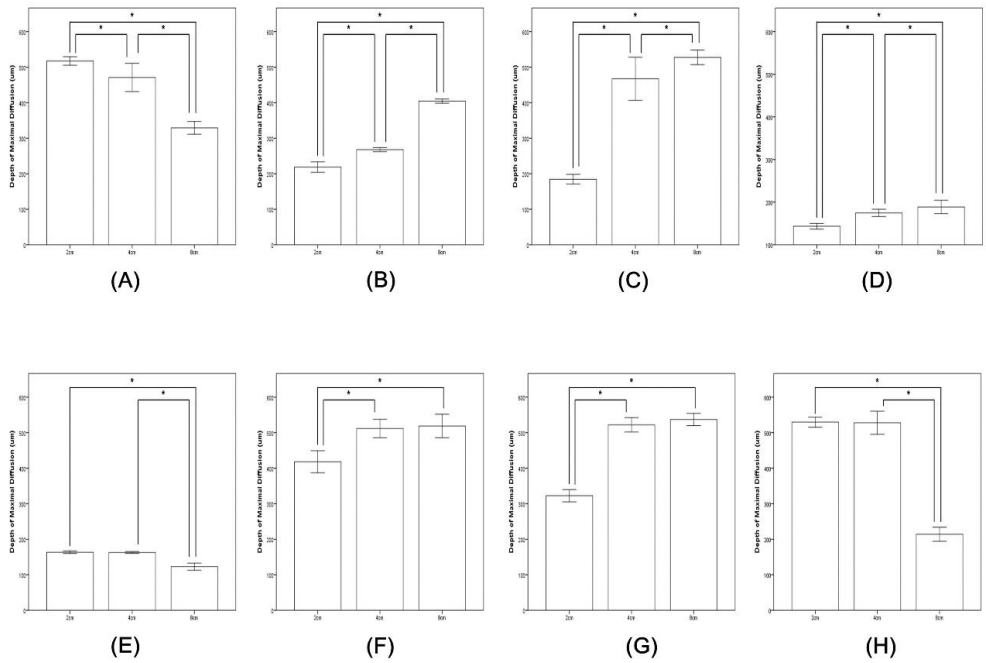

Figure 8. Penetration depth of maximized diffusion (DMD) of doxorubicin in tissue samples (*A*-*H*) according to distance between nozzle and bottom (2cm, 4cm, 8cm). *$p<0.05$ **$p<0.01$ ***$p<0.001$

