UNIWERSYTET MEDYCZNY IM. PIASTÓW ŚLĄSKICH WE WROCŁAWIU
WYDZIAŁ LEKARSKI

Kacper Zieliński

Analyzing the concept of a superhyperthermic capnoperitoneum as a potential vehicle to reduce progression of intraperitoneal metastases

ROZPRAWA DOKTORSKA

PROMOTOR:

dr hab. n. med. Veria Khosrawipour

Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu

Wrocław 2021

First and foremost, I would like to thank prof. Wojciech Kielan for making this work possible. I would like to thank prof. Maciej Ugorski from the Department of Biochemistry and Molecular Biology and prof. dr Jacek Bania from the Department of Food and Hygiene and Consumer Health for providing the laboratory environment to perform these experiments. I would like to express my gratitude toward dr hab. Tanja Khosrawipour from the Department of Surgery, University Hospital of Düsseldorf, Germany for the opportunity to perform histological analyses. Finally, I would like to thank dr Thomas Siegert from the Center for Astrophysics and Space Sciences in San Diego, California for his advice and counselling.

The supervisor, dr hab. Veria Khosravipour, for all help during the current cooperation, valuable substantive comments, as well as support and patience, thank you.

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Abbreviations:

C	Celsius
CO2	Carbon dioxide
CRS	Cytoreductive surgery
DMEM	Dulbecco's modified Eagle's medium
EM	Electromicroscopy
FBS	Fetal bovine serum
H&E	Hematoxylin and Eosin
HIPEC	Hyperthermic intraperitoneal chemotherapy
HT-29	Human colorectal cancer cell line
IPC	Intraperitoneal chemotherapy
KJ	KiloJoule
kV	KiloVolt
LDH	Lactate dehydrogenase
MTS	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PIPAC	Pressurized intraperitoneal aerosol chemotherapy.
PM	Peritoneal metastases
SEM	Scanning electron microscopy

1. Introduction

1.1. Background

Advanced peritoneal metastasis (PM) is an ongoing challenge in cancer therapy. While many attempts have been made to find new strategies in PM treatment, these concepts offer only little improvement in overall outcome and mortality rates [1-5]. Intravenous chemotherapy has only a limited local effect since the chemotherapeutic concentration in the peritoneum remains small with respect to systemic loss [6,7]. Therefore, intraperitoneal chemotherapy (IPC) was introduced to improve local drug availability [8,9]. However, IPC has exhibited only limited efficacy in the treatment of advanced PM [10,11]. Some variations of peritoneal IPC, particularly hyperthermic intraperitoneal chemotherapy (HIPEC) in combination with cytoreductive surgery (CRS), raised hopes in patients with early PM disease [12]. While only a small group of patients qualifies for HIPEC and CRS, namely those with limited disease and good overall health, more patients are recruited than truly match inclusion criteria as it is the only potentially curative treatment [13]. However, there is still controversy that many patients might not benefit from HIPEC and CRS, due to local tumor recurrence as well as the high extent of associated morbidity and mortality. Most recently, pressurized intraperitoneal aerosol chemotherapy (PIPAC) has been proposed as an alternative option for patients with advanced PM without a favourable prognosis for CRS and HIPEC [14-16]. In PIPAC, a liquid chemosolution is injected into the abdominal cavity by means of a spray-device, producing a chemoaerosol that covers the abdominal cavity [17,18]. While some positive results have been documented [14], and multiple attempts to improve [19,20] and extend the therapy [21,22] have been performed, current outcome analysis does not suggest any fundamental changes in PM treatment.

1.2. Looking at therapeutic options derived by a gas-based intraperitoneal methods.

1.2.1. Extreme gas-based hyperthermia

In HIPEC procedures, effective hyperthermia is restricted to chemotherapeutic solutions with temperatures of up to 42 - 43° Celsius (C). As these liquid solutions are introduced into the abdominal cavity, the introduced heat within the fluid gradually covers the abdominal organs which have core temperatures of around 40°C with little distribution inhomogeneity as previously shown by Rettenmaier et al. [23]. While the main temperature will adapt to the core body temperature, the gradient shows that the fluid temperature remains relatively constant. The difference in the inflow temperature, medium perfusate temperature and the core body temperature is around 2 – 4°C [23]. This relatively low temperature gradient reduces the risk of overheating and diminishes the risk for severe local organ damage. After all, increased core body temperatures are a positive predictor of postoperative complications as recently demonstrated by Goldenshluger M et al. [24]. Although the sensitivity of cancer cells to increasing hyperthermia has been extensively demonstrated [25 - 27], it is crucial to keep core body temperatures at a constant level to prevent organ damage. This inability to further increase the temperature of applied IPC is one of the key limitations in the HIPEC approach. Hyperthermia not only directly increases apoptosis, but also improves local drug penetration rates, achieves higher chemosensitivity and exerts other beneficial effects [28 - 30]. However, due to their respective physical properties, the application of H2O based hyperthermic liquid solutions is limited. H2O has a heat-capacity of 4.186 kJ/litre °C, which is the highest heat-capacity of any commonly known substance. This de facto means that a large amount of energy is transferred to any contacting object, causing a quick rise in surrounding temperatures. In contrast, air has a heat capacity of 0.00081 kJ per litre with a density of 1.127 kg/m3 at 40°C and at atmospheric pressure [31-33]. An object should maintain its temperature for a longer duration if the surrounding medium is air instead of water. At the contact area between the object and the air, there is a relatively long-lasting stable temperature gradient. By means of this gradient, the surface of the object displays high temperatures while deeper tissues retain their original temperature. Thus, to analyse the feasibility and efficacy of super-hyperthermia as a potential

treatment option for PM, we will investigate this method's hyperthermic signature, its physical and structural effects on the peritoneal tissue as well as its technical feasibility in clinical applications.

1.2.2. Dehydration by continuous intraperitoneal gas flow

PM has proven to be an extremely aggressive disease: While it can originate from different gastrointestinal or gynaecological tumor entities, the extension of an initial cancer to the peritoneum is a bad prognostic sign. Statistically, patients with PM do not survive the first year after diagnosis, due to the rapid progress of cancer cells in the abdomen [11, 34]. Interestingly, the cancer cells are often confined to the peritoneum or only infiltrate a few millimetres into the peritoneum [35,36]. Clinical experience indicates that in a first step, cancer cells preferentially spread within the peritoneal cavity. In many cases, they do not leave the peritoneal cavity at all, which suggests that tumor cells have important interactions with the peritoneal surface and consider the peritoneal milieu a favourable habitat to grow and infiltrate [37, 38]. This observation can be explained by immunological considerations since the immunological response in the peritoneal cavity is less protective than in solid visceral tissues [39] due to limited vascular accessibility. In fact, the glucose and protein-rich cavitary fluid can further enhance this effect. Since we must assume that the abdominal cavity offers great growth potential for local tumor cells, we made this compartment the focus of our studies. Thus, the main aim is to investigate whether changing the biological properties of the compartment could delay or even halt PM progression. By means of dehydration, we aim to alter the biological properties of this compartment as to make it less hospitable for growing tumor cells. This study is in stark contrast to all previous attempts which included the use of cytostatics. When using cytostatics, the chemotoxic drug effects are limited to a rather short period of time and the overall beneficial characteristics of the compartment remain the same. Dehydration, on the other hand, might be a tool to halt cancer growth in the peritoneal cavity by purely biological means. To analyse the feasibility of such a concept, we investigated the sensitivity of colon cancer cells to different levels of dehydration vs. a normal level of hydration vs. a cytotoxic environment created by oxaliplatin. Further, the effects exerted on colon cancer cells were visualized at the electron microscopic (EM) level, while the effects on the peritoneum were evaluated

at the histologic level. Based on our current data and taking all relevant physical aspects into account, laparoscopically achieved dehydration of the abdominal cavity is a technically feasible and safe tool to treat PM

1.3. The assumed discriminative cytotoxic effects of physical principles on peritoneal surface malignancies

IPC attempts to improve cytotoxic effects by increasing local drug availability in the target tissue. The assumed discriminative nature of the therapy is - to a significant degree- based on the idea that applied chemotherapeutics tend to target malignant cells by accumulation and other means [40-42]. When performing IPC using liquid solutions, the applied chemotherapy passes through the peritoneal surface by mere particle diffusion [43-45]. PM extent in patients qualifying for IPC is limited to the peritoneal cavity [46,47]. Interestingly, we observe PM cases with extensive intraperitoneal spread yet without any distant metastasis, indicating extraordinary cancer cell biology. There seems to be an extracellular interaction between tumor invasion and cellular matrix properties which favours a horizontal tumor spread rather than a vertical spread [48, 49]. While horizontal tumor spread is clearly more visible during laparoscopy, the vertical spread can be assessed following histological examination of PM nodules. As depicted in figure 1, a single peritoneal nodule further invades the peritoneal tissue during its local growth. However, most of the peritoneal surface which falls victim to this invasive tumor growth shows extremely scarce cell density. In fact, much of this first layer is comprised of extracellular matrix, collagen, and other fibres [50, 51]. A few hundred micrometres deeper in this layer, we find either primary extracellular matrix (adventitia) or cell rich tissue, in most cases in the shape of smooth muscle fibres. Hence, we have an area of few hundred micrometres of peritoneum which are scarcely populated with cells and at the same time resistant to external factors due to their composition of extracellular fibres. Thus, any increased cellular toxicity within this thin layer could target primary PM. Therefore, we have focused our work on creating localized, targeted, and superficial therapeutic concepts which can increase superficial cytotoxicity.

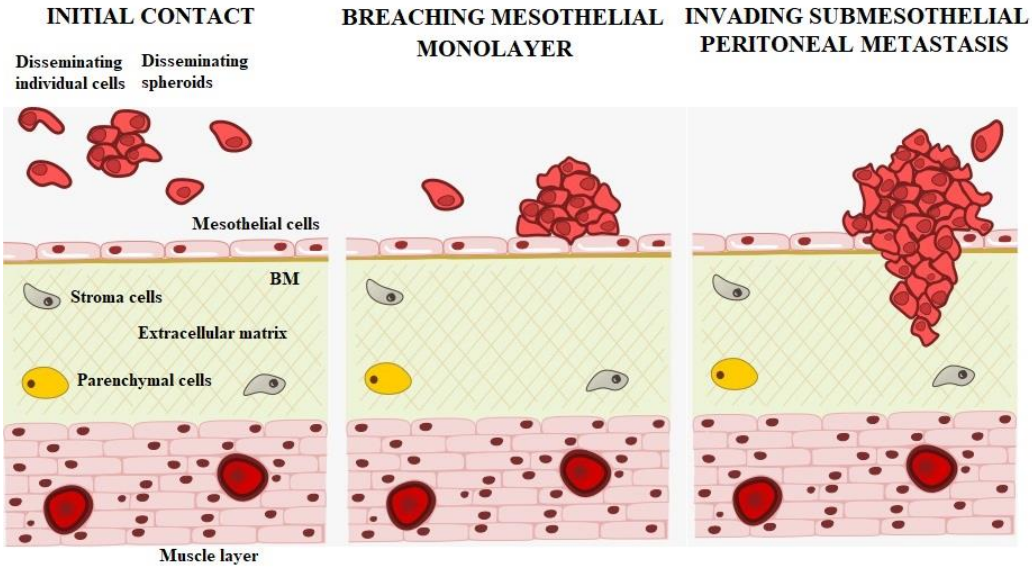

Figure 1 Model of horizontal and vertical spread of PM in the peritoneal cavity. The process is divided into 1) initial contact of individual cancer cells with the peritoneum followed by 2) local infiltration into the mesothelial monolayer and finally 3) invasive growth into deeper tissues.

1.4. Underlying physical principles and assumptions for our hypotheses

1.4.1. Hyperthermia and thermal capacity

Thermal capacity is a physical property of matter and defined as the amount of energy that must be supplied to a given mass to produce a unit change in the mass's temperature [52]. The most common unit used for thermal capacity is Joule per Kelvin. It is important to note that the heat capacity of a particular material is an extensive property. If the material is homogenous, its heat capacity can be calculated as the sum of its parts (assuming a homogenous construction):

$$C_{sum} = \sum_{n=1}^{N} C_n = C_1 + C_2 + C_3 + \cdots C_N$$

The intensive property of the same material is its specific heat capacity. The specific heat capacity of a material can be described in relation to its volume, its mass or even its molar mass. It is important to consider that specific heat capacity is not a strict constant. It can slightly change when a material is heated, depending on the start temperature of the specific material [53]. However, variations could be ignored when working with objects in a narrow range of pressure and temperature.

1.4.2. Laws of Thermodynamics

First law of Thermodynamics

The first law of thermodynamics is also called the law of energy conservation. No energy is added or subtracted in a closed system [54, 55]. This is important when we observe any heating process of a gaseous medium within the abdominal cavity. Since the heat capacity of 1 litre of gaseous medium is 1000 times smaller than the heat capacity of water (respectively abdominal tissue), even a high temperature gas should not be able to cause a substantial temperature increase of the entire abdominal cavity. In fact, in a closed system, the temperature of the gas should significantly cool down while the heat of the surrounding abdominal tissue would only slightly warm up.

