Establishment of a new valid animal model for the evaluation of hyperthermic intraperitoneal chemotherapy (HIPEC) in pediatric rhabdomyosarcoma

Benedikt Wagner[1], Anna Adamus[1], Dörthe Sönnecken[1], Reza Vahdad[1], Paul Jank[1], Carsten Denkert[1], Andreas Mahnken[1], and Guido Seitz[1]

[1]University Hospital of Giessen and Marburg Campus Marburg

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Abstract

Background: Cytoreductive surgery in combination with hyperthermic intraperitoneal chemotherapy has been established as a novel treatment approach for peritoneal sarcomatosis. Despite promising clinical reports, there is still a lack of knowledge regarding optimal drug usage and local effects. Therefore, we intended to establish a murine animal model for further evaluation. Procedure: Alveolar rhabdomyosarcoma cells were xenotransplanted into NOD/LtSz-scid IL2Rγnullmice (n=100). The mice received a continuous intraperitoneal lavage with isotonic saline solution as control- or with cisplatin (30 or 60 mg/m2) as treatment group for 60 minutes at 37 or 42 °C (6 subgroups, each n=16). Tumor spread was documented by an adapted peritoneal cancer index and MRI (n=4). Tumor and tissue samples, harvested at the end of the perfusion, were evaluated regarding morphology, proliferation and apoptosis (H&E-, Ki-67-, Cleaved Caspase 3-staining, TUNEL-assay). Results: Extensive peritoneal sarcomatosis in over 91% of the cases was observed. HIPEC was feasible without acute side effects. Ki-67 staining revealed concentration- or temperature-dependent effects of cisplatin-based HIPEC on the tumors. While Cleaved Caspase-3 showed only sporadic apoptotic effects. TUNEL-assay detected concentration- or temperature-dependent apoptotic effects at the outer tumor surface. MRI scans confirmed the observed tumor dissemination. Conclusion: This is the first animal model for evaluation of HIPEC in pediatric RMS in mice. Cisplatin-based HIPEC had early effects on the proliferation whereas circumscribed apoptotic effects could be detected at the tumor surface. This model allows further insights on the possible efficiency of HIPEC in RMS. Further studies using other drug combinations and treatment will follow.

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Benedikt R. Wagner[1*, +], Anna L. Adamus[1+], Dörthe Sönnecken[1], Reza Vahdad[1], Paul Jank[2], Carsten Denkert[2], Andreas H. Mahnken[3, 4], Guido Seitz[1]

[1] Department of Pediatric Surgery, University Hospital Marburg, Baldingerstrasse,

35033 Marburg, Germany.

[2] Department of Pathology, University Hospital Marburg, Baldingerstrasse, 35033

Marburg, Germany.

[3] Department of Radiology, University Hospital Marburg, Baldingerstrasse, 35033

Marburg, Germany.

[4] Center for Tumor Biology and Immunology, Core Facility for Small Animal MRI,

Hans-Meerwein Str. 3, 35043 Marburg, Germany.

+ Dres. Wagner and Adamus contributed equally to the first authorship of this paper.

* Corresponding author:

Benedikt Wagner, Department of Pediatric Surgery, University Hospital Marburg,

Baldingerstrasse, 35033 Marburg, Germany.

Tel.: +49 (0) 6421/58-66226

Email: bwagner@med.uni-marburg.de

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HIPEC animal model for rhabdomyosarcoma

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Alveolar rhabdomyosarcoma, hyperthermic intraperitoneal chemotherapy (HIPEC), Cisplatin, cytoreductive surgery (CRS), animal model

Abbreviations

CRS	Cytoreductive surgery
DSRCT	Desmoplastic small round cell tumor
HIPEC	Hyperthermic intraperitoneal chemotherapy
PCI	Peritoneal carcinomatosis index
PS	Peritoneal sarcomatosis
MRI	Magnetic Resonance Imaging
RMS	Rhabdomyosarcoma

Abstract

Background: Cytoreductive surgery in combination with hyperthermic intraperitoneal chemotherapy has been established as a novel treatment approach for peritoneal sarcomatosis. Despite promising clinical reports, there is still a lack of knowledge regarding optimal drug usage and local effects. Therefore, we

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Procedure: Alveolar rhabdomyosarcoma cells were xenotransplanted into NOD/LtSz-scid IL2Rγnullmice (n=100). The mice received a continuous intraperitoneal lavage with isotonic saline solution as control- or with cisplatin (30 or 60 mg/m^2) as treatment group for 60 minutes at 37 or 42 °C (6 subgroups, each n=16). Tumor spread was documented by an adapted peritoneal cancer index and MRI (n=4). Tumor and tissue samples, harvested at the end of the perfusion, were evaluated regarding morphology, proliferation and apoptosis (H&E-, Ki-67-, Cleaved Caspase 3-staining, TUNEL-assay).

Results: Extensive peritoneal sarcomatosis in over 91% of the cases was observed. HIPEC was feasible without acute side effects. Ki-67 staining revealed concentration- or temperature-dependent effects of cisplatin-based HIPEC on the tumors. While Cleaved Caspase-3 showed only sporadic apoptotic effects. TUNEL-assay detected concentration- or temperature-dependent apoptotic effects at the outer tumor surface. MRI scans confirmed the observed tumor dissemination.

Conclusion: This is the first animal model for evaluation of HIPEC in pediatric RMS in mice. Cisplatin-based HIPEC had early effects on the proliferation whereas circumscribed apoptotic effects could be detected at the tumor surface. This model allows further insights on the possible efficiency of HIPEC in RMS. Further studies using other drug combinations and treatment will follow.