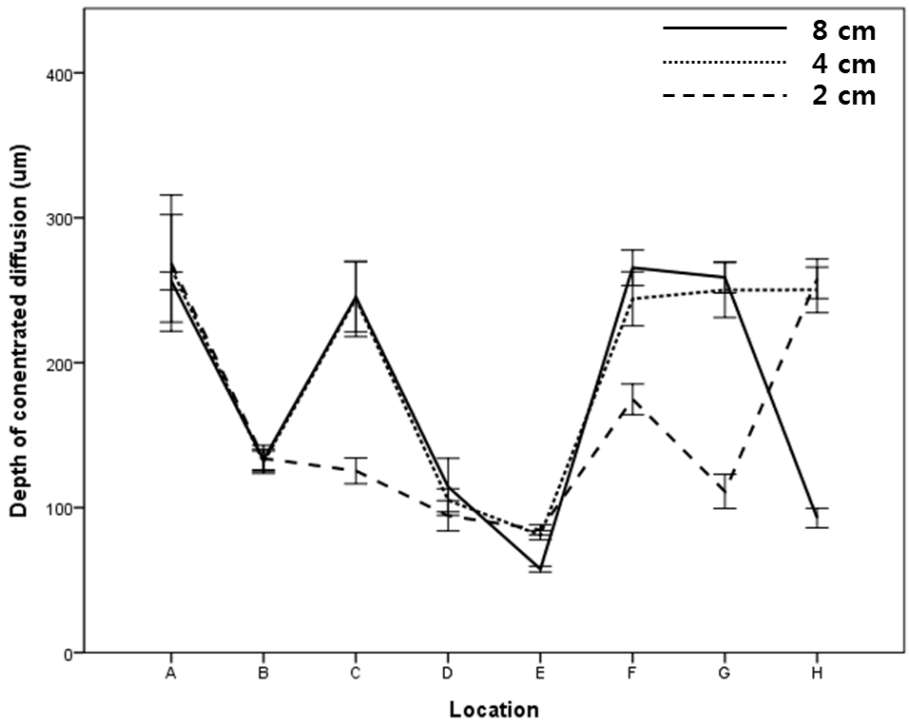

Figure 9. Line chart of DCD at different positions according to distance between nozzle and bottom.

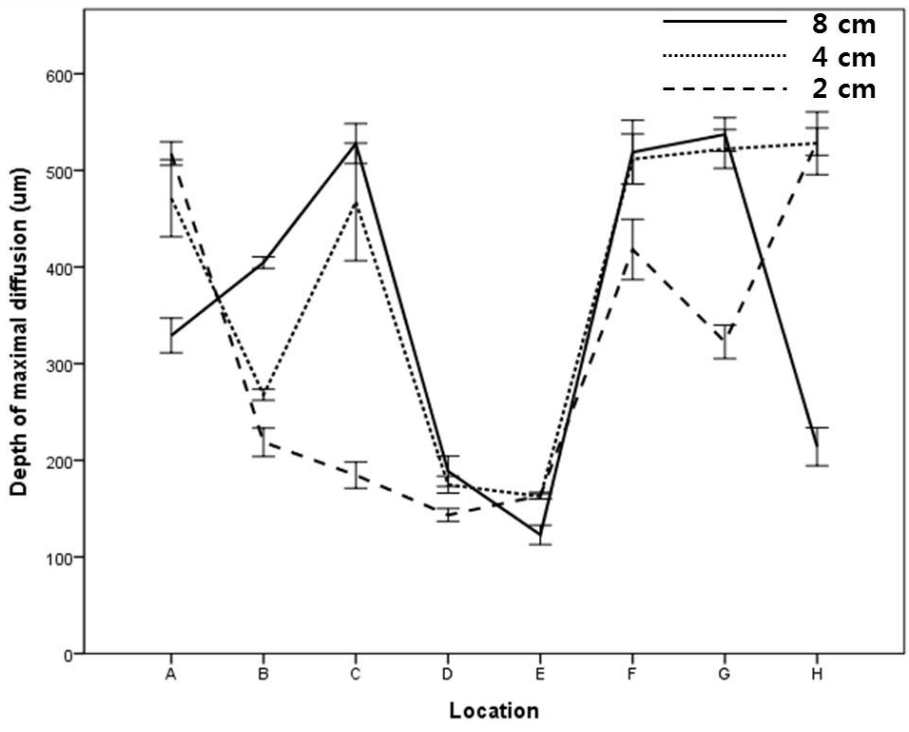

Figure 10. Line chart of DMD at different positions according to distance between nozzle and bottom.

Table 1. Comparison of depth of concentrated diffusion (DCD) at various positions according to the distance between the nozzle and the bottom

Positions	Distance between the nozzle and the bottom			P value
	2 cm	4 cm	8 cm	
A	268.7 ± 23.5*	265.1 ± 18.5*	256.4 ± 3.1*	0.875
B	133.9 ± 4.5*	131.3 ± 3.8*	133.2 ± 3.5*	0.733
C	125.4 ± 4.5	244.1 ± 12.9*	245.3 ± 12.1*	0.066
D	94.4 ± 5.2*	105.1 ± 3.9*, †	114.4 ± 9.8†	0.039
E	84.7 ± 1.8*	80.9 ± 1.6*	57.6 ± 1.1	0.027
F	174.8 ± 5.3	244.1 ± 9.3	265.5 ± 6.1	0.027
G	111.2 ± 5.9	250.2 ± 9.5*	258.8 ± 5.4*	0.051
H	257.9 ± 6.8*	250.2 ± 7.8*	92.8 ± 3.4	0.051

All data were analyzed using Kruskal-Wallis test with post-hoc Mann-Whitney U test including the Bonferroni correction.
Values labeled with the same characters are not significantly different.
All values were shown as mean ± standard deviation (μm).