Second law of Thermodynamics

We must also emphasize the principles of the second law of thermodynamics which describes the natural tendency of spatial homogeneity of energy or, more specifically, temperature [55, 56]. When two systems interact in proximity to each other, these systems reach a thermodynamic equilibrium at some point.

Thermal conductivity and Fournier's law

Thermal conduction is defined as the transport of energy. In this case, energy is conveyed in the shape of a temperature gradient. It is important to distinguish this from convection which will be further discussed below. Thermal conduction can be described by a vector Cond (p, t) with P for position and t time. According to Fournier's law of heat conduction, heat transfer through a material is proportional to the temperature gradient as well

as the area at the right angle to that gradient through which the heat flows [57, 58] (visualized in figure 2). Modification of these factors result in changed heat conduction.

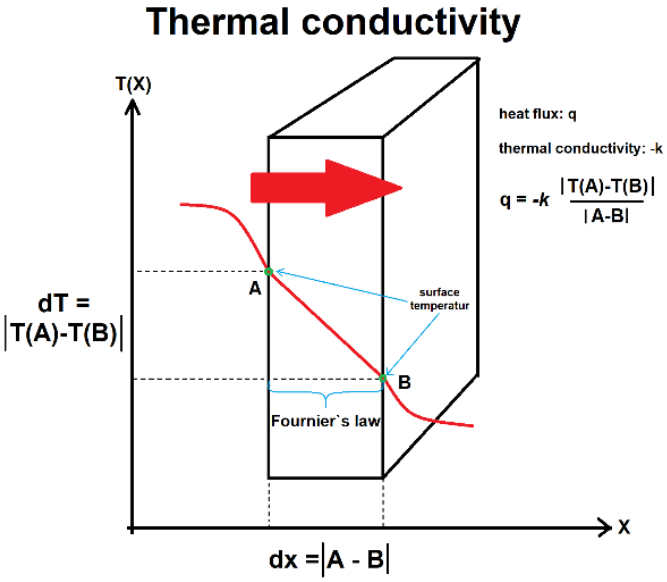

Figure 2 Thermal conduction though a homogenous material according to Fournier's law. q is the local heat flux density calculated by the material conductivity k and the temperature gradient.

1.5. The intraperitoneal cavity from a thermodynamic point of view

There is a standard fluid model which is used for hyperthermic intraperitoneal chemotherapy and similar heated lavage procedures. After filling the intraperitoneal cavity with heated fluid, the energy in the temperature difference is conducted toward the peritoneum. This heat will heat up the peritoneum as well as the underlying tissues and organs. Due to blood circulation, part of the heat is distributed throughout the body. In HIPEC procedures, a limited fluid volume of 3,5 – 4,5 litres enter the abdominal cavity. This fluid with a temperature of 41-43°C [59, 60] is slightly warmer than the core body temperature of around 36-39 ° [61, 62]). Some versions of HIPEC procedures add a circulatory system with an in and outflow enhancing conversion, further transporting heated liquid into the peritoneal cavity. With the circulation system in place, an intraperitoneal electronic temperature probe is placed to monitor temperature fluctuation. Meanwhile, the regional heat is transported through the body by the circulatory blood stream and some heat is lost to the surrounding atmosphere by radiation [63] as demonstrated in figure 3.

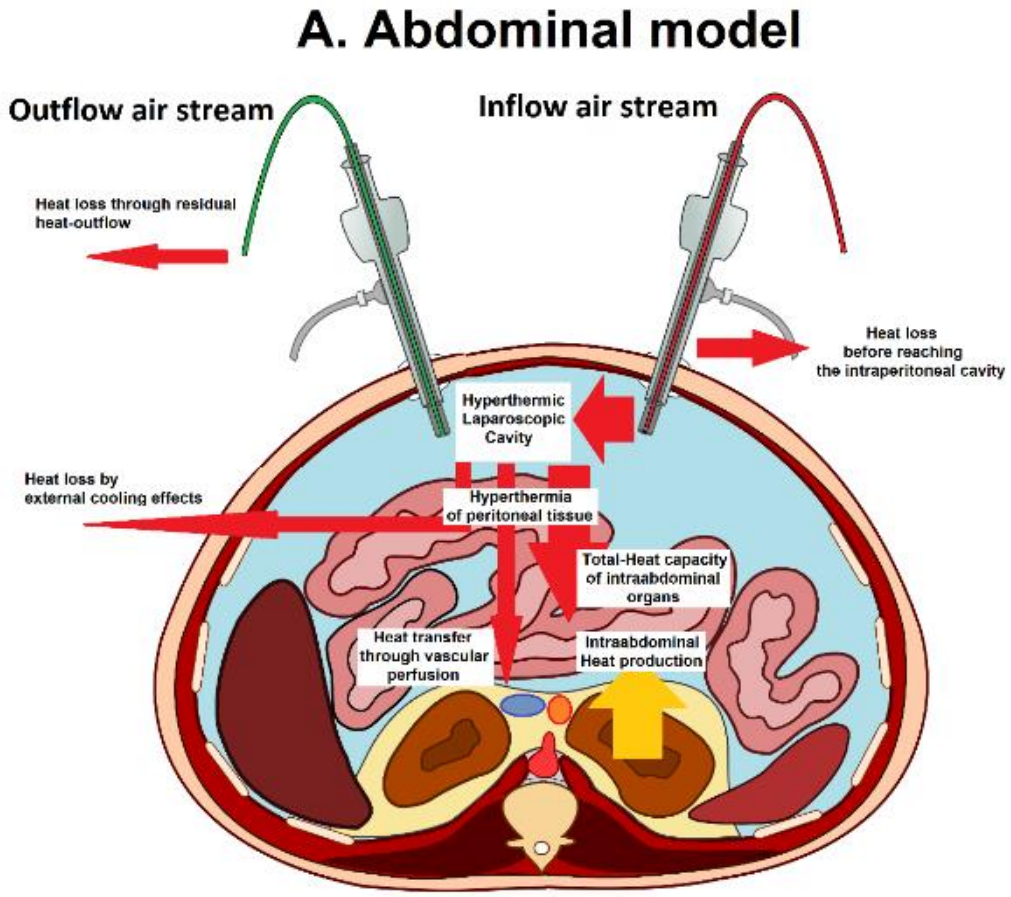

Figure 3 Abdominal model of suspected heat transfer and conduction in a gas-based hyperthermic intervention. The intervention is based on a continuous stream of hyperthermic gas from 2 trocars with an in and outflow. Expected heat loss and conduction is indicated by predominantly red arrows. The yellow arrow indicates the continuous internal heat production of the body regardless of the intervention. The heat conduction pathway is practically identical to the fluid pathway model.

We do not expect any particular effects in these models since the observed temperature changes between inflowing hot fluid and the target tissue are within a very narrow field of 41-43°C. Since water-based fluids have a high specific heat capacity, only minimal changes in observed temperatures along the inflow tube, target tissue and exit tube are conceivable. The transporting medium also has the same heat conducting and storing capacities as the target tissue in the abdomen. This aspect resolves many challenging equations for this approach while also possibly limiting its potential. However, this is not the case when assessing capnoperitoneum from a thermodynamic perspective. After initial insufflation of 4L CO2 (usually at room temperature or below approx. 27°C) in the standard laparoscopic approach, we often observe condensation of water drops on the laparoscopic camera. This phenomenon indicates that the capnoperitoneum reaches high levels of humidity and the temperature of the intraperitoneal CO2 adapts to that of the surrounding tissues. Assuming that the construction of a hyperthermic capnoperitoneum is attempted, this would require heating the incoming insufflating gas up to temperatures exceeding 41-43° C. Since we would expect that the energy transport in a low dense medium (e.g., air) is much lower than that of a liquid (e.g., water), we would also expect that the total flow rate is higher than in a fluid model such as HIPEC. We should mention that depending on the operational protocol during hyperthermia, there could also be the possibility of temperatures far beyond 42-43° C impacting the peritoneal surface. This should be achievable without increasing the total heat dosage on the peritoneal cavity as demonstrated in figure 4.

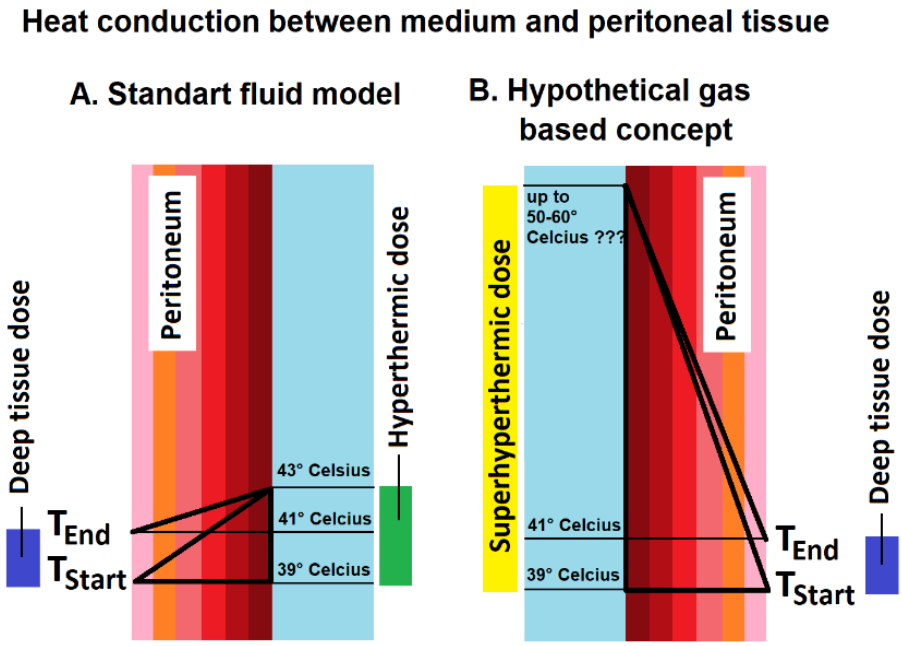

Figure 4: Heat conduction between medium and peritoneal tissue in a fluid model (like HIPEC) and a gas-based hyperthermic model. In the gas-based model, we assume that a larger heat gradient and thus higher superficial hyperthermia is achievable without changing the total applied heat dose.

1.6. The complexity of hyperthermia in a capnoperitoneum model and initial study attempts

A few attempts have been made to adequately understand the complexity of hyperthermia in a capnoperitoneum model for a possible clinical application. One of these first attempts, from a technical point of view, was conducted by Jung DO et al. in 2016 [64]. In their study, hyperthermia in a capnoperitoneum was attempted in combination with PIPAC. During the procedure, which lasted for about 85 minutes, the capnoperitoneum temperature was constantly kept at around 38.8 - 40.2 °C. The temperature probe itself was placed into the capnoperitoneum. The temperatures detected in the airstream were slightly higher than the core body temperature which varies at around 36 - 39°C [64]. Zhao J et al. 2016 [65] used a more biological approach and concluded, from a cell culture model, that a hyperthermic CO2 capnoperitoneum can reduce proliferation and migration of colon cancer cells. In their study, Zhao et al. exposed cells to a 100% CO2 environment and separately heated up the fluid medium to 43°C. By doing so, they found that the combination of CO2 and heat at 43° was highly effective and cytotoxic. However, they were unable to offer any ideas on how this temperature level could be achieved in an actual laparoscopic model. Many similar cell culture experiments were conducted, all yielding similar results [66 - 68]. However, all these experiments lacked a perspective on any actual application model. The closest to an actual working model was presented by Peng Y et al. 2017 [69] using a mouse model. While insufflating a mouse abdomen to 43°C for 3 hours they observed suppression of peritoneal colon cancer cells dissemination as well as some peritoneal tissue damage. To the best of our knowledge, there is currently no clinical data on hyperthermic capnoperitoneum in humans. However, there is some data on hypothermic vs. normothermic capnoperitoneum in humans.

A systemic cochrane database review showed that normothermic insufflation into the peritoneal cavity did not result in improved patient outcomes. Thus, there is no clear evidence in support of heated (normothermic) gas insufflation, with or without humidification, compared to cold gas insufflation in laparoscopic abdominal surgery (70). All these studies underline the urgency to establish a basic conceptual understanding of air carrier based hyperthermic applications.

1.7. Transfer of physical principles in an adequate and sensitive thermal abdominal model

While we must acknowledge that a standard model used in experiments cannot exactly mimic the actual abdominal cavity, a standardized and established model is required to conduct some initial analyses. However, we must remember that patients' abdomens are not standardized either with respect to different statures and weight categories, resulting in different sizes of intraabdominal organs. These size variations may result in altered characteristics in a hyperthermic model. The most important aspect is to ensure that any potential danger and challenges arising in a hyperthermic gas-based model are easily detected and recognized as such. In fact, these are two potentially dangerous aspects in hyperthermic gas applications. First, there is the danger of a local heat build-up in the abdominal tissue far beyond tolerance levels. This is difficult to mimic in an anatomic abdominal model. Since there is a large surface and a constant blood flow which transports local heat out of the abdomen, the local heat can easily reach critical levels without being detected. Secondly, the abdominal organs as well as the abdominal wall surrounding the capnoperitoneum have an enormous heat storing capacity. Thus, superficial build-up of a heat gradient might not be observed due to a relatively large energy storing capacity by the underlying tissues. To detect the thermodynamic effects on the peritoneal surface, we have constructed an abdominal box model with a better temperature isolation and a lower heat storing capacity than is found in a regular abdomen during laparoscopy. This will ensure detection of more sensitive effects and potential dangers in this newly developed concept (figure 5).