Introduction

Peritoneal sarcomatosis (PS) of rhabdomyosarcoma (RMS) is rare in children and has been reported only in smaller case series. The prognosis of these children is still sobering and it represents a therapeutic challenge due to high probability of tumor recurrence. In the past, treatment of PS in childhood was limited to aggressive surgical excision, radiotherapy, and chemotherapy with a palliative intention. Due to recent advances, cytoreductive surgery (CRS) in combination with hyperthermic intraperitoneal chemotherapy (HIPEC) have been established as a novel treatment option for PS and might prolong remission . Based on the therapeutic success in the treatment of PS in adults CRS and HIPEC were used successfully in a phase-I-trial in children and young adults with PS of RMS and others . Hayes-Jordan*et al.* confirmed an improvement of overall survival after CRS and HIPEC in children with PS of desmoplastic small round cell tumor (DSRCT) in combination with radiotherapy whereas it remains unclear if either CRS or HIPEC or radiotherapy or the combination of all determines the therapeutic success . Besides therapeutic benefits HIPEC was well tolerated in terms of toxicity and side effects in a case series of children (<5 years) with PS of RMS .

Despite recent promising clinical reports and due the rarity of CRS and HIPEC in PS in children, there is still a lack of knowledge regarding the best choice of chemotherapeutic drugs and concentration, optimal drug combination, treatment duration and local effects like penetration depth of chemotherapy during HIPEC. To answer these questions, we intended to establish a murine animal model of PS of RMS for further evaluation.

Methods

Cell Culture Procedure

The human alveolar RMS cell line RH-30 (No. ACC-489, DSMZ, Braunschweig, Germany) was obtained from the German biological resource bank ´DSMZ´(https://www.dsmz.de). The RH-30 cells were cultured in Dulbecco's modified Eagle's medium plus Ultraglutamine 1 (Lonza, Verviers, Belgium) with 10% fetal calf serum (FCS) (PAN Biotech GmbH, Aidenbach, Germany) and 1% antibiotic-antimycotic solution (Gibco, Paisley, UK) under humidified conditions at 37 °C and 5% CO_2 atmosphere. All cells were mycoplasma negative. Every second day the culture medium was changed, and confluent cancer cells were treated with 0.05% trypsin – 0.02% EDTA (Lonza, Verviers, Belgium).

Animal model

Eight- to eleven-week-old NOD/LtSz-scid IL2Rγnullmice, weighing 25 to 30g, were used for all experiments. This immunodeficient mouse strain was initially described and established by Shultz *et al.* . The animals were initially sourced from Jackson Laboratories (Bar Harbor, ME, USA) for the establishment of an own breeding and subsequently obtained from our own animal facility. A total number of 100 animals were used for all the experiments. Animals were kept under specific pathogen-free conditions, fed an autoclaved standard diet and given free access to sterilized water. All animal experiments were approved by the local government ethics committee for animal studies (Regierungspräsidium Gießen, V54 – 19 c 20 15 h 01 MR 20/14, No. G38/2017). To ensure a comparable tumor growth, only RH-30 tumor cell passages 2-5 were used for the xenotransplantation. The RH-30 tumor cells were grown and trypsinized as described above and then resuspended in 0.9% sterile sodium chloride solution (Ecolav® B.Braun, Melsungen, Germany). A total number of 2 x 10^6 RH-30 tumor cells were injected into the lower left flank of the animals.

Intraperitoneal lavage/HIPEC

Intraperitoneal lavage treatment was performed 21 days after xenotransplantation (RH-30). In the control group, a continuously intraperitoneal lavage with isotonic saline solution (37 or 42 °C, each group n=16) was used for 60 minutes. In the treatment group, animals were treated with intraperitoneal cisplatin (30 or 60mg/m^2) for 60 minutes at 37 or 42 °C (each group n=16). The groups (each group n = 16) with the corresponding dosages and temperatures are given in Table 1. An inflow catheter was introduced into the right and an outflow catheter into the left lower abdomen. The lavage was administered continuously into the abdominal cavity via the inflow catheter (perfusion rate: 180 ml per hour). The fluids of the outflow were trapped by drainage bags. The lavage fluids were heated up to 37 or 42 °C using a water bath. Figure 1 gives an overview about the experimental setup.

All experiments were performed under general anesthesia and prophylactic pain treatment according to the guidelines of the local government ethics committee for animal studies (Regierungspräsidium Gießen, V54 – 19 c 20 15 h 01 MR 20/14, No. G38/2017). Initial sedation was performed in a whole-body chamber with 4-5% isoflurane (AbbVie Inc., Ludwigshafen, Germany). Further anesthesia and prophylactic pain management were performed with 0.05 mg/kg body weight fentanyl (Hameln Pharma plus GmbH, Hameln, Germany) + 5 mg/kg body weight midazolam (F. Hoffmann-La Roche AG, Basel, Switzerland) + 0.5 mg/kg body weight dexmedetomidine (EVER Valinject GmbH, Gröbenzell, Germany) subcutaneously and maintained after 10-15 min by a continuous supply of 2% isoflurane mixed with oxygen (Linde AG, Hanover, Germany). Body temperature of mice was monitored using an anal temperature probe. After 60 minutes of intraperitoneal lavage, a median laparotomy was performed. Tumor dissemination was documented by the peritoneal carcinomatosis index (PCI) according to the principle of Jacquet and Sugarbaker *et al.* (2006) and adapted for the animal model (Fig. 2) . Tumors and its corresponding regions were photographed using the camera of a 3 mm 0° laparoscope (KARL STORZ GmbH & Co. KG, Tuttlingen, Germany).

Finally, the animals were sacrificed by administration of a lethal dose of isoflurane.