Table 2. Comparison of depth of maximized diffusion (DMD) at various positions according to the distance between the nozzle and the bottom

Positions	Distance between the nozzle and the bottom			P value
	2 cm	4 cm	8 cm	
A	517.5 ± 6.1	471.2 ± 19.9	329.1 ± 8.9	0.27
B	218.7 ± 7.4	267.9 ± 2.8	404.6 ± 2.9	0.27
C	184.6 ± 6.8	467.4 ± 30.4	527.7 ± 10.3	0.27
D	143.4 ± 3.4	174.8 ± 4.4*	188.7 ± 7.8*	0.27
E	163.4 ± 1.7*	162.7 ± 1.3*	122.8 ± 5.1	0.61
F	418.2 ± 15.6	511.7 ± 13.1*	518.9 ± 16.5*	0.61
G	322.5 ± 8.7	522.2 ± 10.1*	537.2 ± 8.7*	0.39
H	529.8 ± 7.1*	528.1 ± 16.3*	214.1 ± 9.9	0.66

All data were analyzed using Kruskal-Wallis test with post-hoc Mann-Whitney U test including the Bonferroni correction.
Values labeled with the same characters are not significantly different.
All values were shown as mean ± standard deviation (μm).

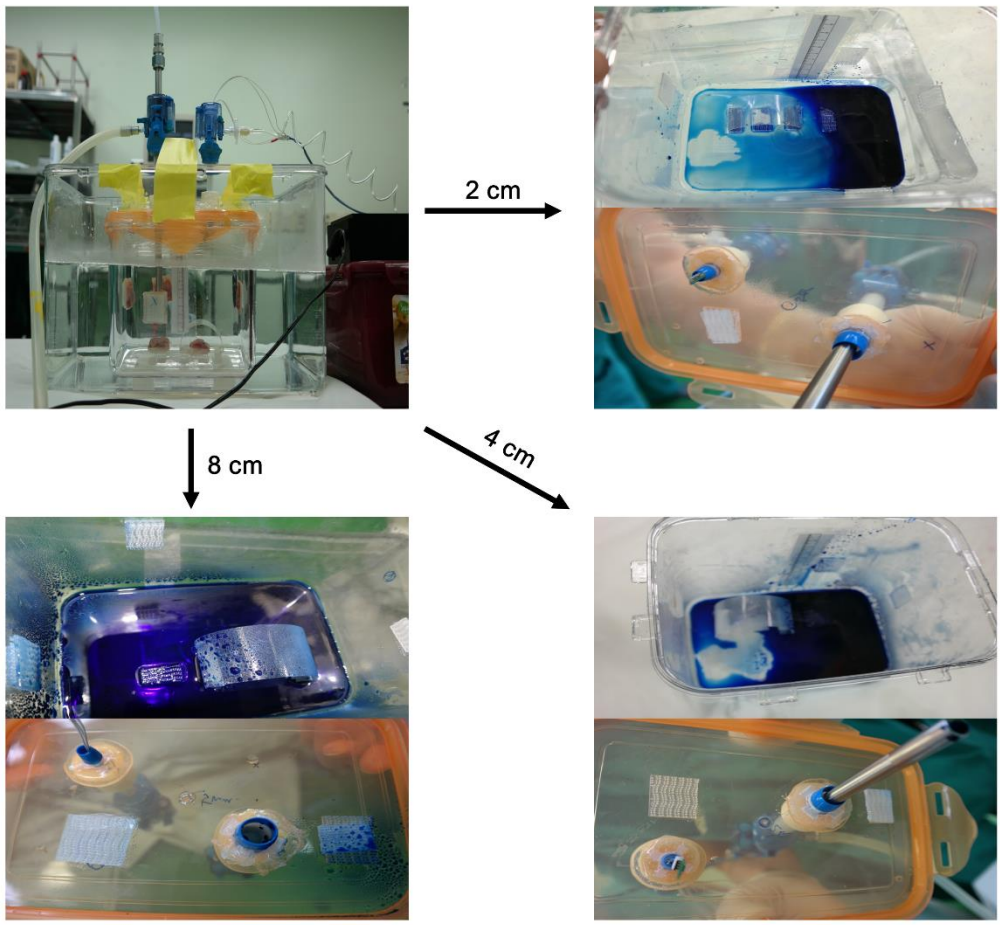

Figure 11. Laparoscopy-like ex vivo experiment with fresh peritoneum tissue to investigate the spatial distribution pattern of sprayed methylene blue during PIPAC. Distance between nozzle and bottom was 2cm, 4cm and 8cm.

Table 3. The number of counted highest DCD and DMD values in the three ex vivo models

Ex vivo models	A	B	C	D	E	F	G	H	Number of the highest values (%)
Depth of concentrated diffusion (DCD)									
2cm-					√			√	2/8 (25)
4cm-			√	√	√		√	√	5/8 (62.5)
8cm-			√	√		√	√		4/8 (50)
Depth of maximal diffusion (DMD)									
2cm-	√				√			√	3/8 (37.5)
4cm-				√	√	√	√	√	5/8 (62.5)
8cm-		√	√	√		√	√		5/8 (62.5)

2.5 Discussion

PIPAC has been known to treat PM more effectively than IV chemotherapy, IPC and HIPEC that overcoming the limited and less homogeneously distribution of solutions and lowering systemic toxicity [58].