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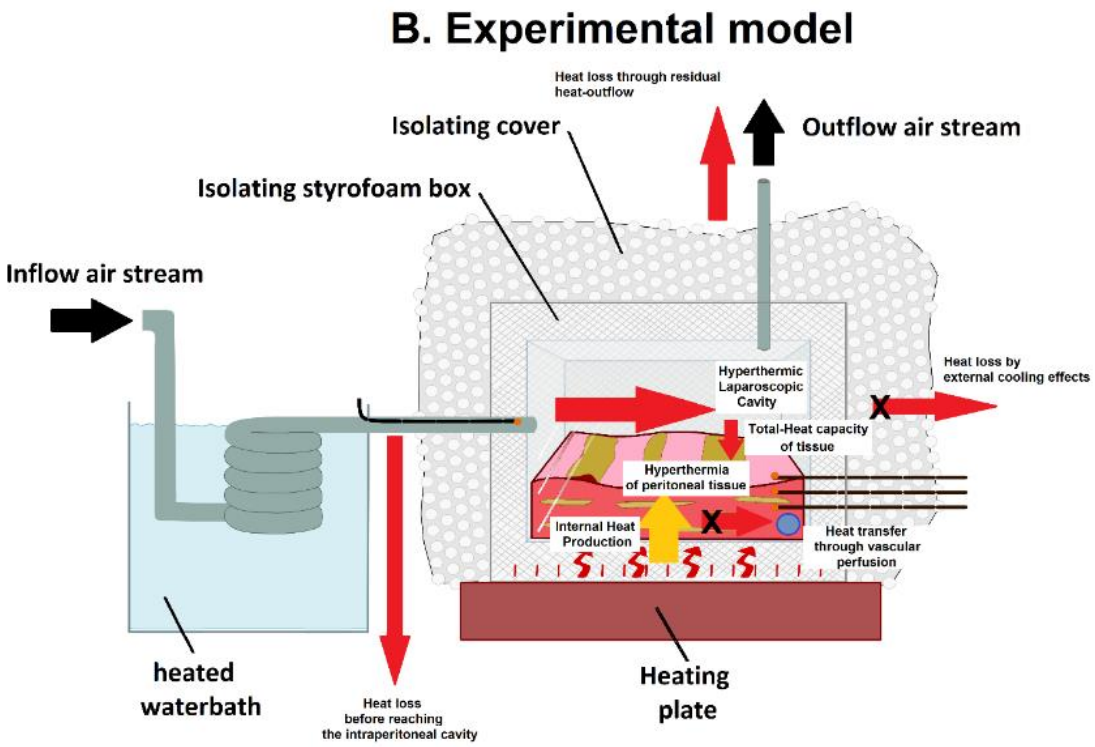

Figure 5: Experimental model of suspected heat transfer and conduction in a gas-based hyperthermic intervention. Similar to the prior abdominal model this system is based on a continuous stream of hyperthermic gas with 2 trocars allowing in and outflow. Expected heat loss and conduction is indicated by red arrows. Two red arrows reflecting heat transfer present in the abdominal model but not in the experimental model are marked as X (first conduction through blood stream and second external cooling and convection). The yellow arrow indicates the internal heat production of the body represented by the water bath which continues regardless of the intervention.

1.8. Dehydration

Evaporation

Evaporation is observed when a molecule near the surface absorbs enough energy to overcome vapor pressure [71]. At that moment, the molecule leaves the fluid and escapes into the surrounding gas. Evaporation continues until some equilibrium is reached, usually when the evaporation of that particular liquid is the same as its condensation. Evaporation could be used as a mean to dehydrate the peritoneal surface. During open abdominal procedures, e.g., laparotomy, we sometimes observe this phenomenon when the intestine is left uncovered. In such an instance, the intestinal surface „dries out ". Rehydration can be achieved by covering the intestine with wet tissue drapes during surgery, or by rehydration in the abdominal cavity after laparotomic closure. However, during these usually short cases, the peritoneal cavity and its superficial cellular layers experience a condition which never occurs in the abdomen. Thus, the question arises as to whether this effect be used for therapeutic purposes. A prolonged and intense exposure to dehydration effects could fundamentally change the intraperitoneal microenvironmental as to stress and discourage cancer cell growth. We know from previous considerations on hyperthermia and our understanding of horizontal and vertical PM spread that the superficial peritoneal layer is, to a large degree, comprised of extracellular matrix (see figure 1). This matrix is usually much more resilient to physical stress factors such as heat compared to cells. Unfortunately, we currently lack any knowledge on dehydration of the peritoneum while we at least have some data on hyperthermic procedures on the peritoneum. The relevance of exposure of the intestinal loops to „open air" is sometimes noticed by the operating surgeon but is a phenomenon that has not been subjected to further analysis. Therefore, its significance remains completely unclear. To the best of our knowledge, there is no clinical data available on this phenomenon, and it remains unknown whether this could be used for clinical purposes. As for our study, dehydration of the peritoneal surface requires sufficient surface evaporation. For therapeutic purposes, this evaporation should occur in a laparoscopic approach rather than in open abdominal surgery. Therefore, potential dehydration of the peritoneal cavity using evaporation effects should be further investigated due to its significant antitumoral potential.

1.9. Theoretic concepts of evaporation

Evaporation effects occur on the surface of a liquid. To escape the liquid and overcome liquid-phase intermolecular forces, molecules require sufficient kinetic energy, which determines the rate of evaporation. The kinetic energy of molecules is proportional to the temperature of a particular liquid. Therefore, evaporation occurs more quickly in higher temperatures [72]. Molecules with the highest kinetic energy are most likely to leave a liquid, leading to a slight cooling of the residual liquid after a while as more molecules with higher kinetic energy are leaving. Evaporation is an endothermic process in which energy (in form of heat) is practically absorbed [72].

Vapor pressure and evaporation equilibrium

In enclosed areas, evaporating molecules accumulate as a vapor above the liquid. Some molecules return to the liquid. At some point, the process of escape and return reaches an equilibrium [73]. In such a system, the vapor pressure is directly related to the vapor pressure of the substance as calculable by the Clausius-Clapeyron Relation. Where V1 and V2 are the vapor pressure at temperatures T1 and T2, Δ Enthv is the enthalpy of vaporisation and K is the universal gas constant.

$$\ln\left(\frac{V_2}{V_1}\right) = -\frac{\triangle Enth_v}{K}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)$$

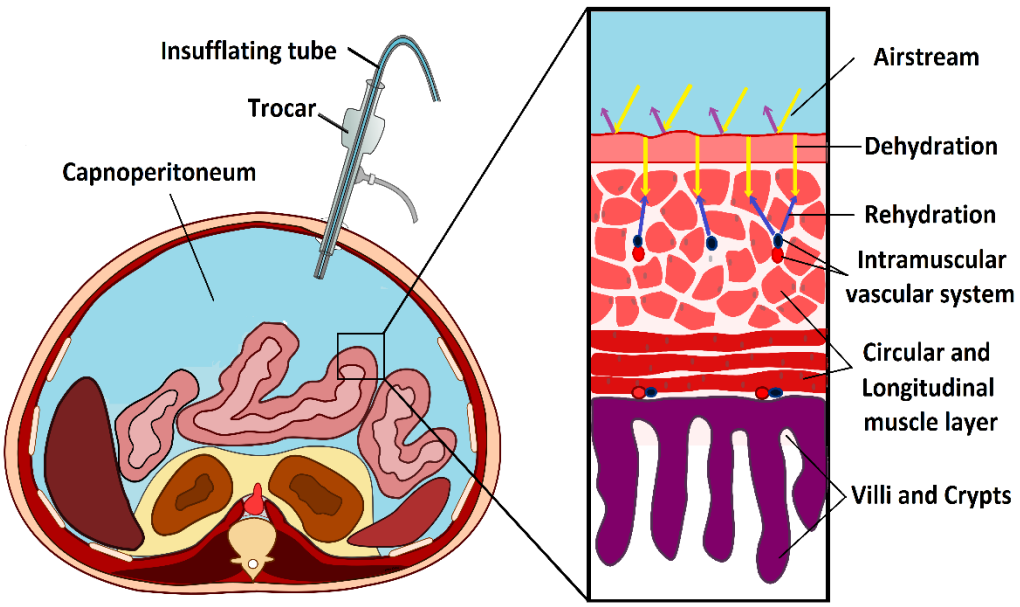

Figure 6 Dehydration model of the visceral peritoneal tissue using a laparoscopic approach. The potential dehydration effect on the peritoneal surface could kick in during a constant gas-based hyperthermic flow. The possible and assumed dehydration and rehydration mechanisms are indicated in the magnification of the small intestine.

Factors influencing the evaporation rate

Until today, many factors have been identified which influence evaporation rates. In fact, some attempts have been made to create mathematical models to predict the interactions of these factors with each other in an evaporation model [72, 74]. Some of these influencing factors should be more thoroughly discussed as they are relevant for our considerations.

Concentration

The concentration of a particular substance in air influences evaporation. With increased concentration of a substance in the air, that given substance will evaporate more slowly. This is relevant in our model when considering the insufflation of a gaseous medium. CO2, which is classically used during laparoscopic procedures, is usually applied in its „dry“ form, which means that its humidity in the gaseous state is 0%.

Convection

The flow rate of air is another important factor. Convection describes movement in a gas in which warmer particles move up and cooler particles move down. In fact, the concentration of a given substance will decrease if the continuous air stream has a low humidity.

Surface area

In general, a substance with a larger surface area evaporates faster since there are more surface molecules per unit of volume that can potentially escape.

Substance temperature

As the temperature of a substance increases, the mean kinetic energy of the molecules increases as well, resulting in a faster surface evaporation rate.

Theoretic concept of evaporation

Evaporation occurs faster if the surrounding gaseous medium has a lower pressure.

Minerals dissolved in liquid

Intermolecular forces influence the liquid state and can increase the level of kinetic energy, causing molecules to escape from the liquid medium. It is important to mention that the principles of evaporation are used in industrial processes like printing, coating, drying of a variety of materials as well as in laboratory analyses such as spectroscopy and chromatography. However, the primary interest in physics and engineering have been combustion and pre-combustion aerosol vaporization for car engines. Outside of these specific settings, there is only little data available on the effects and principles of the evaporation of fluids. For this experiment, I used empiric data and weather models provided by the United States National Weather services as they too incorporate considerations on factors influencing evaporation rates in their forecast measurements.

Unaccounted effects

There are additional affects which must be mentioned yet cannot be accounted for in such a model. One of these factors is the actual extent of evaporation. While we try to capture the role of evaporation on the peritoneal surface during laparoscopic procedures, there is currently no knowledge on its effects in a laparoscopic model when constant gas flow is applied. The role of humidity levels on heat transfer between a gaseous carrier and the peritoneal surface is a closely related matter. Additionally, the effects of laminar flow and flow volume still remain unclear. However, while these factors currently assume an inferior role, they may be of interest in future studies. These effects relate to the influence and changes in humidity on the capnoperitoneum. From clinical experience, we know that soon after insufflation of the abdominal cavity with „dry“ CO2 gas, the capnoperitoneum becomes saturated with H2O. This can be observed when entering the abdomen with the endoscopic camera during laparoscopy. Whenever the camera is colder than the abdominal cavity, we see condensation on the camera optic. The speed and extent of evaporational effects are difficult to quantify, and the significance of laminar flow remains unclear. The insufflation of the abdominal cavity creates a capnoperitoneum of different sizes and dimensions. The insertion of single trocars with an in- and outflow at different locations and within a formed cavity causes a non-laminar abdominal gas-flow. While a non-laminar gas-flow within the abdomen might increase evaporational effects, it may also influence temperature conduction. At this time, the extent of these effects might not be fully detectable. This issue is also observed with fluid procedures such as HIPEC [75 - 77]. During abdominal perfusion, it is unclear if local pockets form with relative flow stagnation. While this effect should be presumably less significant when compared to fluid applications, it must still be discussed.

1.10. Future studies

While the current study focuses on several major aspects of hyperthermic gaseous carriers for intraperitoneal hyperthermia and dehydration, we must acknowledge that this dissertation cannot fully cover all relevant aspect and questions with respect to this novel concept. By means of this work, we have aimed to answer the most urgent and relevant

questions. To implement this concept in the clinical setting and establish this new operative model, many more aspects must be investigated in the future.

From a biological and antitumoral perspective, there are two major aspects that require more careful investigation:

1) First, we aim to create a technically feasible superficial heated surface extending beyond 43° Celsius (see figure 4). For this purpose, we must focus on the following aspects:

1. Analysis of the temperature gradient at different intraperitoneal locations in a hyperthermic model with temperatures extending beyond 43°C
2. Analysis of heat build-up in target tissues during gas-based hyperthermic applications
3. Analysis of locally applied, close range hyperthermic air and its conduction through the tissue (especially the small intestine)
4. Cellular response to short-term hyperthermia in an HT-29 cell model using viability and cytotoxicity tests

2) Second, we aim to investigate whether dehydration is a feasible tool serving as an add-on therapy during laparoscopic procedures. For this purpose, we must further pursue the following considerations:

1. Is dehydration of the abdominal cavity a practical concept?
2. Does partial dehydration of colon cancer cells have a cytotoxic effect?
3. How can we measure and document the impact of dehydration on surrounding tissues?
4. How can we estimate the maximum water volume that can be extracted from the abdominal cavity in such a procedure?