Magnetic resonance imaging (MRI)

Exemplary magnetic resonance images of four animals were taken before, during and after HIPEC with and without the administration of a Gadolinium-containing contrast agent (dosage: 0.1 ml/kg body weight; Gadovist, Bayer Vital GmbH, Leverkusen, Germany) into the tail vein. MR imaging was performed with the experimental 7 Tesla magnetic resonance imaging machine (ClinScan 70/30 USR, Bruker BioSpin MRI GmbH, Ettlingen, Germany). The system consists of a magnetic tube with an inner diameter of 30.2 cm and a preparation plate with a rail for attaching an animal bed. The examined animals were positioned and fixed in the center of the MRI for the exclusion of movement artefacts. They were continuously sedated with an isoflurane-oxygen mixture (AbbVie Inc., Ludwigshafen, Germany) via an anesthetic mask and the respiratory

rate was recorded. The MRI examination protocol includes several horizontal and frontal measurements for morphological imaging.

Histological analysis and Immunohistochemistry

After laparotomy, healthy control (liver, spleen, peritoneum) and tumor tissues were harvested for the histological work-up. The tissues were fixed in 3.7% formalin, paraffin-embedded (Tissue-Tek® TECTM blocking station, Sakura Finetek Co., Ltd., Japan), cut into 3-5 µm thin tissue sections (Leica SM 2000 R Sliding Benchtop Microtome, Leica Camera AG, Wetzlar, Germany), stretched (paraffin stretching bath type 1052, Gesellschaft für Labortechnik mbH, Germany) and mounted on SuperFrost® Plus slides (Gerhard Menzel B.V. & Co. KG, Germany). Subsequently, the sections were dried and fixed over night at 60 °C (heating cabinet type BK3064, Dipl. Ing. W. Ehret GmbH, Germany), deparaffinized with xylol (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and rehydrated in a descending ethanol alcohol series (Otto Fischar GmbH + Co. KG, Saarbrücken, Germany). A standard hematoxylin (MEDITE GmbH, Burgdorf, Germany) and eosin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) staining was carried out for the evaluation of necrosis and histological changes. For immunohistochemical analysis of proliferation and apoptosis protein marker expression, the tumor tissues were labeled with primary antibodies detecting Ki-67 (#M7240, Dako Cytoformation, Glostrup, Denmark; dilution 1:100) as proliferation marker or Cleaved Caspase-3 (#9661, Cell Signaling, Beverly, USA; dilution 1:400) as marker for apoptosis induction. Primary antibodies were diluted in a 2% BSA solution and incubated for one hour at room temperature, followed by another one-hour incubation with a secondary antibody-labeled, polymer-horseradish peroxidase (HRP) (Dako Envision+ Kit; Dako, Glostrup, Denmark). AEC (3-amino-9-ethylcarbazol) served as chromogenic agent. All sections were counterstained with Mayer's hemalum solution (Merck KGaA, Darmstadt, Germany). Each section was digitalized as a whole slide image (WSI) by using a PreciPoint M8 microscope with an integrated scanner and analyzed with the microscopy software ViewPoint Light (PreciPoint, Freising, Germany). The Ki-67 proliferation index was recorded via automated analysis of WSI with a Ki-67 quantifier module of a pathology software (Cognition Master Professional Suite: Ki67 Quantifier, VMscope GmbH, Berlin, Germany).

TUNEL assay

For investigations on induced apoptosis in tumors and surrounding tissues, terminal desoxyribosyltransferasemediated dUTP nick-end labeling test (In Situ Cell Death Detection Kit, Fluorescein, Roche Diagnostics GmbH, USA) was performed. For the TUNEL method, the 3 µm tissue sections were also deparaffinized and rehydrated in a descending alcohol series, followed by a permeabilization step in permeabilization buffer (0.1% Triton X-100, 0.1% tri-sodium citrate dihydrate) for 8 min at room temperature and twice washing step with PBS. The enzyme and labelling solutions provided in the TUNEL-assay kit were diluted with each other in a ratio of 1:10 and 30-50 µl of this TUNEL reaction mixture was added and the samples were incubated for 60 min at 37 °C. Afterwards the samples were washed thrice with PBS. For positive control of apoptosis, a preparation was treated with 0.3 mg/ml DNase I (Roche Diagnostics GmbH) in PBS with magnesium and calcium for 15 min at room temperature and washed twice with PBS. For a negative control, only the labelling solution without enzyme was added to the preparation. The slides were mounted and nuclear counterstained with VECTASHIELD® HardSet Antifade Mounting Medium with DAPI (VECTOR Laboratories, Burlingame, USA). Fluorescence microscopic images were captured on a wide field microscope (Leica DM5500, Leica, Wetzlar, Germany).

Statistical analysis

All experiments were replicated at least three times, and data sets were expressed as means +- standard deviations (SD). Statistically significant differences were compared using the unpaired Student's t-test. P values: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ were considered statistically as significant. All analyses were performed

with the software Microsoft Excel 2017 and Graphpad Prism Version 8 (http://www.graphpad.com/scientific-software/prism/).

Results

Initially, 100 NOD/LtSz-scid IL2Rγnullmice were used for this study. Three weeks after being xenotransplanted with RH-30 cells, tumor growth was observed in 91 mice. No tumor dissemination occurred in 9 mice. Five mice showed extraperitoneal tumor growth after failed intraperitoneal injection. No mouse was prematurely sacrificed due to tumor progression. Sixteen mice died prematurely during treatment and four mice were used for the MRI scans. The remaining 66 mice were finally included into the statistical analysis.

Conventional laparotomy after treatment revealed an extensive peritoneal dissemination of RMS in 91% of the cases. Tumors could easily be distinguished from surrounding tissue by their specific morphology. The histological examination confirmed alveolar rhabdomyosarcoma cells in the observed tumors. The largest tumor masses (> 3 mm size) were typically found in the epigastric region on the greater curvature of the stomach. Smaller tumors (1 – 3 mm size) were detected in the right and left upper region, perihepatic and perisplenic or in the left lower abdomen. Disseminated small tumor nodes (< 1 mm size) occurred at the mesentery of the small and large intestine. Other tumor localizations like the flank, pelvis or peritoneum of the abdominal wall only showed small tumor spots. Mean PCI was 8.03 (Fig 2).