PIPAC can achieve 200-times higher tissue concentration after implementation than IV chemotherapy especially with using only about 10% of the dosage of chemotherapy agents during IV chemotherapy [59]. Moreover, due to low plasma levels of the chemotherapy agents the hepatic and renal toxicities are minimal, with no significant creatinine increased after PIPAC implementation and the level of AST/ALT did not increase after PIPAC implementation [60, 61], inducing lower Common Terminology Criteria for Adverse Events (CTCAE) and the 4-tied peritoneal regression grading system (PRGS) remain improved or unchanged that reflecting tumor regression [62]. And the air collecting at the surgeon and anesthesiologist position after PIPAC procedure found the level of chemotherapy agents was below the tolerance level and comply with working safety law in Europe [55]. The changes in the molecular markers of the PM tissue during PIPAC implementation also have been studied [63]. The gene expression of peritoneal tissue taken before and after PIPAC implementation were also different and changes of gene expression associated with treatment response such as downregulation of the whole gene panel predicts survival rate of patients along with serum cancer antigen 125 (CA125) levels, PCI score and ascites volume changes, suggesting the PIPAC effective in treating patients with PM [63]. The novel PIPAC approach may be a promising new treatment for solid tumors with PM, it offers hope to patients with terminal illness as a palliative treatment.

A lot of research found PIPAC can reach relatively uniform distribution of aerosolized agents compared with conventional IPC and HIPEC [59, 64], according to the data of in vivo and ex vivo experiments of PIPAC found still lack in reach homogeneous delivery of chemotherapy drug within abdominal parietal and visceral tissue[37, 38].