2. Materials and methods

2.1. Abdominal model

Tissue experiments were performed in an ex vivo styrofoam model using commercially available tissue samples (local pork supplier, Żerniki Wielkie). Fresh post-mortem parietal peritoneum samples of the swine (5 x 7 x 8 cm) were placed at the bottom of the box (figure 7). Two trocars of 5mm in diameter were placed (Kii Balloon Blunt Tip System; Applied Medical Resources Corporation) in the centre and at the side of the box. The box was additionally isolated with bubble wrap. The thickness of the styrofoam box was 3cm on each side and the internal volume was 4 litres. The model box was placed on a heater (Lighted Tissue Bath XH-1003,). Temperature probes (Digital thermometer, FisherbrandTM Tracebale, Pittsburgh, USA) were placed at multiple sites (figure 7). At the exiting site of the incoming tube, probes were placed in the air at 3mm distance from the peritoneum (probe 1), as well as at 2mm and 5mm (probes 2 and 3, respectively) into the peritoneum. The incoming airflow was kept constant at 15 litres per minute. Prior to entering the box, the air was directed through a heated water bath to regulate incoming air temperature at this flow rate.

2.2. Experimental design

By means of the underlying heater, the temperature in the box was kept constant at an equilibrium of 37°C. All temperature probes indicated this temperature for 5 minutes. After stabilizing the temperature in the box, airflow in the incoming trocar was conducted at different temperatures. Experiments were conducted at a wide range of temperatures. Air temperature was measured along a wide temperature span ranging from 45°C to 72°C. Applied temperatures were placed into 5 groups (cohorts). Medium temperatures for these groups were 47°C, 50°C, 60°C, 66° and 69°C. Temperature increases at probes 2, 3 and 4 were measured for 1 hour at an airflow of 15 litres per minute. Temperatures were measured and medium temperatures were calculated for each of the 5 groups.

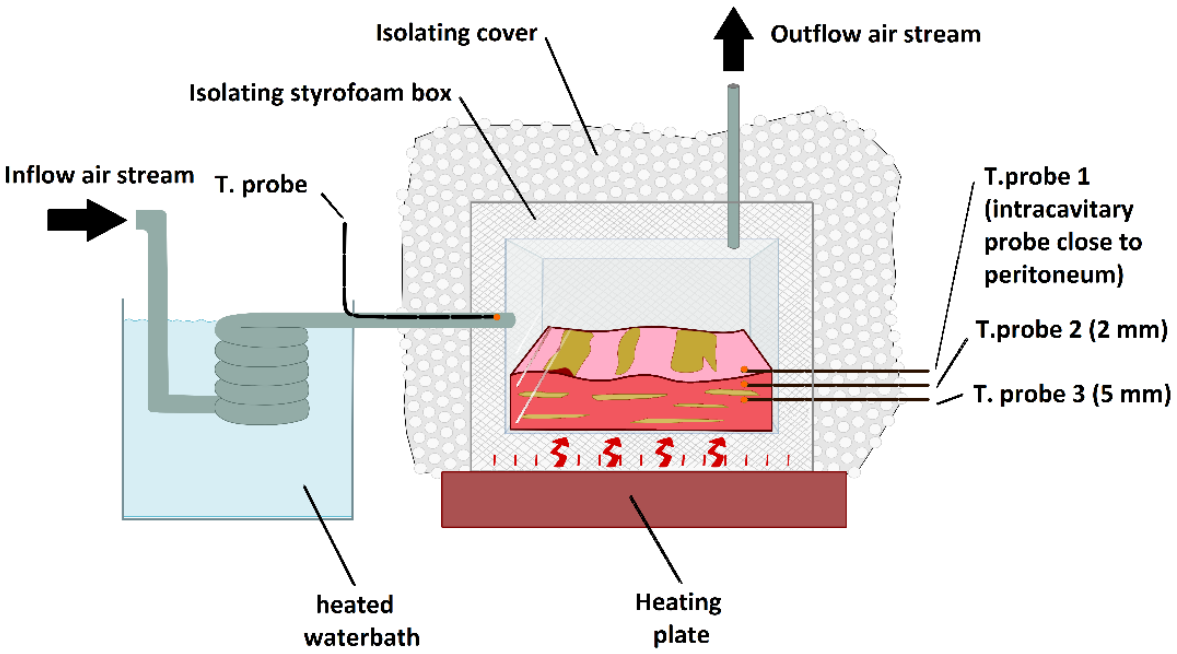

Figure 7: Experimental abdominal box model. Abdominal cavity represented by a 4 litre styrofoam box with an additional isolation cover. Position of temperature sensors as illustrated.

Local heat exposure

Fresh post-mortem small intestinal samples (12cm length) were placed at the bottom of the box. The head of the temperature probe was placed inside the small intestinal lumen. Both sides of the lumen were closed. One group was treated with heated 0.9% saline at 68°C, 70°C and 72°C and the saline was poured into the box thereafter. In the second group, air was heated to 70°C, travelled through the plastic tube and impacted the small intestinal samples at 1 cm distance and 15-liter flow rate for 50 seconds. Then, the temperature increase was measured using the temperature probes. The peritoneal tissue was removed from the ex vivo model and embedded in paraffin before standard hematoxylin and eosin staining was conducted. Untreated small intestinal samples were used as control.

Cell cultures

The human colorectal cancer cell line HT-29 was obtained from the CLS (Cell Lines Service GmbH, Eppelheim, Germany). HT-29 cells were grown in Dulbecco's modified

Eagle's medium (DMEM - high glucose, Sigma-Aldrich, Poznan, Poland) and supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Poland), 2 mmol/L glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich) in a humidified 5% CO2 incubator (NuAire CO2 Incubator, Biogenet, Warszawa, Poland) at 36^{o} C. Cells (1.4x10^{5}/ well) were seeded in 24-well plates (TC Plate 24 Well, Standard, F, Sarstedt AG & Co. KG, Germany) and incubated for 48 hours. Cells subjected to EM analysis were seeded on sterile slipcovers.

In vitro short-interval hyperthermia

Cells were seeded in 24-well plate at a concentration of 2x10^{5} cells/well in 1ml of medium. After 48h of incubation, medium was changed for 10sec and replaced with 2ml of heated medium at the following temperatures: 37°C (Control), 42°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C. After 10sec, medium was again replaced with 1ml of medium heated to 37°C and cells were incubated for another 24h. For positive control, Oxaliplatin was used at a concentration of 1.2mg/ml and added to the well for 1h, then standard medium was applied and incubated for a further 23h. Then, cytotoxicity and viability testing were performed. Before these experiments were conducted, a previous experiment was performed to ensure that heated medium contained its temperature when placed in a 24 well plate for 10 seconds.

Viability test

An MTS test (colorimetric CellTiter 96® AQueous One Solution assay, Promega, Poland) was used to measure cell viability after heat or oxaliplatin treatment. The test was performed according to the manufacturer's instruction. The medium was removed from each well and replaced by 0.3mL of fresh DMEM. Next, after 1h of incubation at 37°C at 5%CO2, an MTS-based reagent was added to each well and absorbance was detected at 490nm using a microplate reader (Tecan, Basel, Switzerland). Cells treated with medium heated to 37°C were used as control. The percentage of viability was referenced to the control for all groups.

Cytotoxicity assay

The extent of cytotoxicity caused by heat or oxaliplatin was measured by release of lactate dehydrogenase (LDH) into the supernatants using Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific). 50µl of medium was taken from each well. The test was performed according to the manufacturer's protocol. Cytotoxicity levels were calculated as the percentage of LDH released from test samples cells compared to LDH released by lysis buffer treated cells and normalized to the spontaneous release from control cells. As reference, colour reaction was measured spectrophotometrically on a microplate reader (Tecan, Basel, Switzerland) at 490nm and 680nm.

In vitro cell dehydration

Cells were divided into 3 groups. In the first group (dehydration), medium was removed from the wells and plates were placed back into the cell incubator for 15minutes at 90% humidity. After cell dehydration, wells of this first group were subjected to cellular analyses by scanning electron microscopy (SEM) while the other wells were refilled with 0.5ml of medium. The second group was treated with oxaliplatin (Medoxa, medac GmbH, Wedel, Germany) with 7.36µl per well for 45 minutes. The oxaliplatin concentration in each well was 0.24 mg/ml, corresponding approximately to a common HIPEC application amount of 960 mg/4 litre (480mg/m²). The third group received no treatment and was used as a control for proliferation assay and EM. After the experiments, cells were incubated at 36 °C and 5% CO2 for 24 hours. Then, the MTS proliferation assay was performed.

Electron microscopy

Dehydrated HT-29 cells were subjected to cryogenic SEM analysis. The probes were washed with Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich) and fixated in 2.5% glutaraldehyde solution (Sigma-Aldrich). After fixation, samples were washed in PBS, rinsed in ultrapure (sterilized through 0.1 µm filter) deionized water, mounted on a cryo-shuttle using OT/colloid graphite mixture and plunged in liquid nitrogen. Frozen specimen was then quickly transferred to a cryo-preparation chamber (Cryo Quorum PP3010T) and sputtered with a conductive layer of platinum at 140°C. Then, specimens were transferred to the microscopy chamber while maintaining

a temperature of -140°C (Auriga60, Zeiss). Samples were observed at 2kV of acceleration voltage using In Lens and SE2 secondary electron detectors.

Ex-vivo dehydration

Tissue experiments were performed in an ex vivo model, which was previously described in detail, using commercially available tissue samples (local pork supplier, Żerniki Wielkie). Fresh post-mortem swine peritoneum samples of small intestine (12 cm in length) were placed at the bottom of the plastic box mimicking the abdominal cavity. Two trocars of 5 mm in diameter were placed (Kii Balloon Blunt Tip System; Applied Medical Resources Corporation) in the centre and at the side of the box, which was then tightly sealed. Next, samples were subjected to a laminar CO2 flow at a rate of 10 litres per minute and 37°C for 10 minutes. Then, samples were removed from the *ex vivo* model and embedded in paraffin before standard hematoxylin and eosin [78] staining was conducted [79].

2.3. Operative model for laparoscopic dehydration of the abdominal cavity

Evaporation

The model used for our dehydration study was based on an assumed volume of 4 litres (V_B = 4 L), which mimics the abdominal capnoperitoneum during laparoscopy. The abdominal cavity model was based on half of a rotational ellipsoid with semi-minor axis a and semi-major axis ($c = 2a$) (Figure 1A). The intraperitoneal evaporation rate was based on the inner surface of the abdomen, the known empirical constant term of the evaporation coefficient and the known maximum humidity ration of water in air at 40°C [80 - 82]. A standard tube cross section of a 12-mm trocar was assumed as an in and outflow gate with a cross section area of 1 cm^2. The values of a (and c), and the surface, S_{tot}, of the abdominal model were calculated. Based on these, the mass of evaporated water per unit time, g, given a scalable stream of air, L (in units of L/min), was analysed (Figure 1 B). The volume of a full rotational ellipsoid is provided by:

$$V_{RE} = \frac{4\pi}{3} a^2 c \tag{1}$$

such that the half volume (= volume of the abdominal model) is:

$$V_B = \frac{4\pi}{3} a^3, \tag{2}$$

accounting for the fact that $c = 2a$. Solving equation (2) for a provides:

$$a = \sqrt[3]{\frac{3V_B}{4\pi}} \approx 0.99 \text{ dm.} \tag{3}$$

The surface area of the half-ellipsoid is provided by the area of the bottom ellipse:

$$A_E = \pi ac = 2\pi a^2 \approx 6.1 \text{ dm}^2, \tag{4}$$

plus half the surface of the rotational ellipsoid:

$$0.5A_{RE} = \pi a\left(a + \frac{c^2}{\sqrt{c^2 - a^2}}\right) arcsin\left(\frac{\sqrt{c^2 - a^2}}{c}\right) = \pi a^2 \left(1 + \frac{4\sqrt{3\pi}}{9}\right) \approx 10.4 \text{ dm}^2 \tag{5}$$

such that:

$$S_{tot} = A_E + 0.5A_{RE} \approx 16.5 \text{ dm}^2. \tag{6}$$

The amount of water that evaporates per unit time due to a constant air stream is estimated by:

$$G = e_C S\,(x_S - x). \tag{7}$$

In this equation, e_C is the evaporation coefficient in units of mass per surface and time, S is the surface of the liquid exposed to the air stream, x is the humidity ratio of air (set to zero in our setup), and x_S = 0.04 kg/kg is the maximum humidity of saturated air at 40°C. The evaporation coefficient is an empirical function of the air speed, and takes the form:

$$e_C = f + dv, \tag{8}$$

Where v is the velocity of the air stream through the abdomen, and f and d are to-be-determined constants that depend on the geometry and experimental setup. Typically, f and d are measured to determine the influence of wind evaporation on lakes, and generally combined to e_C-values of the order of 0.1 to 1.0, given wind velocities of a few m/s. Our experiment allows for a maximum air stream of L_{max} = 40 L/min. We estimate the maximum speed of streaming air in our model by approximating the rotational ellipsoid by a cylinder with radius r and height (length) h, to obtain

an average cross section, A_C. This provides an equation system for two variables, r and h, given the known surface and volume:

$$V_B = \pi r^2 h \text{ and } S_{tot} = 2\pi r^2 + 2\pi r h. \quad (9)$$

The system of equations has three solutions (in units of dm), $(r/h)_1$ = (-1.82/0.38), $(r/h)_2$ = (0.55/4.21), and $(r/h)_3$ = (1.28/0.78). Clearly, the first solution is unphysical, the second solution approximates the abdomen as a 'longer tube', and the third one with the proportions of a 'tin can'. Solutions 2 (A_{C2} = 0.79 dm^2) and 3 (A_{C3} = 5.15 dm^2) serve as a lower and upper bound for the streaming velocity, such that:

$$\boldsymbol{V_{max} = \frac{L_{max}}{A_C} a^3 = 0.13\ldots\ 0.84\ \text{m/s}.} \quad (10)$$

Thus, the parameters f and d will be at most of the order of unity, like evaporation on a lake. Since without an air stream, no evaporation will be present, f will be zero. Hence d is the only unknown parameter, we have provided estimates for different values of d, ranging between 10^{-4} and 10. Finally, the mass of evaporated water per unit time is given as:

$$g \approx dvA_C x_S \approx dLx_S. \quad (11)$$

This is also equivalent to the maximum amount of water transported out of the abdomen, as we assume complete saturation after initial dry air input. Figure 1B shows the relation of equation for different values of d.

