# Conditioning of the abdominal cavity reduces tumor implantation in a laparoscopic mouse model

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**Abstract**

*Purpose* The addition of 4 % $O_2$ and 10 % $N_2O$ to the $CO_2$ pneumoperitoneum (PP), together with slight cooling and humidification (conditioning), contributes to reducing adhesions by preventing mesothelial damage. We investigated the effect of peritoneal damage during laparoscopy on tumor implantation.

*Methods* In Experiment 1, different tumor cell concentrations were injected into control mice without PP and into mice with 60-min dry $CO_2PP$ (mesothelial damage). In Experiment 2, tumor cells were injected into control mice (group I) and in mice with mesothelial damage (group II). In groups III to VI, mesothelial damage was decreased by adding humidification, humidification + 10 % $N_2O$, humidification + 10 % $N_2O$ + 4 % $O_2$, and conditioning, respectively.

*Results* In Experiment 1, the tumors increased with the number of cells injected and with mesothelial damage in the abdominal cavity ($p = 0.018$) and abdominal wall ($p < 0.0001$). Experiment 2 confirmed that 60 min of dry $CO_2PP$ increased the number of tumors in the abdominal cavity and wall ($p = 0.026$ and $p = 0.003$, respectively). The number of tumors was decreased in the abdominal cavity by conditioning ($p = 0.030$) and in the abdominal wall using humidified $CO_2$ ($p = 0.032$) or conditioning ($p = 0.026$).

*Conclusions* Tumor implantation was enhanced by peritoneal damage (60 min of dry $CO_2PP$ and desiccation), but this was prevented by conditioning. If confirmed in humans, conditioning would become important for oncologic surgery.

**Keywords** Laparoscopy · Pneumoperitoneum · Mouse model · Tumor implantation · Conditioning of the abdominal cavity · Humidified gas

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## Introduction

Laparoscopy has become the gold standard of treatment for benign conditions. Its benefits over laparotomy include better cosmesis, less pain, faster recovery of bowel function, and shorter hospitalization. Less immunologic depression [1, 2] is also well documented but its significance remains unclear. However, its suitability for cancer surgery remains a subject of debate because of concerns about tumor metastasis and growth. In addition to these advantages of laparoscopic surgery, image magnification techniques have improved our visualization of metastatic or recurrent

disease and allowed for better dissection in challenging areas such as the paravesical and pararectal spaces, with limited bleeding from small vessels because of pressure from the pneumoperitoneum (PP) [3]. Furthermore, faster recovery and no large abdominal incision permit earlier postoperative chemotherapy or radiation, with fewer radiation complications from bowel adhesions [3]. The potential disadvantages are a risk of port site metastasis (PSM) and increased tumor spread. Port site metastasis was suggested to be caused by "the chimney effect" and the aerosolization of cancer cells through leaks around the trocars [4]. The retraction and bulging of mesothelial cells, exposing the underlying basal lamina, caused by $CO_2$ PP raised concerns about the attachment of tumor cells [5]. Mathew et al. observed more dissemination of radiolabeled adenocarcinoma cells during laparoscopy with $CO_2$ PP than with gasless laparoscopy [6]. Champault et al. [7] identified intact cells in the gas that escaped during laparoscopy in six of nine patients. However, current evidence on the role of aerosolization in the development of PSM is inconclusive since other authors have shown that the aerosolization of cancer cells is not responsible for PSM. Whelan et al. [8] were unable to demonstrate aerosol formation in any of the in vivo or in vitro models. In a rat model injected with CC531 cells, Wittich et al. [9] concluded that aerosolization is not a relevant factor in the pathogenesis of the PSM. Similarly, Iwanaka et al. [10] found that $CO_2$ PP was not essential for the development of PSM.