Anesthesia and intraperitoneal lavage were feasible without acute side effects in 87% of cases. 16 mice died during treatment due to respiratory failure. All these 16 mice had additionally lower body weight compared to those mice which tolerated the intraperitoneal lavage well. HIPEC at 37 °C resulted in mild hypothermia (body temperature 34 – 36 °C) only in isolated cases (n=6). Body temperature could be normalized by using a physical heat source. Hyperthermia (body temperature > 39 °C) during HIPEC at 42 °C did not occur due to fast heat losses in anesthetized mice.

MRI with or without contrast agent during HIPEC was technically feasible. One mouse died during MRI scans due to breathing arrest. MRI scans confirmed the observed tumor dissemination and could illustrate distribution of the lavage during HIPEC for the first time (Fig. 3).

By H&E-staining, no cell morphological changes were evident in the tissues of the tumors (Fig. 4A) and the representatively examined control organs (liver, spleen, and peritoneum) (Supplemental Figure S1).

Evaluation of tumor proliferation by Ki-67 immunohistochemistry and digital image analysis revealed early temperature-dependent effects after intraperitoneal lavage on the tumors (Fig. 4B). There was a significant difference in tumor proliferation after having used isotonic saline solution heated up to 42 °C compared to the group, in which 37 °C warm isotonic saline solution was infused. Early concentration- or temperature-dependent effects of cisplatin-based HIPEC on the tumors could be revealed. Significant effects on tumor proliferation were shown in each HIPEC group compared to the control group (saline solution, 37 °C) dependent on either Cisplatin-concentration, temperature of the lavage or the combination of both. Comparing the HIPEC groups with each other there was no significant difference in the proliferation index (Fig. 4C).

Cleaved-Caspase 3 staining revealed no early apoptosis induction. There were no differences in the sub-groups and no temperature- or chemotherapy-dependent effect was seen especially at the outer tumor surface. Only sporadic apoptotic effects scattered all over the tumor without clear focus were seen (Fig. 5A).

Using the TUNEL-assay method early apoptotic effects at the outer tumor surface could be detected. Immunofluorescence microscopy revealed an increase in apoptotic cell layers dependent on the treatment. Five to ten cell layers of the tumor surface have been affected dose and temperature dependent after HIPEC. The penetration depth of the treatment was approximately 30 to 40 µm of the outer tumor surface. Lavage heated up to 42 °C compared to 37 °C warm lavage (saline solution or Cisplatin) tends to result in more apoptotic affected cells visually observed. The most affected and apoptotic cell layers or the deepest penetration of the lavage was seen in the HIPEC sub-group using 60mg/m^2 Cisplatin heated up to 42 °C (Fig. 5B)

Discussion

PS of RMS is rare in children and still represents an oncological and therapeutic challenge . Due to recent promising clinical reports HIPEC (in combination with CRS) has been established as a novel treatment option for PS of RMS . Besides significant improvement of the technical performance of HIPEC in children there is still no consistent treatment plan regarding the best choice of chemotherapy and concentration, optimal drug combination and treatment duration. Additionally, it remains unclear whether CRS or HIPEC are the main factors for treatment success. Here, we established a feasible and reproducible murine model for the evaluation of HIPEC in pediatric RMS for the first time. At this juncture there is no animal model available allowing repeatable experimental studies of HIPEC within the context of disseminated intraperitoneal pediatric RMS.

We were able to demonstrate a constant and reliable intraperitoneal tumor growth of human alveolar RMS (cell line RH-30) in NOD/LtSz-scid IL2Rγnullmice. This model resembles pediatric RMS in terms of organ invasion and intraperitoneal tumor spread very well. To evaluate the tumor dissemination, we adapted the peritoneal carcinomatosis index (PCI) modelled on Jacquet and Sugarbaker *et al.* for this animal model. Furthermore, we developed a feasible and easy way for the application of liquid hyperthermic intraperitoneal chemotherapy in mice . MRI scans confirmed the tumor growth and showed the performance of HIPEC *in vivo* for the first time. Besides our animal model there are only few recent comparable HIPEC mouse models but using non pediatric tumor entities like adult ovarian cancer or performing HIPEC as pressurized aerosol . Especially pressurized intraperitoneal chemotherapy (PIPAC) has not been applied in children.

Using TUNEL-assay method we demonstrate different penetration depth (30 - 40 µm) of the HIPEC treatment depending on cisplatin and/or hyperthermia. Early concentration- or temperature-dependent antitumor effects of cisplatin-based HIPEC on the tumors could be revealed immediately after treatment. The observed penetration depth of cisplatin-based HIPEC is considerably more superficial as those published by Goodman *et al* . . However, cisplatin has a favorable area under the curve ratio (maximal the chemotherapeutic dose, minimal systemic toxicity) for the intraperitoneal application but there is no clear statement regarding the penetration depth of cisplatin-based HIPEC for pediatric RMS till now . In the past, cisplatin was used as single therapeutic agent in different concentrations for HIPEC in children suffering from intraperitoneal RMS . Whereas the synergy between heat and drug cytotoxicity could be confirmed, the optimal concentration of cisplatin for HIPEC remains unclear . Until now cisplatin concentrations for HIPEC derive from those for intravenous chemotherapy (15-30 mg/m^2) knowing that higher cisplatin dosage with respectively higher effectiveness and tolerable toxicity are feasible . As final experiment ending immediately after HIPEC it was not possible to capture late anti-tumor effects after intraperitoneal chemotherapy lavage knowing that cellular signal cascades and apoptosis pathway need more time .