Maximum transport capacity

The operative model for calculating the maximum possible mass of water removed in a closed laparoscopic model is based on the previously explained calculations with the maximum humidity of saturated air at a temperature about 40°C with x_S = 0.04 kg/kg. The density of dry air is set at $D \approx 1.127$ kg/m^3 at 101.3 kPa and 40°C. L is expressed as units of litres per hour. Then, the maximum mass of the removed water from the abdominal cavity (T_{max}) at any given volume passing the abdominal cavity during laparoscopy (V_{pass}) is as demonstrated below:

$$T_{max} = x_S D\ L \quad (12)$$

and approximated to (Figure 2):

$$T_{max} = 2.7\ gram/hour \quad (13)$$

Graphic design

For the graphics provided, multiple graphic programs were used. Among these programs were Inkscape 1.0.1,2020, GNU, USA and programs provided by Windows office 2019, Microsoft.

Statistical analysis

Experiments were performed at least in triplicate. For the cellular experiments, each well was considered as a single value, corresponding to the subgroups. The student t-test was used to compare groups. Probability (p) values were considered as follows: *=$p<0.05$, **=$p<0.01$, and #=$p>0.05$, with p-value <0.05 considered to be statistically significant. Data are presented as the mean standard deviation unless otherwise indicated.

3. Results

3.1. Hyperthermia

3.1.1. Temperature of incoming tube (Probe 0.)

Data was collected from the peritoneal samples (T_1, T_2 and T_3) as well as from the sensory input at the incoming tube (T_0). At the very beginning of the experiment, it became evident that the temperature in the water bath was not identical to the temperature measured at the end of the incoming tube. Dysfunction of temperature sensors could be excluded as a potential explanation for this discrepancy. The temperature at the end of the incoming tube was 5 - 7 C° lower than in the water bath. The total length of the warming tube was set at 3 meters. The tip of the warming tube was placed into the experimental box, while the last 50 cm of the tube remained outside of the water bath. A decrease in the air speed streaming through the tube did not significantly change this discrepancy. However, after we reduced the length of the tube outside of the water bath from 50 cm to 30 cm, we observed that the measured discrepancy was reduced by 3 - 5 C°.

When referring to hyperthermic insufflation, it is important to consider that the actual hyperthermic temperature is the temperature at the exiting point of the incoming tube. Even when using a short tube of 30 cm, a significant temperature loss is detected before the end of the incoming tube is reached. Data from the probes $T_{1\text{-}3}$ was successfully recorded after each testing phase for a period of 0 to 60 minutes. The 15 previously measured temperatures were further categorized into 5 groups and visualized in figures 8 to 10.

3.1.2. Temperature at the experimental cavity (Probe 1.)

Data from the cavity probe showed a slow medium temperature increase when insufflation was performed at lower (47°C) vs. higher temperatures (69°C). The temperature increases after about 30 minutes and then seems to reach a plateau, or, in some cases, slightly increase. This plateau seems to be the maximum cavitary temperature achievable. Our reference temperature of 45°C is reached and surpassed by the highest medium insufflation temperature of 69°C.

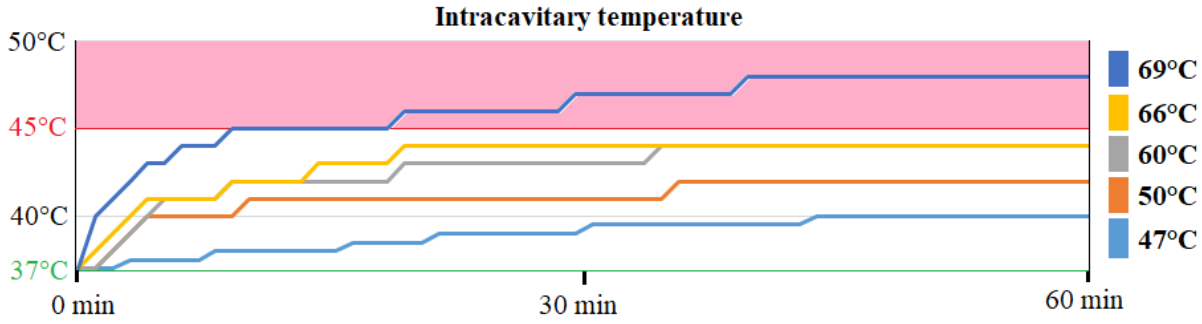

Figure 8 Medium intracavitary temperature increase during hyperthermic air insufflation in the experimental model. Different insufflation temperatures are marked with the provided colours (47°C, 50°C, 60°C, 66°C and 69°C, respectively). The temperature increase is constantly recorded over period of 60minutes.

3.1.3. Temperature of peritoneal tissue at 2mm depth (Probe 2.)

Data from the superficial tissue sample showed a slow medium temperature increase when insufflation was performed compared to data from the intracavitary sample. The medium temperature increases continuously and does not seem to reach a clear plateau within the observed timeframe. Our reference temperature at 45°C is reached but not surpassed by the highest insufflation temperature of 69°C.

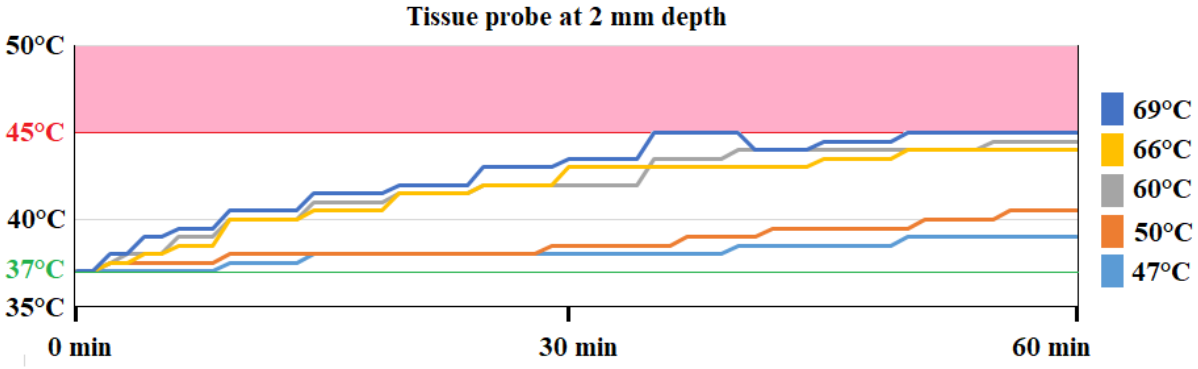

Figure 9 Medium temperature at 2mm intraperitoneal tissue depth. Temperature increase during hyperthermic air insufflation in the experimental model is recorded at this location. Different insufflation temperatures are marked with the provided colours for 47°C, 50°C, 60°C, 66°C and 69°C, respectively. The temperature increase is constantly recorded over period of 60 minutes.

3.1.4. Temperature of peritoneal tissue at 5mm depth (Probe 3.)

Data from the last and deepest cavity sample showed an even slower medium temperature increase compared to the previous two probes (T_1 and T_2). The temperature increases after about 30 minutes and then seemed to reach a plateau. This indicated that maximum medium cavitary temperature seemed to be reached. Our temperature reference of 45°C is not reached nor surpassed by the highest insufflation temperature of 69°C.

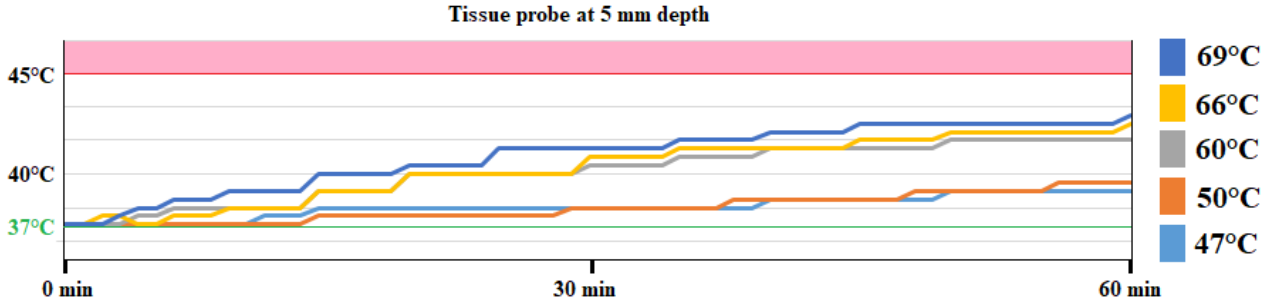

Figure 10 Medium intraperitoneal tissue temperature at 5 mm depth. Temperature increase is recorded during hyperthermic air insufflation at this location. Different insufflation temperatures are marked with the provided colours for 47°C, 50°C, 60°C, 66°C and 69°C, respectively. The temperature increase is constantly recorded over period of 60 minutes.

3.1.5. Temperature measured at different time points and distinct locations in the experimental model.

A major temperature drop is detected when comparing the outflow temperature of the incoming tube with the cavity temperature, an observation which could be of clinical relevance. Although this drop remains constant even after one hour of hyperthermic insufflation, the difference becomes less. A further temperature decrease is noted when comparing A and B probes with those located at points C and D. The temperature jump from point A to B is quite dramatic while the jump from point B to C is far smaller and less intense. Furthermore, the temperature at the far distant point D seems to stabilize within a temperature frame above 37°C but still below 45°C. This possible equilibrium seems achievable despite the large temperature difference at the incoming tube A.

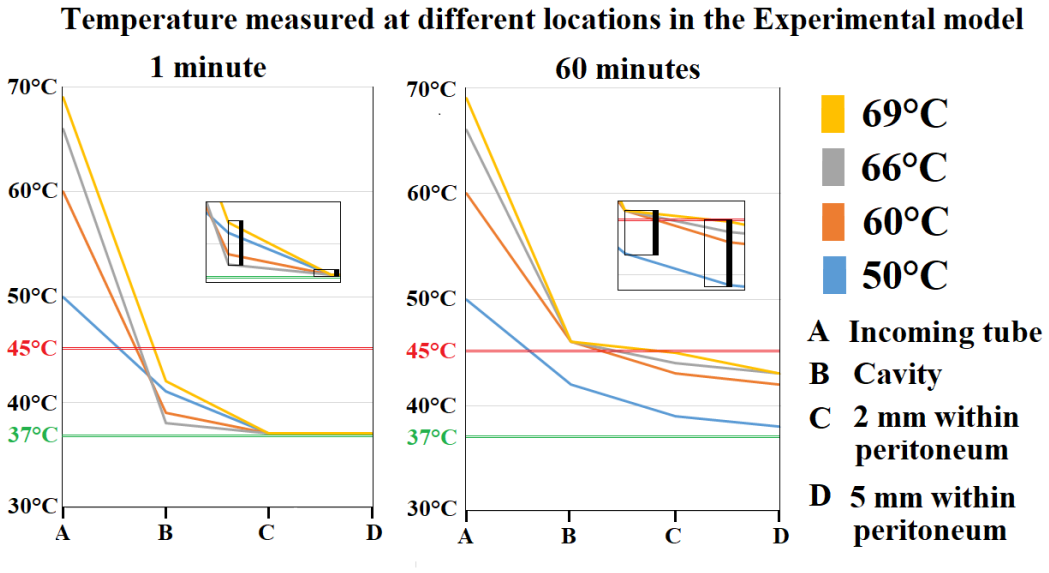

Figure 11. Temperature distribution in the experimental model after starting hyperthermic insufflation at 1 min and 60 min. The graphs demonstrate different insufflation temperatures of the incoming tube. Temperatures were measured at the insufflation point (A), cavity temperature 5 mm close to the peritoneum (B), temperature at 2mm depth in the peritoneum (C) and at 5mm depth into the peritoneum (D).