The effects of PP on mesothelial cells and the role of the entire peritoneal cavity as a cofactor in adhesion formation have become well established over the last decade. Non-humidified $CO_2$ PP causes desiccation at the peritoneal layer [11] producing mesothelial cell bulging up, intercellular clefts increase in size, and the underlying basal lamina becomes visible [12]. In a laparoscopic mouse model, we demonstrated that the driving mechanism was acute inflammation in the entire peritoneal cavity [13]. This acute inflammation is the net result of detrimental and beneficial factors. The duration of mesothelial hypoxia caused by $CO_2$ PP [14], the duration of hyperoxia [15], the severity of desiccation [16], mechanical trauma [17], and bleeding [18] have all been identified as detrimental factors, whereas the addition of 4 % $O_2$ [15] and 10 % nitrous oxide ($N_2O$) to the $CO_2$ PP [18], the use of humidified gas [16, 19], and a lower PP temperature [20, 21] have all been identified as beneficial factors. Although the relationship between acute inflammation and peritoneal cell retraction and bulging, which exposes the basal membrane, is demonstrated only for the duration of $CO_2$ pneumoperitoneum, we assume that the same holds true for other factors investigated for adhesion formation and for $CO_2$ resorption.

It seems logical that mesothelial cell retraction would facilitate tumor implantation. Thus, we conducted this study to confirm whether tumor cell implantation, like adhesion formation, increases with factors causing acute inflammation and mesothelial damage/retraction. We also evaluated whether the same factors that reduce acute inflammation inhibit tumor cell implantation.

## Methods

### Tumor cell line

CT-26 (colon adenocarcinoma), a syngeneic cell line from BALB/c mice, was used. Cells were cultured in complete medium (RPMI-1640 medium supplemented with 10 % FCS, 1 % penicillin/streptomycin and 1 % L-glutamine) at 37 °C in a humidified environment of 5 % $CO_2$ until they reached 90–95 % confluence. On the day of tumor injection, cells were washed with PBS and detached with Versene buffer (0.48 mM EDTA in PBS). Subsequently, following dispersion in complete medium, cells were counted, and diluted to obtain the number of cells needed for injection in 1.0 ml of RPMI-1640 medium without any supplement. The viability of injected cells was evaluated by trypan blue exclusion (over 90 %) before i.p. injection, while the remaining cells were cultured with complete medium for 3 days to demonstrate attachment and growth.

### The laparoscopic mouse model

The laparoscopic mouse model was validated for adhesion formation. BALB/c mice were used because of the important PP-enhanced adhesions, as inbred strains have less variability [22]. The experimental conditions were strictly controlled, as established previously. Mice were anesthetized with i.p. 0.08 mg/g pentobarbital ($T_0$). Exactly 10 min later ($T_{10}$), the preparation was started, with shaving, positioning on the operating table, and intubation with a 20-gauge catheter. Mice were ventilated with a Mouse Ventilator (MiniVent, Type 845, Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) using a tidal volume of 250 μl at 160 strokes/min (to prevent hypercarbia/acidosis especially during PP [23] with humidified room air to prevent cooling) [21].

Laparoscopic surgery was standardized. A midline incision was performed caudal to the xyphoides at $T_{20}$ and a 2-mm endoscope with a 3.3-mm external sheath for insufflation (Karl Storz, Tuttlingen, Germany) was introduced into the abdominal cavity. The incision was closed gas-tight around the endoscope to prevent leakage.

PP was created at 15 mmHg insufflation pressure using the Thermoflator Plus (Karl Storz) and a water valve to damp pressure changes [14]. Humidification was achieved using the Storz Humidifier (204320 33, Karl Storz).

The body temperature of the mice was strictly controlled by correct timing of the preparation and surgical procedures, keeping everything set up in a chamber at 37 °C. Desiccation was induced with a controlled flow of gas through the abdominal cavity, at 23 ml/min using a 26-gauge needle at a pressure of 15 mmHg.

### Adhesion formation in the laparoscopic mouse model

These experiments on tumor implantation were conducted under established experimental conditions with known effects on adhesion formation. Adhesions at opposing surgical lesions are known to be enhanced by 60 min of $CO_2$ PP, by desiccation (using a 23 ml/min flow of dry $CO_2$, obtained by placing a 26-gauge needle in the abdomen under 15 mmHg pressure) and a higher body temperature (at 37 °C induced in a heated chamber on a homeothermic pillow) [16, 21]. Adhesions were reduced by adding 10 % $N_2O$ to the $CO_2$ [18], the mechanism of which is unknown, although it is the single most effective treatment, and by adding 4 % of oxygen to prevent the mesothelial hypoxia of pure $CO_2$ [15], at a lower body temperature of 32 °C [16, 21], and finally, by preventing desiccation using humidified gas [16, 19].