Several limitations should be pointed out in this study. Due to ethics committee approval CRS before HIPEC was not performed. Focusing the feasibility of a HIPEC animal model for pediatric RMS, we did not do any cytoreductive surgery before HIPEC compared to existing clinical studies. The recommendation of administration of HIPEC after CRS is generally based on clinical studies for adults whereas it remains unclear if either CRS or HIPEC or the combination of both determines the therapeutic success in children . Nevertheless, the observed intraperitoneal tumor growth in our animal model was a disseminated miliary tumor spread (maximal 3 to 4 cm in diameter) compared to an incomplete tumor resection in children. In combination with HIPEC residual tumor of 2,5 cm can be tolerated with the same outcome due to local tumor control through tumor penetration of HIPEC . All the few existing data regarding CRS and HIPEC in children were lifted from restricted clinical case reports . Ethical considerations constrain the systematic *in vivo* study of HIPEC in children regarding optimal drug concentration and combination. With the establishment of an appropriate animal model this limitation can now be overcome. Our model allows valid additional insights on the possible efficiency of HIPEC in pediatric RMS to improve existing treatment strategy and manage the therapeutic challenge.

In summary, we were able to establish the first animal model for evaluation of HIPEC in pediatric rhabdomyosarcoma in mice. Using TUNEL-method early cisplatin- and hyperthermia-dependent apoptotic effects

at the tumor surface (penetration depth 30 – 40 µm) can be detected. Additionally, statistically significant reduction of early tumor proliferation depends on cisplatin or hyperthermia. This model allows valid additional insights on the possible efficiency of HIPEC in RMS so further studies using other drug combination, concentration and cytoreduction will follow.

Conflicts of Interest

The authors declare no conflict of interest.

Authors Contributions

GS conceived the project, edited, and revised the manuscript. BW, AA and DS performed the research and analyzed the data. BW and AA drafted the article. BW, AA, and GS wrote the article. All authors have read and approved the final version of the manuscript.

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Legends

Fig. 1 Schematic illustration of the HIPEC procedure. Saline solution and fluid containing cisplatin were heated up through a water bath (37 or 42 °C) and circulated continuously into the abdominal cavity (perfusion rate: 180 ml per hour), through an inlet catheter into the right and an outlet catheter into the left lower abdomen. The temperature and flow are constantly monitored, and the outflow fluid is trapped by a drainage bag.

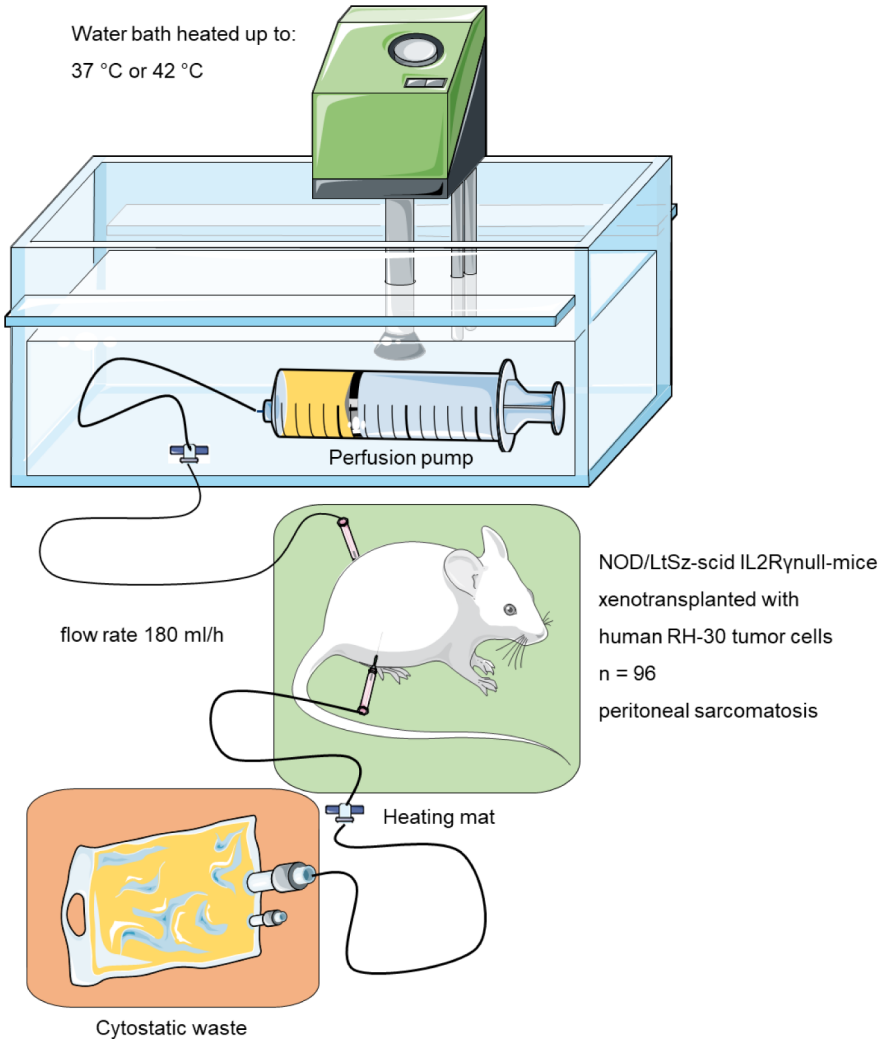
Fig. 2 Overview about the peritoneal carcinomatosis index (PCI). **(A)** Total and PCI of the respective organs was recorded according to the principle of Jacquet and Sugarbaker *et al.*(1996), which has been adapted for the animal model. PCI lesion size score: 0 = no tumor; 1 = tumor [?] 1 mm; 2 = tumor > 1 mm [?] 3 mm; 3 = tumor > 3 mm. **(B)** Exemplary photographic representation of the main tumor localizations. There are up to five intra-abdominal tumor sites, which are preferentially located on the large curvature of the stomach, peri-hepatic, peri-splenic, mesenteric and peritoneally on the abdominal wall.