3.1.6. Results of short-term hyperthermia on small intestine via air and lavage

The performed experiments show that there is a significant difference when comparing the medium on which hyperthermic exposure was performed. While the hyperthermic lavage rapidly heats up the entire tissue, the exposure to hyperthermic air only slowly heats up deeper tissues. In the observed timeframe of 50 seconds, the hyperthermic air curve appeared nearly flat, despite exposure to high temperatures of 70 °C at the opposite site (figure 12). The direct hyperthermic temperature of 70°C corresponds to the outer wall temperature of the small intestine and the inner wall temperature as recorded in figure 12. Therefore, we conclude that our model hypothesis is confirmed. This means that by means of hyperthermic insufflation, a large temperature gradient can be created which leads to high surface temperatures while deeper tissues retain their original temperature.

As we saw in the previous figure (figure 11), this gradient cannot be recreated when attempting to heat up the intraperitoneal cavity with hyperthermic air. This is because air temperature in the cavity rapidly drops down without direct exposure.

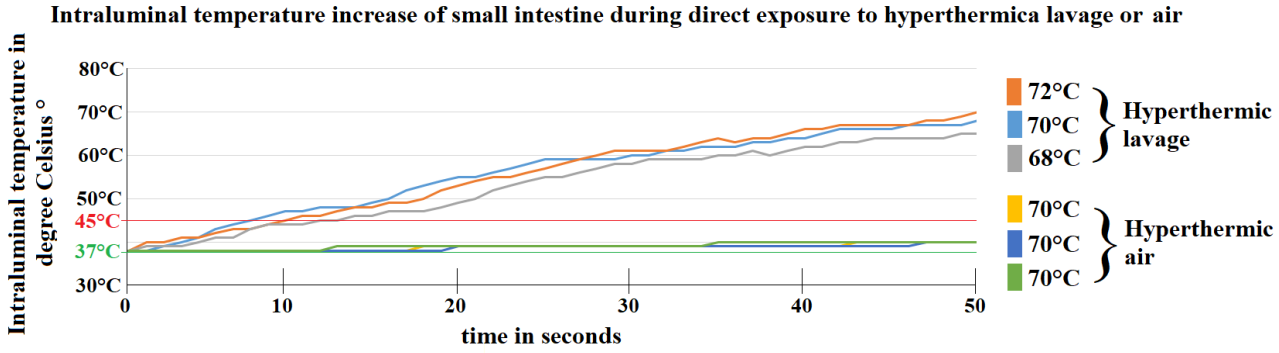

Figure 12 Development of intraluminal temperature of small intestine after direct exposure to hyperthermic lavage or air at 70° for 50 seconds. The temperature probe is in direct contact with the inner wall and about 2mm of tissue separates the probe from direct exposure to the hyperthermic medium.

3.1.7. Results of short-term in vitro hyperthermia using colon cancer cells.

The performed viability test shows that in a short-term exposure of 10 seconds, significant effects on viability can be observed with temperatures of 70°C and higher (figure 13). Temperatures below 60°C seem to have a rather paradox beneficial effect. However, these effects are not statistically significant and should therefore be dismissed. Viability decreases with temperatures of 65°C and higher. The observed effect on viability grows with each temperature jump. Temperatures at 70°C have similar effects on the viability as oxaliplatin treatment. Temperatures beyond that, namely at 75° and 80°C, outweigh the effects of Oxaliplatin treatment by far.

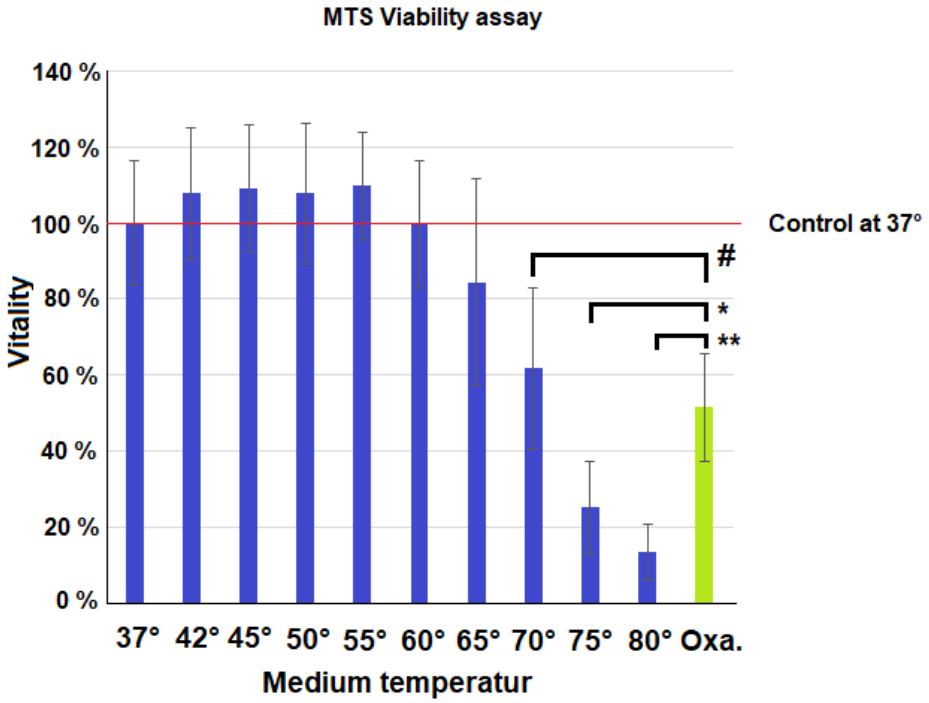

*Figure 13.: Measuring cell viability following hyperthermia. In-vitro exposure of colon cancer cells (HT-29) to short-term hyperthermia (10 seconds). Exposure to temperature levels from 37°C (control) in 5°C step increments until 80°C. An additional control with Oxaliplatin at 37°C was conducted to compare vitality levels with chemotherapy. Significance levels in the graphic: # = p> 0.05, *= p< 0.05, **= p< 0.01.*

3.1.8. Results of short-term hyperthermia on colon cancer cells via cytotoxicity assay

The performed cytotoxicity results are similar to those in the viability tests. Temperatures below 60°C do not seem to affect cytotoxicity (figure 14). However, with temperatures of 65°C and higher, rapidly increasing signs of cytotoxicity were observed. At temperatures between 65° and 70°C, cytotoxicity is significantly lower compared to oxaliplatin treatment. A slight increase in these temperatures between 75° and 80°C has proven to be more toxic than oxaliplatin. Despite an enormous heat tolerance, there seems to be a very narrow range at which point cells cannot manage additional temperature increases. Once this point is reached, massive cytotoxicity can be observed.

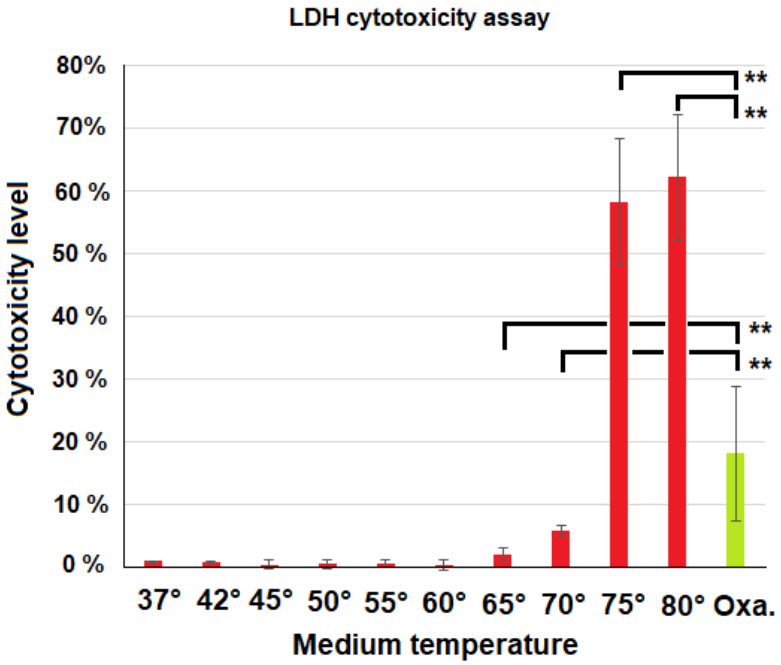

*Figure 14.: Measuring cytotoxicity following hyperthermia: In-vitro exposure of colon cancer (HT-29) cells to short-term hyperthermia (10 seconds). Exposure to temperature levels of 37°C (Control) in 5°C step increments until 80°C. An additional control with Oxaliplatin at 37°C was conducted to help compare vitality levels with chemotherapy. Significance levels in the graphic: # = p> 0.05, *= p< 0.05, **= p< 0.01.*

3.2. Histopathological examination of small intestine peritoneal samples

Hematoxylin and eosin staining shows potential changes after short-term exposure to heated air stream at close range, including detectable changes in the superficial layer. While this effect is limited to less than 50µm of the surface, it still reaches the first layer of the subperitoneal tissue (figure 15). In our analysis, no structural damage was visible on this level, as the changes seemed to be unspecific and could also indicate denaturation or possibly dehydration.

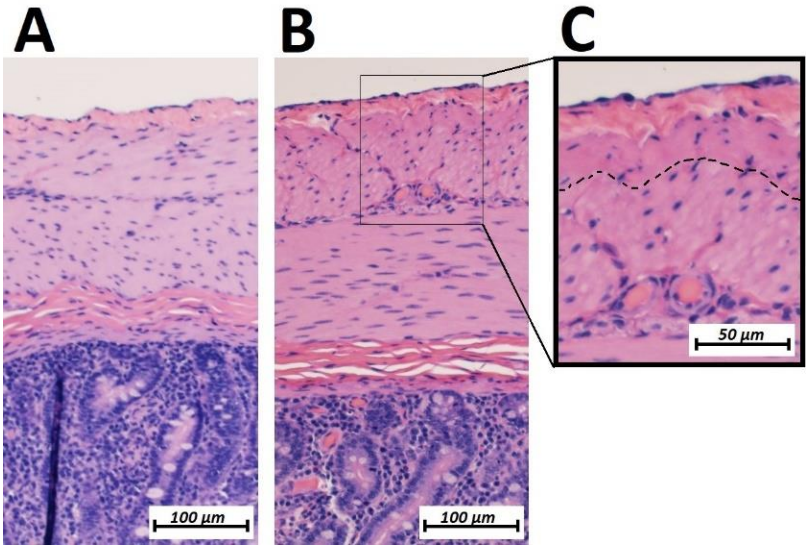

Figure 15 Hematoxylin and eosin staining of small intestine treated with a close-range heated air stream for 50 seconds. A) control, B) treated tissue and C) magnification of potential changes from heated air stream.

3.3. Dehydration

3.3.1. Cytotoxic effects of dehydration and oxaliplatin on HT-29 cells:

Cellular dehydration was only partial. Humidity was kept at 90% for the entire 15-minute duration of the treatment. Cell viability was significantly reduced after dehydration and treatment with oxaliplatin compared to control with 73.5% (Dehyd.) and 77.8 % (Oxa.), respectively ($p<0.05$, Figure 16). However, the highest decrease in vitality (down to 41.6%), was caused by a combined effect of dehydration and oxaliplatin ($p<0.01$).

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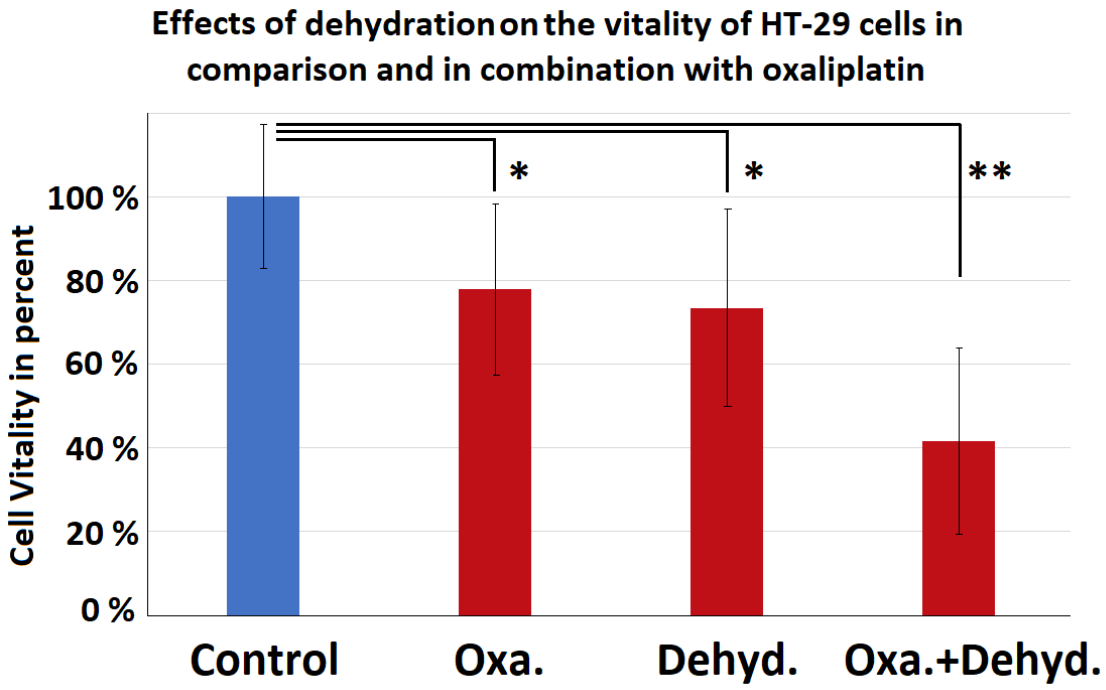

Figure 16 In vitro cell vitality of colon cancer cells following short-term partial dehydration for 15 minutes (Dehyd.), oxaliplatin (Oxa.) treatment, both, and untreated control group.

