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Irradiation Does Not Increase the Penetration Depth of Doxorubicin in Normal Tissue After Pressurized Intra-peritoneal Aerosol Chemotherapy (PIPAC) in an *Ex Vivo* Model

VERIA KHOSRAWIPOUR[1,2], ALEXANDER BELLENDORF[3], CAROLINA KHOSRAWIPOUR[2], YOUSEF HEDAYAT-POUR[4], DAVID DIAZ-CARBALLO[5], ECKART FÖRSTER[6], RALPH MÜCKE[4], BURAK KABAKCI[2], IRENÄUS ANTON ADAMIETZ[4] and KHASHAYAR FAKHRIAN[2,4]

[1]*Departments of General Surgery and Therapy Center for Peritonealcarcinomatosis,*
[4]*Radiation Oncology and* [5]*Hematology and Medical Oncology,*
Marien Hospital Herne, Ruhr University Bochum, Herne, Germany;
[2]*Basic Research Laboratory, Department of Surgery, Marien Hospital Herne,*
Ruhr University Bochum, Herne, Germany;
[3]*Department of Nuclear Medicine, University Clinic Essen, Essen, Germany;*
[6]*Department of Neuroanatomy and Molecular Brain Research, Ruhr University Bochum, Herne, Germany*

Abstract. *Aim: To compare the impact of single fractional with bi-fractional irradiation on the depth of doxorubicin penetration into the normal tissue after pressurized intra-peritoneal aerosol chemotherapy (PIPAC) in our ex vivo model. Materials and Methods: Fresh post mortem swine peritoneum was cut into 12 proportional sections. Two control samples were treated with PIPAC only (no irradiation), one sample on day 1, the other on day 2. Five samples were irradiated with 1, 2, 4, 7 or 14 Gy followed by PIPAC. Four samples were treated on day one with 0.5, 1, 2, 3.5 or 7 Gy and with the same radiation dose 24 h later followed by PIPAC. Doxorubicin was aerosolized in an ex vivo PIPAC model at 12 mmHg/36˚C. In-tissue doxorubicin penetration was measured using fluorescence microscopy on frozen thin sections. Results: Doxorubicin penetration (DP) after PIPAC for the control samples was 407 μm and 373 μm, respectively. DP for samples with single fraction irradiation was 396 μm after 1 Gy, 384 μm after 2 Gy, 327 μm after 4 Gy, 280 μm after 7 Gy and 243 μm after 14 Gy. DP for samples with 2 fractions of irradiation was 376 μm after 0.5+0.5 Gy, 363 μm after 1+1 Gy, 372 μm after 2+2 Gy, 341 μm after 3.5+3.5 and 301 μm after 7+7 Gy irradiation. Fractionating of the irradiation did not significantly change DP into normal tissue. Conclusion: Irradiation does not increase the penetration depth of doxorubicin into the normal tissue but might have a limiting impact on penetration and distribution of doxorubicin. Further studies are warranted to investigate the impact of addition of irradiation to PIPAC of tumor cells and to find out if irradiation can be used safely as chemopotenting agent for patients with peritoneal metastases treated with PIPAC.*

Correspondence to: Khashayar Fakhrian, Department of Radiation Oncology, Marien Hospital Herne, Ruhr-University Bochum, Hölkeskampring 40, 44625 Herne, Germany. Tel: +49 023234991531, Fax: +49 023234993306, e-mail: khfmed@yahoo.com

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Pressurized intra-peritoneal aerosol chemotherapy (PIPAC) has been introduced as an innovative approach to improve the treatment of advanced peritoneal carcinomatosis (PC). Using a micropump, the drug-containing solution is delivered into the abdominal cavity in the shape of micro droplets within a 12 mmHg "therapeutic capnoperitoneum" (1-3). Penetration depth of PIPAC has been reported to be 300-600 μm deep with a tissue concentration of 0.03-4.1 μmol/g (4). Despite improvements in the results of the treatment after PIPAC, outcomes of the patients with PC remain poor. Radiation therapy has been currently studied for the treatment of PC (5-8). However, dose-dependent treatment sequelae after irradiation limits the use of radiotherapy for treatment of PC. Low-dose irradiation might enhance the sensitivity of PC to cytotoxic agents (9-11). Theoretically, radiation might have a chemo-sensitizing effect and lead to higher tumor cell killing after PIPAC, provided that irradiation does not increase the

normal tissue drug penetration, which might lead to higher toxicity after PIPAC. We previously demonstrated that single fraction irradiation with higher doses might reduce the doxorubicin penetration (DP) in our *ex vivo* model. This study was conducted to compare the impact of single fractional with bi-fractional irradiation on the depth of DP into the normal tissue after PIPAC in our *ex vivo* model.