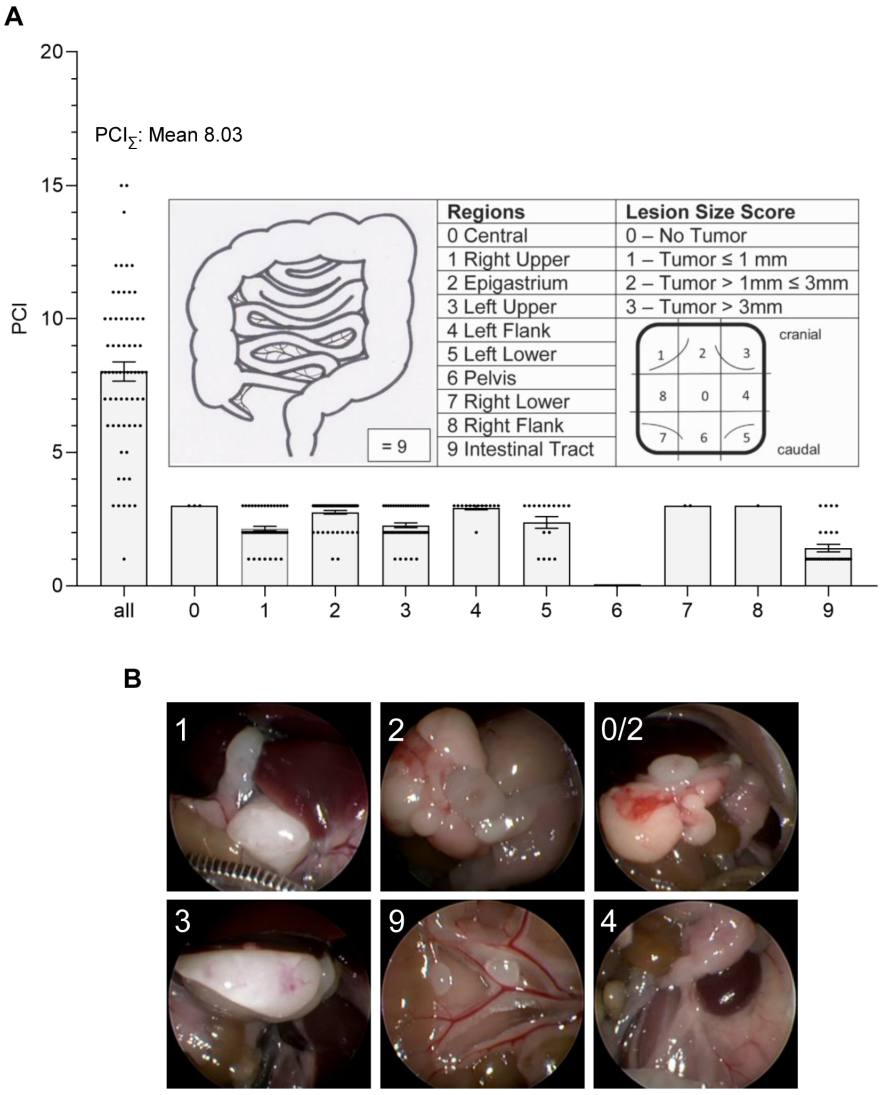
Fig. 3 *In vivo* detection of RH-30 tumors by MR imaging. RH-30 tumors were detected by MR imaging of the abdominal cavity**(A)** without (native) and **(B)** with contrast agent.**(C)** For evaluation of the fluid distribution in the abdominal cavity during peritoneal lavage, MR images were also taken during HIPEC treatment. MR imaging confirmed the observed tumor dissemination depicted in Figure 2. Tumor lesions are indicated by red asterisks.

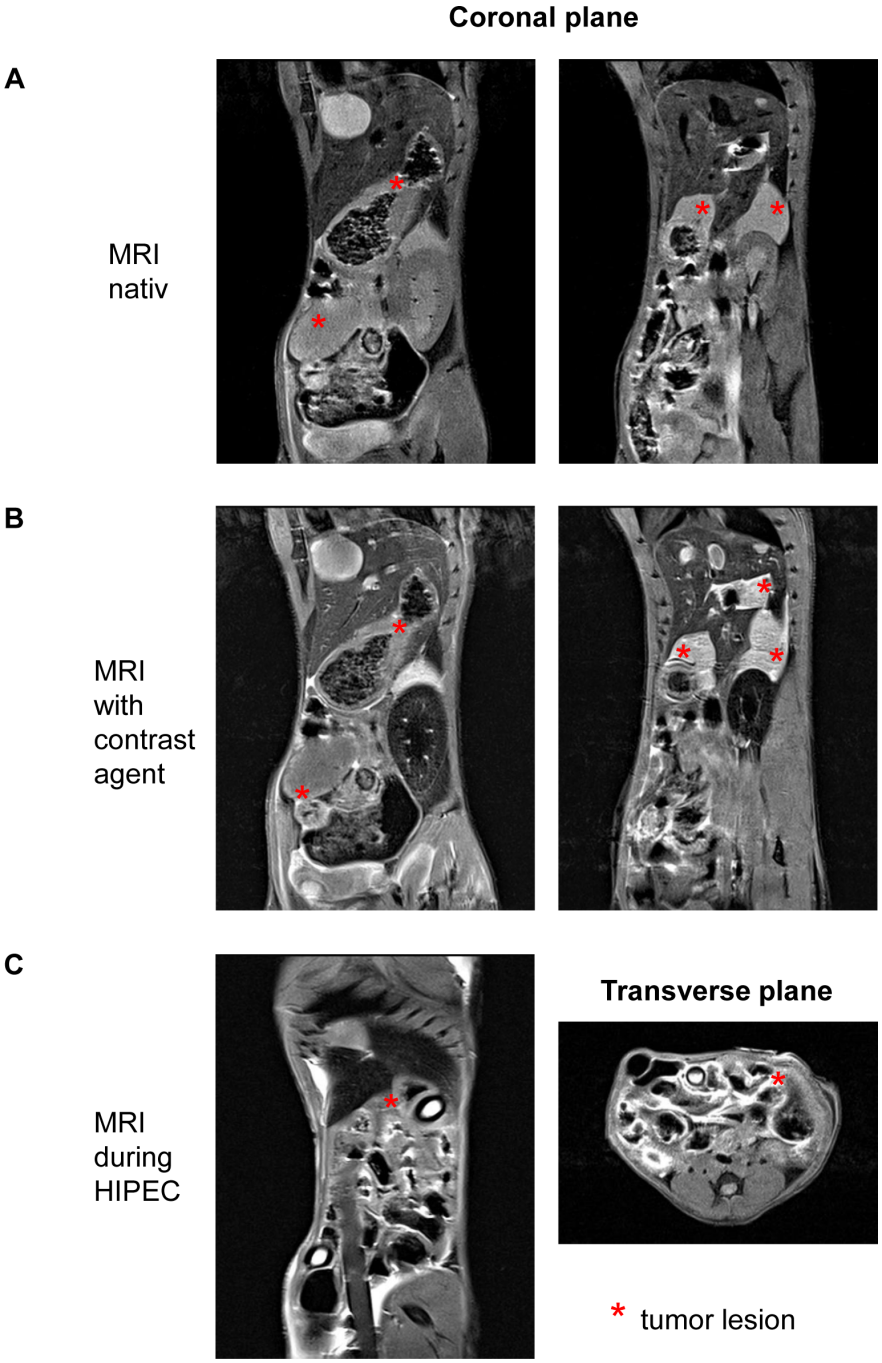
Fig. 4 Evaluation of changes in cell morphology and proliferation marker expression after HIPEC treatment. **(A)**Hematoxylin and eosin staining revealed no cell morphological changes in RH-30 tumors. **(B)**