3.3.2. Electron microscopic analysis of dehydrated Ht-29 cells:

Cryo-SEM was used to study cell morphology following partial dehydration (figure 17). The structural analyses of the extracellular cell surface revealed some major changes which progressed with increased extent of dehydration. Untreated cells had a polygonal shape with sharp boundaries distinguishing them. In partially dehydrated cells, the margins became blurred, and the cells appeared hollow. EM analyses showed that dehydration greatly affects the structural integrity of HT-29 cells. Interestingly, the dehydration effect is heterogeneous. While some areas experience higher dehydration and therefore larger cellular changes, other areas seem less susceptible to dehydration. Higher levels of dehydration even seem to cause instant cellular collapse and degeneration (figure 17).

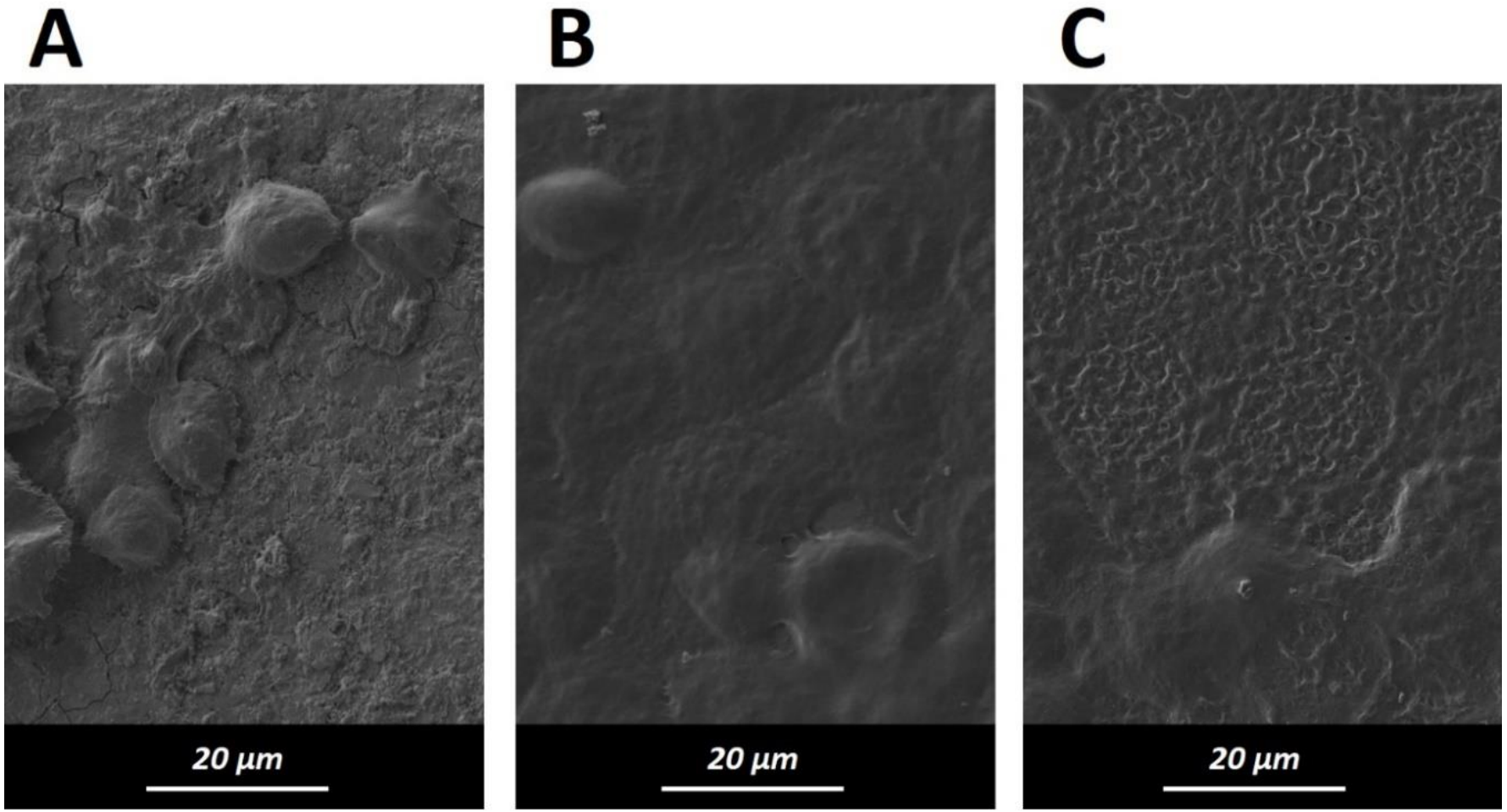

Figure 17 Electron microscopy analyses of HT-29 colon cancer cells at different stages of dehydration, at a 2500x magnification level. A) Untreated, normally hydrated HT-29 cells. B) Cells after short-term dehydration with remaining structural integrity C) Cellular collapse under dehydration.

3.3.3. Histopathological examination of small intestine peritoneal samples

Hematoxylin and eosin staining showed clear morphologic changes after dehydration. A clear limited dehydration of the superficial layer is detectable. While the effect is limited to less than 100 µm of the surface in this experimental setting, it still reaches the first layer of the subperitoneal tissue (figure 18). On this level, no structural damage is visible.

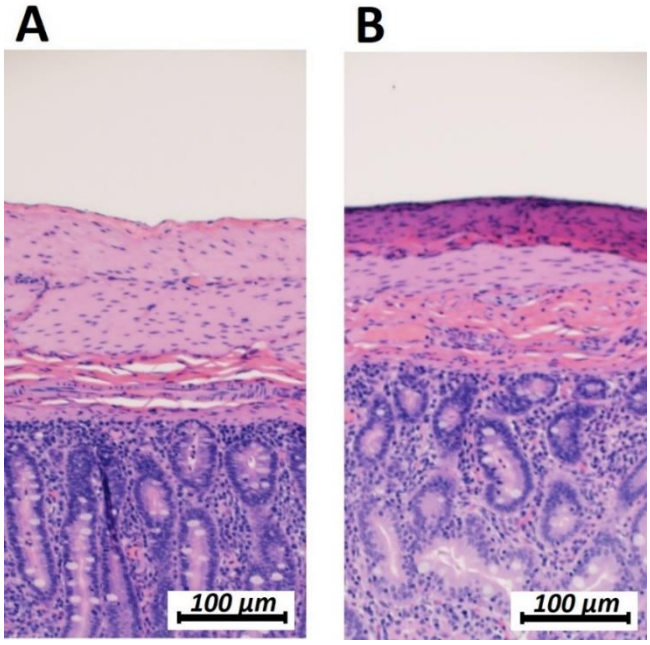

Figure 18. Histology of dehydrated visceral peritoneal tissue at 10x magnification (hematoxylin/eosin staining). A) histology of untreated small intestine B) histology of superficial small intestinal dehydration along the upper adventitia area.

3.3.4. Operative model for laparoscopic intraabdominal dehydration and maximum transport capacity

The calculation of the maximum transport capacity shows that via laparoscopic approach it is technically feasible to remove a few hundred ml of intracavitary fluid per hour from the abdomen (Figure 19 A, B and Figure 20). The relationship to the total volume passing through the abdominal cavity is practically linear. The calculation model for evaporation effects is rather more complex, as previously demonstrated. Water evaporation on a biological surface has not yet been investigated, and there is no available evaporation constant for this model since the evaporation constant "d" is unknown. Figure 19 B shows the equation for different values of *d*. However, the evaporation effect exponentially grows with an increase in applied volumes (Figure 19 B) and reaches with all $d > 10^{-3}$ probably clinically relevant levels of over 10 ml per hour.

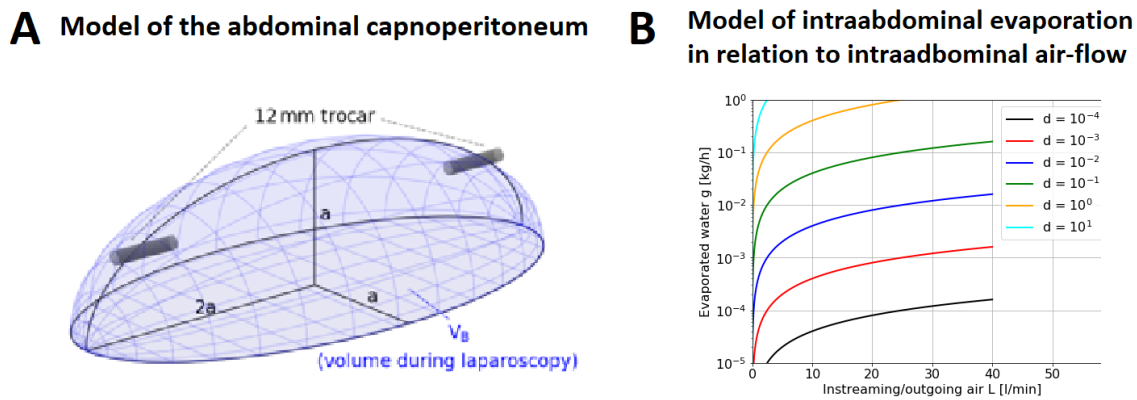

Figure 19A) Exemplified mathematical model of the abdominal capnoperitoneum during laparoscopy with two 12 mm trocars used as a CO2 in- and outflow B) Mathematical model of the intraabdominal evaporation process on a biological surface depending on various evaporation constants (d).

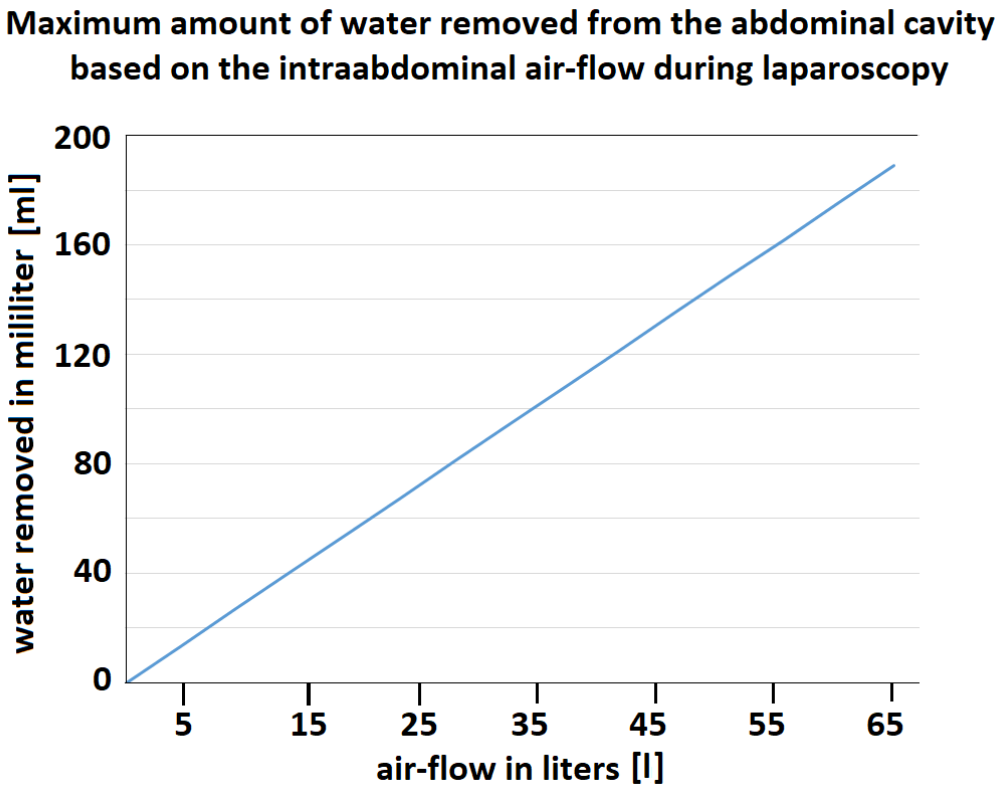

Figure 20. Maximum possible amount of water removed from the intraabdominal cavity and surface, respectively, depending on air flow rate in and out of the abdominal cavity using a laparoscopic model.