The published articles of in vivo and ex vivo experiments all used Capnopen® nebulizer (Capnopen® ; Capnomed, Villingendorf, Germany) developed by Göhler, D et al [37, 38, 65]. The Capnopen® nebulizer spray aerosols with median

diameter of 25μm, deposited within 15-cm diameter circular concentrated area, indicating the MIP developed by Germany did not perform an evenly spray of chemotherapy agents during PIPAC [42]. Published in vivo and ex vivo studies of PIPAC showed the penetration depth of the chemotherapy agents highest at the position opposite the MIP than other tissue samples, with the reported ex vivo experiment maximum penetration measured at the tissue samples located in the position below the aerosol jet (215±79μm) and much lower drug penetration was observed for tissue samples located outside the aerosol jet (34±19μm), in ex vivo experiment even changes of nozzle position still found highest penetration at the location below the nozzle, the tissue penetration changes according to different nozzle position. These results suggesting that to reach evenly distribution of the agents during PIPAC implementation is still needed to be improved [37, 38, 65].

A lot of ex vivo prototype that mimicking human abdominal cavity had been used in exploring the spray and penetration of chemotherapy agents, such as EVA bag with blue ink explored staining status[43], ex vivo bovine urinary bladder model with methylene blue[66] and ex vivo model with plastic box. Since the EVA bag and bovine urinary bladder model are too small to simulate three-dimensional abdominal cavity, we used plastic box to develop human abdominal-like ex vivo model in this experiment.

Since the PIPAC prototype is not available in South Korea, previously we collaborated with department of biomedical engineering in Seoul National University developed new prototype that develop similar sized aerosol droplets with comparably wide sprayed area and comparable penetration depth to the current Capnopen® prototype.

And the laparoscopy-like ex vivo experiments with fresh swine tissues proceeded in Germany have several limitations. First of all, the nebulizer with aerosol jet inserted right center of the ex vivo model, but when proceed the laparoscopy the trocar usually inserts at lower 1/3 of abdomen. Secondly, the selection of the number and spatial positions of the tissue samples are not enough, have not considered the asymmetrical three-dimensional structure human abdominal cavity due to tumor or adhesion caused by inflammation enough [37].

Considering complicated anatomical factors, we redesigned the novel ex vivo model of PIPAC as Figure 5 show, investigated chemotherapy drug penetration of

as many spatial positions as possible to simulate human abdominal cavity with the developed prototype that aerosolizes droplet with a median diameter of 30μm at a flow rate of 30ml/min, under the pressure of 7 bar [57]. Since the distance between nozzle and the bottom of 1cm and 2cm have no obvious difference, in this experiment we selected the distance between nozzle and the bottom 2cm, 4cm and 8cm as nozzle positions.

From the result, we can see with nozzle position changing, the pattern of DCD and DMD of 8 tissue samples vary a lot. Among the three different ex vivo models still high DCD and DMD recorded at the tissue A that located opposite the nozzle. That is in accord with the published data that found highest penetration at the tissue below the nozzle with different distance between nozzle and bottom [37].

According to the published article the highest maximal diffusion found at tissue located below the nozzle (469μm) when distance between nozzle and the bottom was 1cm [37]. In our experiment, the tissue A that located below the nozzle found highest DMD (517.5 ± 6.1μm) at 2cm-ex vivo model. Moreover, the droplet size sprayed by Capnopen® nozzle was about 20μm and the average droplet size tested by Institute of Medical and Biological Engineering, Medical Research Center, Seoul National University College of Medicine was 20.4~32.1μm [57]. Even though the droplet size we tested is larger than published data, the DMD of tissue located below the nozzle was better than published data.

In our study we found according to different nozzle position the highest DCD and DMD values were found at different tissue samples. The pattern of penetration in each ex vivo model also different from the result of published articles. According to different nozzle position apart from the tissue located below the nozzle, at other sample tissues also observed high penetration depth. Moreover, tissue F covered with tunnel-like barrier to simulate tissue with anatomical barrier in human abdominal cavity found high penetration in this ex vivo model different from previous result that seldom detected doxorubicin penetration, suggesting the prototype we used in this experiment better at drug delivery than conventional prototype used in Europe.