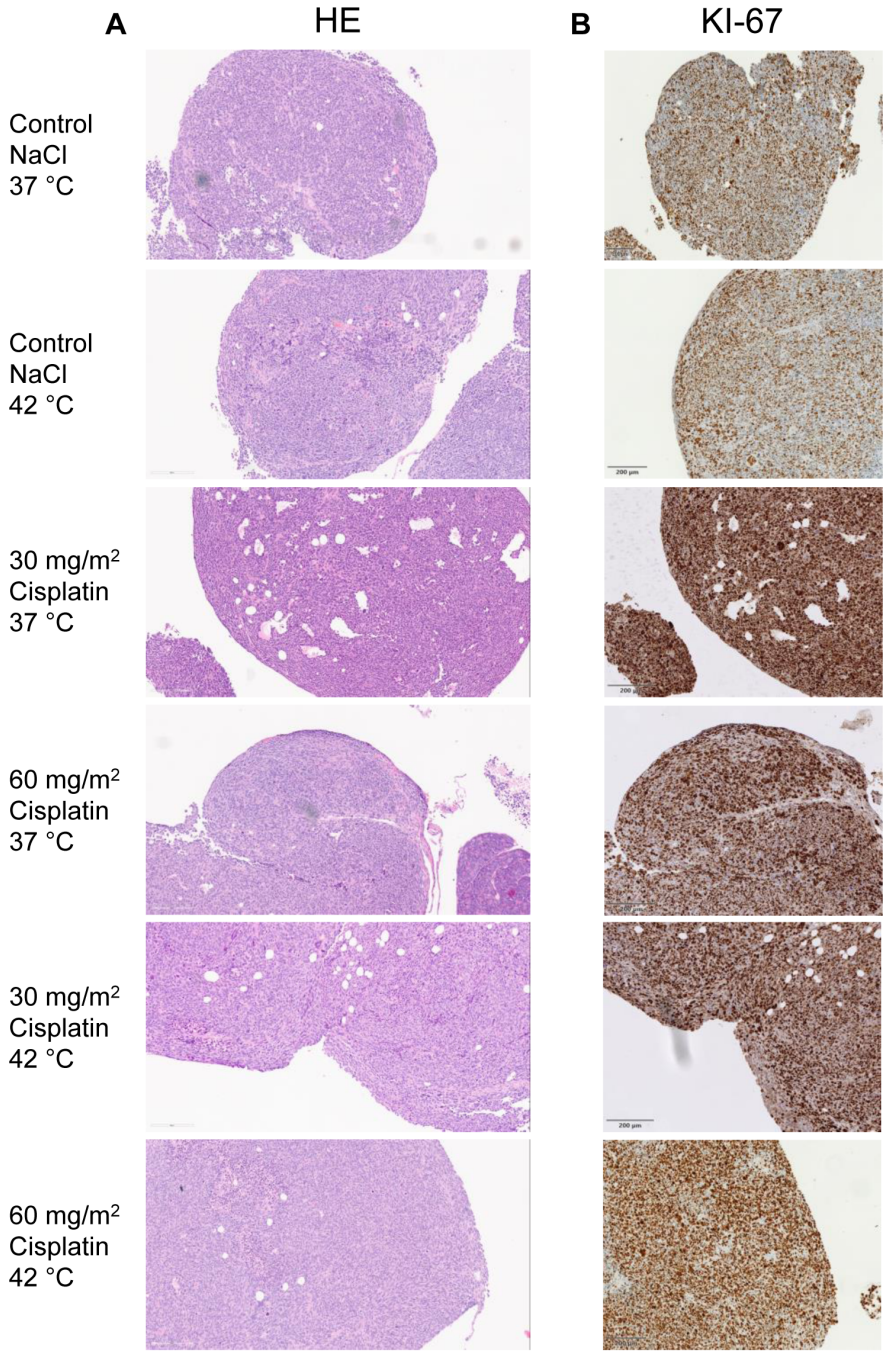
Immunohistochemical analysis of the proliferation marker Ki-67 (brown) showed no obvious changes in protein expression through the individual treatments. **(C)** The detailed examination of Ki-67 protein expression and determination of the Ki-67 proliferation index using a Ki-67 quantifier module of a pathology software (Cognition Master Professional Suite: Ki67 Quantifier, VMscope GmbH, Berlin, Germany) showed a slight reduction in the proliferation capacity.