4. Discussion

The concept of using new physical principles in the treatment of PM [83, 84] and other surface malignancies [21-22] has been promising. Many concepts, including irradiation [85 - 87], high-intensity ultrasound [88, 89] and nanoparticles [20] have been created and tested. Currently, the most frequently applied concept is hyperthermia. In fact, hyperthermia has demonstrated efficacy in enhancing the antitumoral effect both in combination with chemotherapy or radiation without showing any disproportionate additional side effects [90-92]. Until now, hyperthermic intraabdominal procedures were usually applied using fluid solutions. However, fluid solutions are water-based and thus, have their own limitations. This is due to the unique physical properties of water which were previously discussed. However, when changing the applying medium, e.g., using air instead of water, physical properties differ and previous limitations are no longer applicable. In fact, when using gas-based hyperthermic procedures, additional therapeutic features such as dehydration can be added. First attempts have been made to look beyond mere fluid installations. Currently, the use of PIPAC is becoming more and more popular, a therapy which is heavily based on the use of a capnoperitoneum environment during laparoscopy. In PIPAC, an enhanced local drug concentration can be achieved by creating an increased local diffusion gradient by means of aerosolization [93]. The presented data shows that a hyperthermic medium such as air could be introduced into a body cavity at temperatures beyond 43°C without crossing the heat tolerance level in the cavity. This observation has allowed us to establish, for the first time in the scientific field, more advanced models to investigate hyperthermic gas-based intraperitoneal procedures extending beyond temperatures of 43°. The presented data further indicates that a sharp temperature gradient can be created on the peritoneal surface at close range to the outstream air tube without significantly increasing the temperature of areas deeper than 2 mm within a certain timeframe. In fact, our observations indicate that this effect can only be achieved by close contact between target tissue and inflowing gas tube. This is significant, as even the previously mentioned undirected hyperthermic gaseous inflow is unable to create this effect. In this work, we studied the ability to create a local, short term, extreme hyperthermic gradient while considering the extent of short hyperthermia required to create a cytotoxic effect on the superficial cancer layer. The presented cellular data indicates that, for a short period of time, high level

hyperthermia is tolerated by colon cancer cells. However, at some point, there is a sharp increase in cytotoxicity indicating that compensation mechanisms have become ineffective. In fact, with increasing temperatures even for short periods of time, toxicity levels are observed extending beyond those achievable with Oxaliplatin (as used in PIPAC). In our model, these observed effects have been limited to an exposure time of 10 seconds. To establish a better understanding of these effects, it will be necessary to apply different time intervals and compare that data in an applicable in-vivo model. By means of applying temperatures at around 75°C in 10 second time intervals, high cellular toxicity levels can be achieved using the high temperature gradient, while at the same time protecting the underlying tissue from heat damage. Furthermore, it may be possible to achieve structural surface degradation using this temperature gradient. In our model, this effect might be limited to about 50 µm micrometres which indicates that the hyperthermic effects are most likely superficial and leaving the deeper tissues intact. This effect is quite remarkable. Comparing this effect to similar tissue effects created by highly accurate surgical lasers, it becomes evident that while lasers can have a cutting width of up to 25µm, the structural effects and heat conduction achieved by local evaporation by far outweigh effects of the laser [94]. These are some considerations that should be applied in laparoscopy or other endoscopic procedures. The application of air-based hyperthermia is possibly easier to handle than fluid-based hyperthermia in a laparoscopic model. However, as demonstrated by this study, its theoretical aspects are far more complex. Unfortunately, no clinical or animal data is available on hyperthermic (>43°) insufflations during laparoscopy. In fact, the clinical research community has recently become aware that the insufflation temperature might be of interest during laparoscopic procedures. In the last 10 years, multiple studies have tried to illuminate the standard capnoperitoneum model and analyse the effect of normothermic, humidified CO2 vs. cold dry CO2 which is currently standard operational procedure in laparoscopic procedures.

According to recent studies, the use of normothermic, humidified CO2 for pneumoperitoneum in laparoscopic procedures is associated with lesser postoperative pain, lower risk of postoperative hypothermia, and lower analgesic requirements [95, 96]. A metanalysis of current data performed by Dean et al. [96] suggests that warmed, humidified CO2 insufflation during laparoscopic abdominal surgery can improve intraoperative maintenance of normothermia when compared to the use of cold, dry CO2. However, there is also opposing data indicating that relatively mild hypothermia induced

by CO2 gas at room temperature (27°C) and its surface evaporation cooling effects seem unproblematic [70]. In fact, dry cold CO2 will probably remain the standard for laparoscopy in most hospitals worldwide at least for the foreseeable future. Since the core body temperature is around 36 - 39°C, this would mean a temperature difference of 10-11°C which occurs for hours, depending on the length of a given procedure. Thus, it is possible that a temperature increase of 10 - 11°C beyond core body temperature instead of decrease may be equally tolerable. However, it has been acknowledged that there is no experience and only little understanding on the effects of a hypo- or hyperthermic capnoperitoneum due to the physical challenges faced when air is used as a medium, such as extreme low-heat capacity and unique physical qualities [97]. However, more basic research must be conducted to better understand, manage, and possibly optimize extreme-hyperthermic air-based hyperthermia. At this stage, a large animal model is the next step to evaluate safety aspects in a hyperthermic laparoscopic procedure beyond 43° via heated CO2 insufflation. The idea of a therapeutic pneumoperitoneum and thus, changing the biology of the particular body compartment is not a completely new concept. Carlo Forlanini introduced this idea in 1882 when treating pulmonary tuberculosis by means of a pneumothorax [98]. This innovative concept largely benefited the patients since no adequate drugs were available for pulmonary tuberculosis at that time. By means of this new concept, pulmonary tuberculosis became widely manageable, and dr Forlanini was nominated for the Nobel Prize in Medicine over 20 times [99]. Today, we face a similar challenge in PM treatment. For decades, there have been no treatment options that could significantly improve patients' life expectancy. In individual cases with limited disease, CRS combined with HIPEC was evaluated as an effective treatment option in providing long-term survival [100]. However, for patients with extensive disease, beside intravenous treatments and PIPAC, only a few more options are available [11]. The efficacy of these treatments is limited due to the aggressive behaviour of cancer cells within the peritoneal cavity and the limited effect of applied chemotherapeutic drugs, even when locally applied in HIPEC or PIPAC procedures. The metastatic process in the peritoneal cavity is often the limiting factor for patient survival with PM. Cancer cells within the cavity are derived from internal organs, either gastrointestinal or gynaecological, and they are habituated to a fluid-surrounding environment. This aspect does not change irrespective of mutations in the cancer cell genome [102]. Thus, changing the basic biology of the abdominal cavity

presumably targets and interferes with any cancerous process limited to the biological surface. The dehydration of an internal cavity is one of the most fundamental changes possible. Even partial dehydration is not well received by cells, especially when they are unfamiliar with this. In contrast, cellular structures in collagenous and extracellular matrix of cancer cells are barely affected by dehydration and will rehydrate without fundamental changes. Dehydration can be achieved using a continuous gas flow in the abdominal cavity during laparoscopy. Our model has well demonstrated the efficacy of dehydration as a novel treatment for PM and thoroughly illuminated important therapeutic and applicational aspects. High cytotoxicity is achieved with moderate dehydration. Cellular collapse has been observed and histological effects of dehydration on the peritoneal layer were demonstrated. Also, by means of this work we have outlined a basic conceptual understanding of the technical and physical aspects as well as applicational limitations. In summary, this approach needs to be further investigated to evaluate the potential of intraperitoneal dehydration in the management of advanced PM.

5. Conclusion

Dehydration might be a useful tool in changing the intraperitoneal environment to slow down PM progression. Extreme hyperthermia beyond temperatures of 43° can create an extreme temperature gradient along the peritoneal surface which can halt progression of peritoneal metastases. Applicational, biological and technical aspects of this approach must be subjected to further analysis to establish a better overall understanding and facilitate its use in the clinical setting. In fact, when using dehydration, the routine of the laparoscopic procedure remains unchanged, with its established applicational aspects, such as the use of a capnoperitoneum. Moreover, there is no need for any new or modified drug applications. Therefore, the threshold to apply this newly developed concept in the clinical setting is relatively low. The next steps include testing of applicational safety using animal models, and, in a second step, clinical trials. Moreover, further investigations are required to assess whether dehydration can be applied as a solitary or add-on therapy in combination with e.g., PIPAC procedures.

6. Summary

Background:

Changing the intraperitoneal environment can be a tool to treat advanced peritoneal metastasis. Intraperitoneal hyperthermic lavage with fluids is limited to 43°C to prevent critical increase of core body and intraabdominal temperature. However, further temperature increases of the superficial intraperitoneal layer beyond 43°C might be possible. In this study, we aim to explore different aspects of extreme gas-based hyperthermia and its related dehydration effects to evaluate its therapeutic potential in treating peritoneal metastasis.

Materials and Methods:

An experimental abdominal model has been established to investigate heat conduction, heat loss and potential therapeutic limits of gas-based superhyperthermia on peritoneal tissue samples. Temperature sensors have been placed at distinct points. Additionally, short-term effects on colon cancer cell vitality were investigated after heat exposure beyond 43°C. Furthermore, dehydration effects by means of continuous airflow have been analysed on peritoneal tissue in cell culture as well as via electron microscopy.

Results:

The data shows that gas-based hyperthermia via gas insufflation is feasible between 50° and 80°C. However, cytotoxic effects on colon cancer cells are limited to temperatures above 65°C. Dehydration of the abdominal cavity seems to be an additional effect observed due to a constant gas-flow through the abdomen. In contrast to hyperthermia, short-term partial dehydration demonstrates high cytotoxicity on colon cancer cells. The effects of dehydration seem to be limited on the superficial tissue layer (< 500 µm).

Discussion:

Intraperitoneal gas-based hyperthermia beyond 43°C seems to be achievable. In combination with dehydration effects observed due to a continuous gas-flow, this model can achieve a relevant amount of cytotoxicity which can be translated into a therapeutic option to treat advanced peritoneal metastasis. Further studies, including a large animal model, are needed to establish a safety and feasibility protocol as well as to evaluate antitumoral effects of this method.

7. Streszczenie

Tło:

Zmiana środowiska wewnątrzotrzewnowego może być narzędziem leczenia zaawansowanych przerzutów do otrzewnej. Podawanie wewnątrzotrzewnowe płynów jest ograniczone do 43°C, aby zapobiec krytycznemu wzrostowi temperatury głębokiej ciała i temperatury w jamie brzusznej. Jednak możliwy jest dalszy wzrost temperatury powierzchniowej warstwy śródotrzewnowej powyżej 43°C. W tym eksperymencie dążymy do zbadania różnych aspektów ekstremalnej hipertermii gazowej i związanych z nią skutków odwodnienia, aby ocenić jej potencjał terapeutyczny w leczeniu przerzutów do otrzewnej.

Materiały i metody:

Aby zbadać przewodnictwo cieplne, utratę ciepła i potencjalne granice terapeutyczne hipertermii gazowej stworzono eksperymentalny model jamy brzusznej na próbkach tkanki otrzewnej świni. Czujniki temperatury zostały umieszczone w różnych punktach modelu. Zbadano krótkoterminowy wpływ na żywotność komórek raka okrężnicy po ekspozycji na ciepło powyżej 43°C. Ponadto przeanalizowano efekty odwodnienia za pomocą ciągłego przepływu powietrza na tkance otrzewnej w hodowli komórkowej. Do oceny efektów odwodnienia użyto również mikroskopu elektronowego.
Wyniki:

Dane pokazują, że hipertermia oparta na gazie jest możliwa między 50 a 80°C. Jednak działanie cytotoksyczne na komórki raka okrężnicy jest ograniczone do temperatur powyżej 65°C. Dodatkowym, zaobserwowanym efektem stałego przepływu gazów przez jamę otrzewną wydaje się być odwodnienie. W przeciwieństwie do hipertermii krótkotrwałe, częściowe odwodnienie wykazuje wysoką cytotoksyczność w stosunku do komórek raka okrężnicy. Wydaje się, że skutki odwodnienia są ograniczone do powierzchownej warstwy tkanki (<500 μm).

Dyskusja:

Odwodnienie może być użytecznym narzędziem do zmiany środowiska wewnątrzotrzewnowego w celu spowolnienia progresji przerzutów nowotworowych.
Ekstremalna hipertermia powyżej temperatury 43°C stwarza ekstremalny gradient temperatury w poprzek otrzewnej, który może zatrzymać rozrost komórek nowotworowych. Aspekty aplikacyjne, biologiczne i techniczne tego podejścia muszą zostać poddane dalszej analizie celem lepszego ogólnego zrozumienia i ułatwienia zastosowania hipertermii gazowej w warunkach klinicznych.
W rzeczywistości, przy zastosowaniu odwodnienia, rutynowa procedura laparoskopowa pozostaje niezmieniona. Ponadto nie ma potrzeby stosowania nowych lub zmodyfikowanych leków. Dlatego zastosowanie tej nowo opracowanej koncepcji w warunkach klinicznych wydaje się być stosunkowo proste.
Kolejne kroki obejmowałyby testowanie bezpieczeństwa stosowania na modelach zwierzęcych, a w następnym etapie badania kliniczne. Ponadto konieczne byłyby badania oceniające możliwości i wyniki stosowania odwodnienia jako terapii pojedynczej lub dodatkowej w połączeniu np. z procedurami PIPAC.

8. List of figures

9. References

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