Materials and Methods

Ex vivo PIPAC model. Since the experiments were performed in an *ex vivo* model on commercially available tissue samples, no approval of the Local Board on Animal Care was required. Fresh *post-mortem* swine peritoneum was cut into 12 proportional sections. Two control samples were treated with PIPAC only (no irradiation), one sample on day 1, the other on day 2. Five samples were irradiated with 1, 2, 4, 7 or 14 Gy followed by PIPAC. Four samples were treated on day one with 0.5, 1, 2, 3.5 or 7 Gy and with the same radiation dose 24 hours later followed by PIPAC. The *ex vivo* PIPAC model has previously been described (10, 16). A commercially available hermetic sealable plastic box with a total volume of 3.5 lt, mimicking the abdominal cavity, was used. In the center of the top cover of the plastic box, 10- and 5-mm trocars (Kii® Balloon Blunt Tip System; Applied Medical, Rancho Santa Margarita, CA, USA) were placed. Using two trocars, the nozzle of the micropump (MIP®; Reger Medizintechnik, Rottweil, Germany) and a temperature/humidity sensor (XA 1000; Luft-, Mess- und Regeltechnik GmbH, Fellbach, Germany) probe were introduced. The plastic box was situated in a water bath (Typ 3043; Köttermann, Häningsen, Germany) and kept at a constant temperature of 36˚C during the whole procedure. Four fresh tissue specimens of peritoneum (German land race pigs), each measuring 3.0×3.0×0.5 cm, were placed at the bottom of the plastic box in direct extension of the axis of the micropump nozzle in the core of the aerosol jet (Figure 1). The distance between the nozzle of the MIP® and the bottom of the plastic box was 8 cm. The plastic box was then tightly sealed and a constant CO_2 capnoperitoneum of 12 mmHg (Olympus UHI-3, Olympus medical life science and industrial divisions; Olympus Australia, Notting Hill, Australia) was established throughout the entire PIPAC procedure. Three mg of doxorubicin (doxorubicin hydrochloride was purchased from Teva®, 2 mg/ml; Pharmachemie B.V., Haarlem, The Netherlands) was dissolved in 50 ml NaCl 0.9% at room temperature (23˚C) and aerosolized with a flow rate of 30 ml/min (pressure of up to 8 bars). After the aerosol phase, the tissue specimens were exposed to another 30 min of aerosolized doxorubicin (exposure phase).

Microscopic analysis. After treatments, all tissue samples were rinsed with sterile NaCl 0.9% solution in order to eliminate superficial cytostatics and immediately frozen in liquid nitrogen. Cryosections (10 μm) were prepared from different areas of each specimen. Sections were mounted with VectaShield containing 1.5 μg/ml 4',6-diamidino-2-phenylindole (DAPI) to stain nuclei. Penetration depth of doxorubicin was monitored using a Leica TCS SP8 confocal laser scanning microscope (Leica, Hessen, Wetzlar,Germany). The distance between the luminal surface and the innermost positive staining for doxorubicin accumulation was measured and reported in micrometers.

Statistical analyses. Experiments were independently performed four times. A total of ten tissue sections per tissue sample were subjected to DP measurement. The statistical analyses were performed using Sigma Plot 12 (Systat Software Inc., San Jose, CA, USA). The Kruskal-Wallis one way analysis of variance on Ranks was used to compare independent groups. A significant *p*-value was considered in case of $p<0.01$.

Table I. *Comparison of penetration depth of doxorubicin in normal tissue after pressurized intra-peritoneal aerosol chemotherapy (PIPAC) without irradiation (control sample) and single fraction irradiation plus PIPAC*.

Samples	PD (*p*-Value)
CS (PIPAC)	407 μm
d1: 1 Gy + PIPAC	396 μm (*vs*. CS, n.s.)
d1: 2 Gy + PIPAC	384 μm (*vs*. CS, n.s)
d1: 4 Gy + PIPAC	327 μm (*vs*. CS, n.s.)
d1: 7 Gy + PIPAC	280 μm (*vs*. CS, 0.0069)
d1: 14 Gy + PIPAC	243 μm (*vs*. CS, 0.0005)

CS, Control sample; PD, penetration depth; d1, day 1; n.s., non-significant.

Table II. *Comparison of penetration depth of doxorubicin in normal tissue after pressurized intra-peritoneal aerosol chemotherapy (PIPAC) without irradiation (control sample) and fractionated irradiation plus PIPAC*.