This could be caused partly by asymmetrical positioning of tissue samples. But most importantly the nozzle used in this experiment may be the main reason causing this result. The two nebulizers have similar flow rate, similar aerosol size

and deposit aera. But, in this novel nebulizer, aerosol was sprayed at about velocity of 1.38m/s under the pressure of 100psi with larger exit [57], but in published conventional PIPAC the aerosols are sprayed at velocity of 16.67m/s under the pressure of 200psi with smaller exit [65]. According to the liquid dynamics a larger spray outlets with a lower aerosol velocity reduce the aerosol turbulent flow within the nebulizer [67]. In this prototype, owing to lower turbulence the break-up length was very long, the liquid may spray with injection pressure, under the effect of inertia the particles spray in their original direction and the deflect collisions make particles spray into various parts within the peritoneal cavity [68-70]. In conventional prototype the higher turbulence causing chaotic recirculation of liquid within the nozzle, the higher turbulence leading to short break-up length, the circulating particles disseminate in the cavity, particles move outward and collect on the wall of the ex vivo model, quickly sediment to the floor of the model under the effect of gravity [67]. From the model we assume that sample D may harder to be sprayed than sample E, but the result show doxorubicin penetration at sample D better than sample E. This may be interpreted by gravitational and inertia force of nozzle with bigger outlet size that doxorubicin delivered better at sample D than sample E by SNU nozzle. So, using the prototype causing less turbulence could better deliver drug into the ex vivo model (Figure 12).

From the result of our experiment, after quantifying DCD and DMD values we found at 4cm-ex vivo PIPAC model most appropriate for delivery chemotherapy agents. It requires aerosol with the size of 0.5~5μm diameter to reach the effective deposition of gas-like aerosols, but actually more than 97.5% of the aerosols sprayed by the nebulizer larger than 3μm [42, 71, 72]. Due to the gravitational force and effect of mechanical inertia, droplets with different sizes may precipitate and have impact on the peritoneum [65], especially aerosols larger than 3μm likely to precipitate due to gravity and inertia of fluid mechanics [68, 73]. From the results we can speculate that the optional nozzle position should be further determined by in vivo experiments with three-dimensional abdominal cavity structure, since the peritoneal cavity asymmetric due to PM or adhesions caused by surgery, inflammation affects the movement of aerosol agents and twist of aerosol agents within the peritoneal cavity. Particularly, we observed that the closer the nozzle to the bottom of the ex vivo model found more unevenly distribution of aerosols.

This study also has some limitations. First, from Figure 9 we found at tissue sample A, B, D, E found no big different of DCD according to nozzle position, suggesting different nozzle position may not have a lot effect on the DCD, apart from the nozzle position different nozzle injection angle may also help upgrade drug delivery into the ex vivo model. So, the optional position on the ex vivo model is just one of the parameters that including the nozzle injection angle in the ex vivo model. Second, the ex vivo model unwillingly has some limitations since it cannot simulate the three-dimensional structure of the abdominal cavity including tumor location, adhesion and spread. So, we need to plan in vivo experiment with swine model to further evaluate optional nozzle position during PIPAC. Third, the post-mortem swine tissue sample without vascular and lymphatic transport may affect drug penetration into the tissue, and in human patients delayed uptake and lymphatic transport can make up for distribution and accumulation of aerosol. Forth, it should be clinically investigated the role of optional position of the nozzle. To overcome the inhomogeneous distribution of aerosols during PIPAC, the rotation of the nozzle during PIPAC implementation can be considered to change the spray direction [74]. The rotating nozzle is simulating the car washer, rotate the nozzle in the three-dimensional in vivo swine model. Even though rotation of the nozzle is ideal for improving a homogeneous distribution of agents, an automatic device with a circular motion and a conical pendulum should be developed in the future. As an alternative method, the optimal nozzle position should be investigated to maximize the distribution and penetration depth of the agents under various PIPAC conditions.