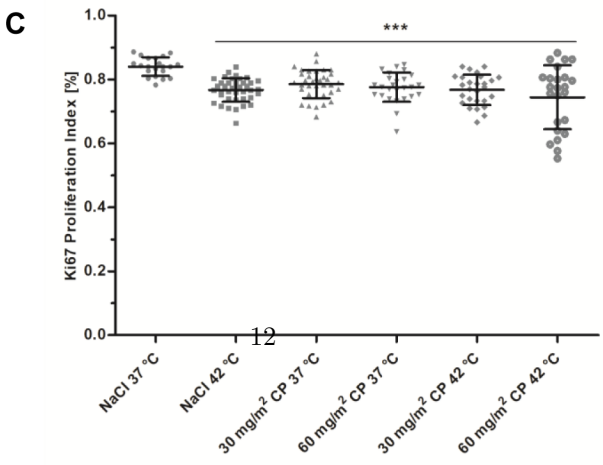
Fig. 5 Investigation of markers for apoptosis induction after HIPEC treatment. **(A)** Apoptosis marker expression of the activated variant of Caspase 3 (black) revealed no HIPEC-specific apoptosis induction. The nuclei were counterstained with hematoxylin (blue). **(B)** Terminal desoxynucleotidyl transferase (TUNEL)-labelled DNA fragmentation as a sign of apoptosis induction (green fluorescence signal) is visible in the marginal areas of the HIPEC treated tumors in a dose and temperature dependent manner. The nuclei were counterstained in blue with DAPI.











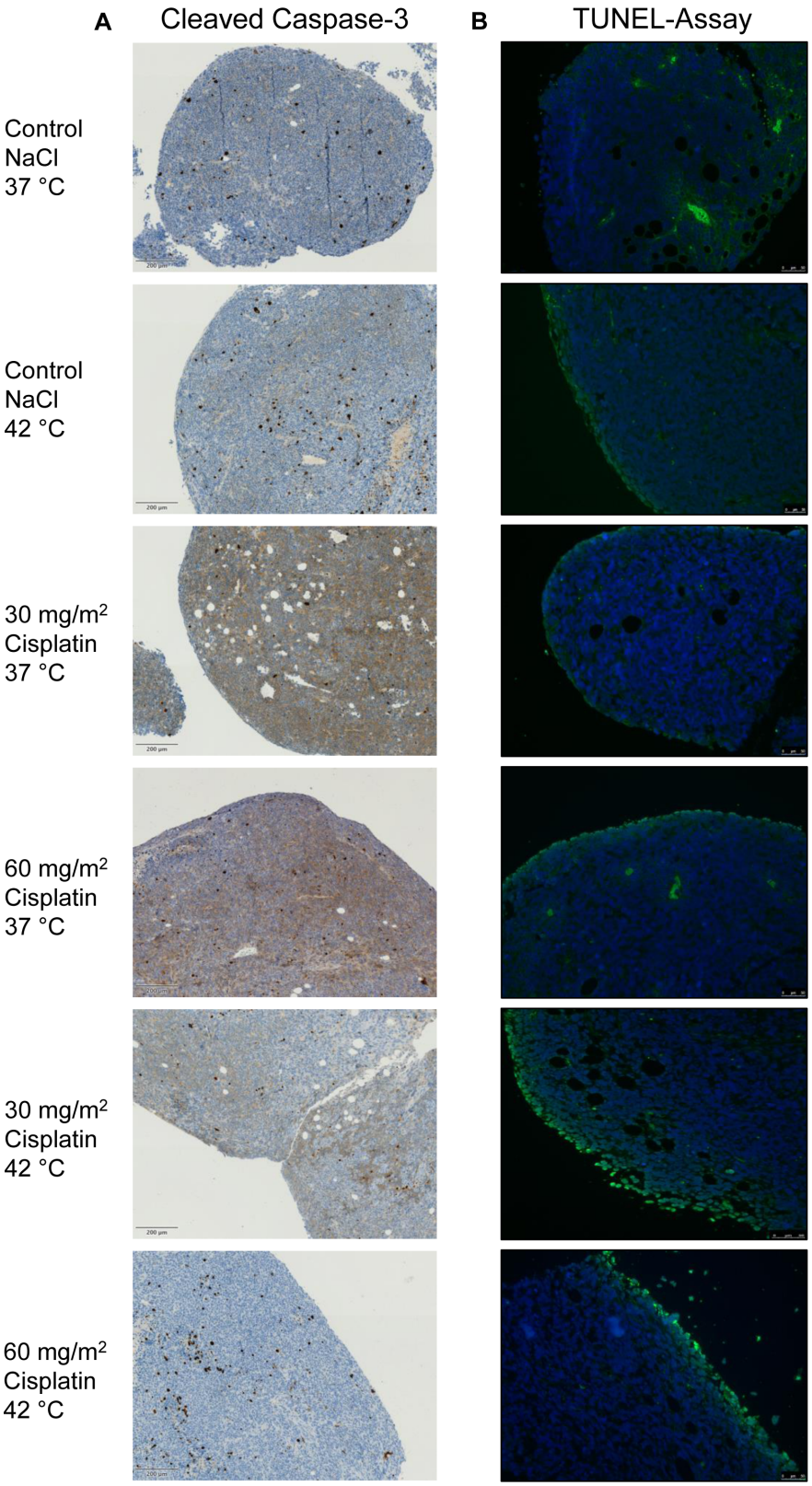


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TABLE 1.pdf available at https://authorea.com/users/410114/articles/519605-establishment-of-a-new-valid-animal-model-for-the-evaluation-of-hyperthermic-intraperitoneal-chemotherapy-hipec-in-pediatric-rhabdomyosarcoma