Samples	PD (*p*-Value)
CS (PIPAC on day 2)	364 μm
d1: (0.5 Gy) + d2 (0.5 Gy+ PIPAC)	376 μm (*vs*. CS, n.s.)
d1: (1 Gy) + d2: (1 Gy + PIPAC)	363 μm (*vs*. CS, n.s.)
d1: (2 Gy) + d2: (2 Gy + PIPAC)	373 μm (*vs*. CS, n.s)
d1: (3.5 Gy) + d2: (3.5 Gy + PIPAC)	342 μm (*vs*. CS, n.s.)
d1: (7 Gy) + d2: (7 Gy + PIPAC)	301 μm (*vs*. CS, 0.0036)

CS, Control sample; PD, penetration depth; d1, day 1; d2, day 2; n.s., non-significant.

Results

DP after PIPAC for the control samples were 407 μm and 373 μm, respectively. DP for samples with single fraction irradiation was 396 μm after 1 Gy, 384 μm after 2 Gy, 327 μm after 4 Gy, 280 μm after 7 Gy and 243 μm after 14 Gy. DP for samples with 2 fraction of irradiation was 376 μm after 0.5+0.5 Gy, 363 μm after 1+1 Gy, 372 μm after 2+2 Gy, 341 μm after 3.5+3.5 and 301 μm after 7+7 Gy irradiation. Fractionating of the irradiation did not significantly change the DP into normal tissue. Lower radiation doses after single or fractionated irradiation (cumulative radiation dose <7Gy) did not significantly change the DP after PIPAC compared to control samples (Tables I-II, Figures 2-3). A cumulative radiation dose of 14 Gy significantly reduced the DP into normal tissue irrespective of fractionation modality (Tables I-II). Single fraction irradiation with 7 Gy significantly reduced the

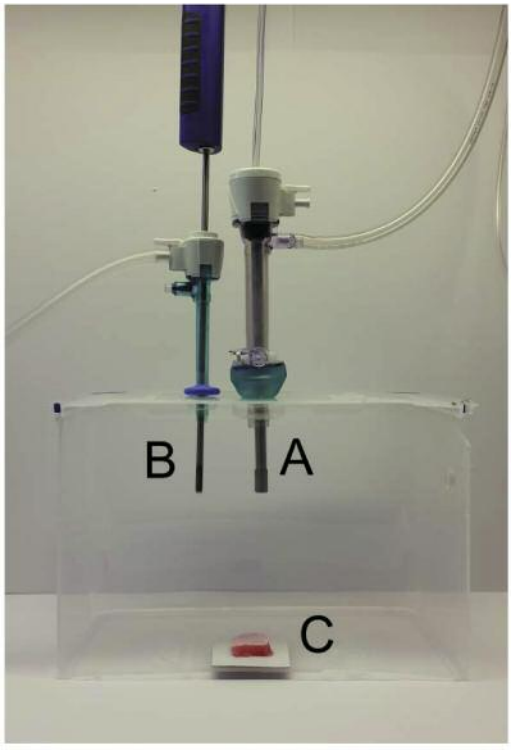

Figure 1. *Laparoscopy-like ex vivo experiment on fresh swine peritoneum to investigate the spatial distribution pattern of aerosolized doxorubicin during PIPAC therapy. For better demonstration, the front wall of the plastic box (ex vivo PIPAC model) has been removed. MIP® in the center of the top in a 10 mm trocar; laterally placed in a 5 mm trocar the temperature/humidity probe. The tissue sample was at the bottom of the box in the center of the aerosol spray jet.*

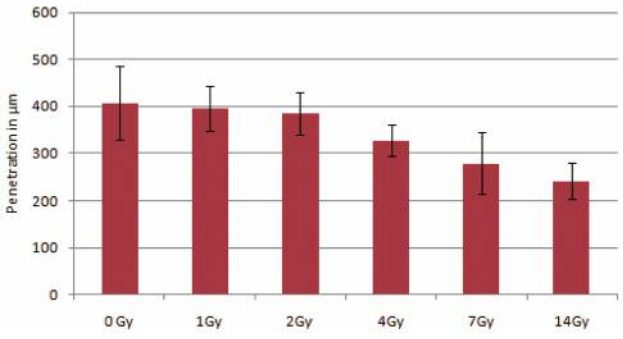

Figure 2. *Tissue penetration depth of doxorubicin after single fractional irradiation.*

penetration depth of doxorubicin into the normal tissue (Table I, Figure 4), whereas irradiation with 7 Gy in two fractions did not significantly impact the penetration depth of doxorubicin (Table II, Figure 3).