The study suggests that the optimal nozzle position for PIPAC may different among PM patients, according to the different nozzle position the pattern of tissue penetration change accordingly. So, the optimal position of nozzle should be further determined by the three-dimensional structure of the human abdominal cavity with intraperitoneal tumors or adhesions. More experiment should be done with this PIPAC prototype on three-dimensional in vivo model.

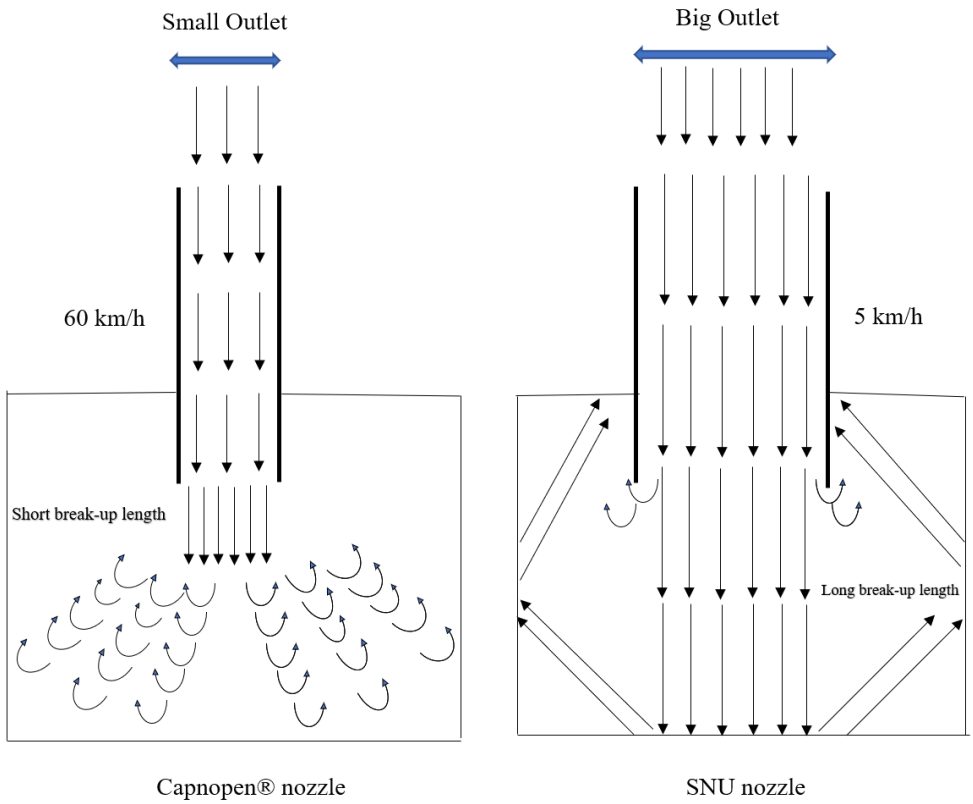

Figure 12. Two different turbulent jets of conventional nozzle and SNU nozzle with different outlets.

2.6 Acknowledgements

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Chapter 3. 초록

배경: 가압복강내에어로졸항암요법은 solid 종양의 복강내 전이종양의 새로운 치료방법으로 잘 알려져 있다. 가압복강내에어로졸항암요법은 현재 주로 유럽에서 잘 사용되고 있다. 그러나 가압복강내에어로졸항암요법은 현재 국내에 도입이 되여있지 않고 시중에서 구매할수 없다. 본 실험을 진행하기 앞서 서울대학교 의공학과와 콜라보를 하여 새로운 PIPAC prototype 을 만들었으며 본 prototype 을 사용해 prototype 의 노즐의 위치가 변함에 따라 조직의 penetration 이 어떻게 변화를 하는지 어떤 위치에 있을 경우에 가장 이상적인 delivery 를 얻어낼 수 있는지 알아보려고 한다.