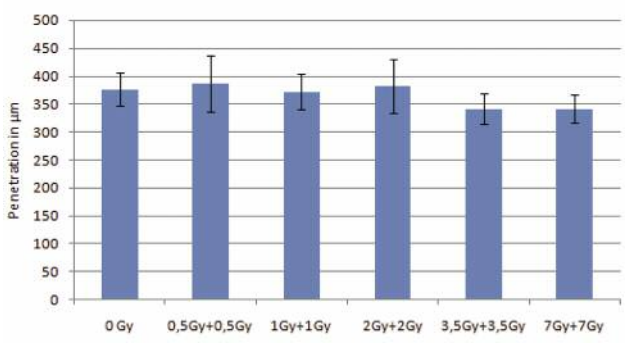

Figure 3. T*issue penetration depth of doxorubicin after bi-fractional irradiation.*

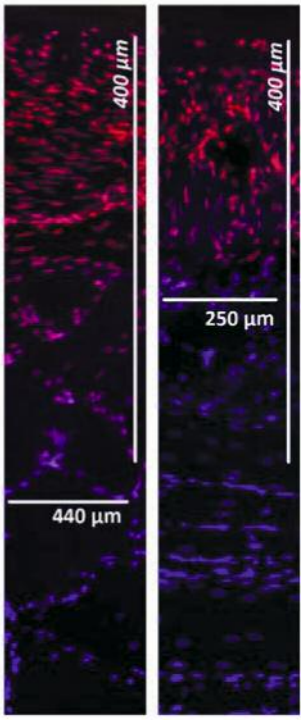

Figure 4. *Microscopic analysis of maximum and minimum penetration depth of doxorubicin into fresh peritoneal tissue samples of German land race pigs. Nuclei (blue) were stained with 4',6-diamidino-2-phenylindole (DAPI). Left side to right: 0 versus 7 Gy.*

Discussion

Despite significant progress in chemotherapeutic regimens, poor response to systemic treatment is observed in a considerable part of patients, which is mainly due to molecular mechanisms and limited drug distribution in the tumor (12-14). The novel PIPAC approach may be a

promising new treatment for PC for the next decade. It offers hope to patients who, in the past, could not escape from a terminal illness. PIPAC therapy has been introduced as an innovative approach to improve the treatment of advanced, multi-resistant PC. Data obtained in animal experiments report a homogenous spatial methylene blue distribution pattern in the abdominal cavity after PIPAC (4, 15). However, recent findings demonstrate controversial results concerning distribution pattern of cytotoxic agents during PIPAC (16). Consequently, further optimizing PIPAC is necessary as it has already demonstrated advantageous results in the clinical setting (17-19). Several authors have reported that increasing intraperitoneal pressure enhances the diffusion of drugs into the tumor, resulting in a higher local depot (20, 21). Adding irradiation to PIPAC might increase the efficacy of PIPAC through several mechanisms. Several studies have shown that low-dose irradiation might enhance the sensitivity of peritoneal cancer tumor cells to cytotoxic agents (9-11). Theoretically, radiation might have a chemo-sensitizing effect and lead to higher tumor cell killing after PIPAC, provided that irradiation does not (i) negatively impact the tissue penetration depth or tissue concentration of cytotoxic agent in the tumor and (ii) increase the tissue penetration depth or tissue concentration of cytotoxic agent in the normal tissue.

We previously demonstrated that higher single fractional irradiation might reduce the penetration depth of doxorubicin into the normal tissue (22). The comparison of single-fractional *vs*. bi-fractional irradiation, in the present study, confirms our previous results that irradiation does not increase the penetration depth of doxorubicin into the normal tissue, whether in single fractional modality or in two fractions. We observed a general reduction in the penetration depth of doxorubicin in the peritoneum on the second day. This might be due to the ongoing loss of *post-mortem* cellular functions. Our data indicate that irradiation does not increase the penetration depth of doxorubicin into the normal tissue. However, one should not forget that our experiments were performed in a *post-mortem* model as the response in the living normal tissue might be totally different. Nevertheless, the described findings can be used for further experimental studies to evaluate the efficacy of adding irradiation to PIPAC.

Conclusion

Our data indicate that irradiation does not increase the penetration depth of doxorubicin into the normal tissue. Higher fractional dose might have a limiting impact on penetration and distribution of doxorubicin into the normal tissue. Further studies are warranted to investigate the impact of addition of irradiation to PIPAC of tumor cells and to find out if irradiation can be safely used as chemopotenting agent for patients with peritoneal metastases treated with PIPAC.

Conflicts of Interest

This study was financed by institutional funds. The Authors have no conflicts of interest or financial ties to disclose.

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