자료와 방법: 8 개의 똑 같은 크기의 신선한 사후 실험돼지의 복막조직(A-H)을 인체의 비대칭구조를 모방하여 만든 ex vivo 모델의 부동한 공간위치에 부착을 하였다. 노즐과 ex vivo 모델의 밑부분과의 거리 2cm-,4cm-,8cm 를 선택했다. 본 ex vivo 모델에서 새로운 prototype 를 사용했으며 본 prototype 은 30-μm 크기의 에어로졸들을 30ml/min 의 유속으로 7 bar 의 압력아래 분사한다. Ex vivo 모델내에 약물의 분포도는 methylene blue 로 축정했으며 눈으로 관찰하여 측정하였다. 조직의 약물 침투는 depth of concentrated diffusion (DCD)와 depth of maximal diffusion (DMD)를 측정하여 confocal laser scanning microscopy 로 관찰하였고 부동한 ex vivo 모델끼리 비교 분석하였다. 각 모델의 가장 높은 DCD 와 DMD 를 카운트한결과 4cm-ex vivo 모델에서 가장 이상적인 약물 delivery 가 측정되었다.

결과: 4cm-와 8cm-ex vivo 모델에서 2cm-ex vivo 모델보다 약물의 분포가 가장 잘 측정되었다. 노즐위치가 다름에 따라 각 조직의 약물침투패턴이 다르며 각 위치에서 가장 높은 약물침투가 측정되는 조직 또한 다르다. 항암제의 약물전달은 4cm-ex vivo 모델에서 가장 훌륭한 DCD 와 DMD 5 (62.5%)이 측정되었다.

결론: 본 prototype 을 사용했을 때 노즐위치에 따본 ex vivo model 의 각 타깃조직의 penetration 패턴이 다르며 노즐위치가 다름에 따라 약물침투가 가장 많이 되는 조직 또한 다르다. 인체 복강의 복잡한 three-dimensional 구조를 고려했을 때 노즐위치를 위아래로 바꾸거나 각도를 바꾸는 방법으로 더 우월한 delivery 를 얻을 수 있을 것이다. 향후 in vivo 실험을 더 진행해야 할 필요성이 있다.

핵심 검색어: Pressurized intraperitoneal chemotherapy, Peritoneal metastasis, Ex vivo, Nozzle
학번: 2017-39829

Chapter 4. Thank You Letters

Greetings,

I would like to take this opportunity to thank you for having me in Gynecologic Oncology part of Seoul National University School of Medicine. It is been 4 years since I entranced into SNU.

First and for most, thank you so much for Jae-weon Kim and Maria Lee having me here and providing opportunities and learning guidance. I have learned a lot through outpatient part and surgeries.

Secondly, thank you so much for Hee Seung Kim and Jung Chan Lee providing opportunities to complete this project together.

During the 4 years in Seoul National University I had a lot of experiences both in research and clinical study including outpatient and surgical part.

And thank you so much for the help of fellows and residents worked together in this project.

During 4 years in SNU I had a lot of meaning time here, grown up a lot and it is a period of time full of thankfulness.

Chapter 5. Abbreviations

PM: Peritoneal metastasis
CT: Computed tomography-scan
MRI: Magnetic resonance imaging
PCI: Peritoneal cancer index
RPD: Random proximal distribution
CRD: Complete redistribution
WCD: Widespread cancer distribution
HIPEC: Hyperthermic Intraperitoneal Chemotherapy
CRS: Cytoreductive surgery
FIGO: International Federation of Gynecology and Obstetrics
IPC: Intraperitoneal Chemotherapy
IV: Intravenous Chemotherapy
GOG: Gynecologic Oncology Group
PIPAC: Pressurized Intraperitoneal Aerosol Chemotherapy
Closed Aerosol Waste System (CAWS)
MIP: Micro junction pump
T-probe: Temperature Probe
DAPI: 4,6-diamidino-2-phenylindole
DCD: Depth of concentration diffusion
DMD: Depth of maximal diffusion
CTCAE: Common Terminology Criteria for Adverse Events
PRGS: Peritoneal Regression Grading System
CA125: Cancer Antigen 125