### Animals

Sixty-six female BALB/cJRj mice, 9- to 10-week old, weighing 20 g were kept under standard laboratory conditions and fed a standard laboratory diet with free access to food and water. The study was approved by the Institutional Review Animal Care Committee.

### Experimental design

In all experiments, cells were injected intraperitoneally exactly 80 min after the initiation of anesthesia ($T_{80}$) and after desufflation of the PP if appropriated, in 1 ml of RPMI-1640 medium without any supplement. The first experiment was designed as a dose-finding curve to estimate the number of cells to be used in further experiments, and to confirm the increase in tumor implantation following 60 min of $CO_2$ PP together with desiccation. The three control groups comprised mice with a BT of 37 °C, which were mechanically ventilated, not subjected to surgery, and were injected with 0.1, 0.3, and $1 \times 10^6$ CT-26 cells without PP (groups I, II, and III, respectively). The three experimental groups were subjected to the same conditions, but following 60 min of PP with dry $CO_2$ and a flow of 23 ml/min (desiccation; groups IV, V, and VI, respectively). These six groups comprised five mice per group.

In the second experiment, $0.3 \times 10^6$ CT-26 cells were used. Groups I and II, like those in the first experiment,

comprised mice with a BT of 37 °C in a control group without PP (group I) and following 60 min of $CO_2$ PP with dry gas and desiccation (group II). The other groups were chosen to evaluate the effect of factors known to be beneficial for preventing adhesion formation. In group III, the $CO_2$ was humidified; in group IV, besides humidification, 10 % of $N_2O$ was added; in group V, 4 % $O_2$ was also added (humidified 86 % $CO_2$ +10 % $N_2O$ +4 % $O_2$); and in group VI, mice were cooled to 32 °C [21]. These six groups comprised six mice per group.

All experiments were performed using block randomization by days. Therefore, a block of animals comprised one animal from each group, which was always operated on, on the same day, avoiding day-to-day variability. Within a block, experiments were performed in random order, varying each day. After 1 week, the total tumors, being those in the abdominal cavity, the abdominal wall, and the bowel mesentery were quantified as explained below.

### Tumor implantation quantification

After 1 week, mice were euthanized with an overdose of anesthesia and tumors were scored blindly and separately by two observers. Abdominal cavities were opened with a xyphopubic vertical midline incision and, without magnification, the number of visible tumors, larger than 1 mm in diameter, were counted. Most tumors were found growing in the abdominal wall and the bowel mesentery. Secondary places were the fat of the pancreas, and the fat tissue close to the bladder, kidney, liver, bowel, cecum, and diaphragm. Tumors were quantified everywhere but especially in the abdominal wall and the bowel mesentery. Figures. 1 and 3 show the tumors in the abdominal cavity, while Figs. 2 and 4 show the tumors in the abdominal wall.

### Statistics

In Experiment 1, the effect of mesothelial trauma (dry $CO_2$ PP and desiccation) was evaluated simultaneously for the three tumor concentrations by a two-way analysis of variance (proc GLM) using the SAS System (SAS Institute, Cary, NC, USA). In Experiment 2, statistical differences between groups were evaluated with the Mann–Whitney test using the GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). All data are expressed as the median, with 25 and 75 % percentiles and minimum and maximum values.

## Results

Experiment 1 was designed for dose finding in control mice and in mice with mesothelial damage induced by 60 min of

**Fig. 1** Tumor implantation in the abdominal cavity after laparoscopic surgery using the CT-26 cell line. Implantation in the abdominal cavity (*box plots*) of CT-26 tumor cells, injected i.p. as 0.1, 0.3, and $1 \times 10^6$ in control mice (no surgery and no pneumoperitoneum; groups: I, II, and III, respectively; *open bars*) or after 60 min of pneumoperitoneum with non-humidified $CO_2$; groups IV, V, and VI, respectively; *dark bars*). Implantation increased with the number of cells ($p < 0.001$) and with peritoneal damage ($p = 0.018$)
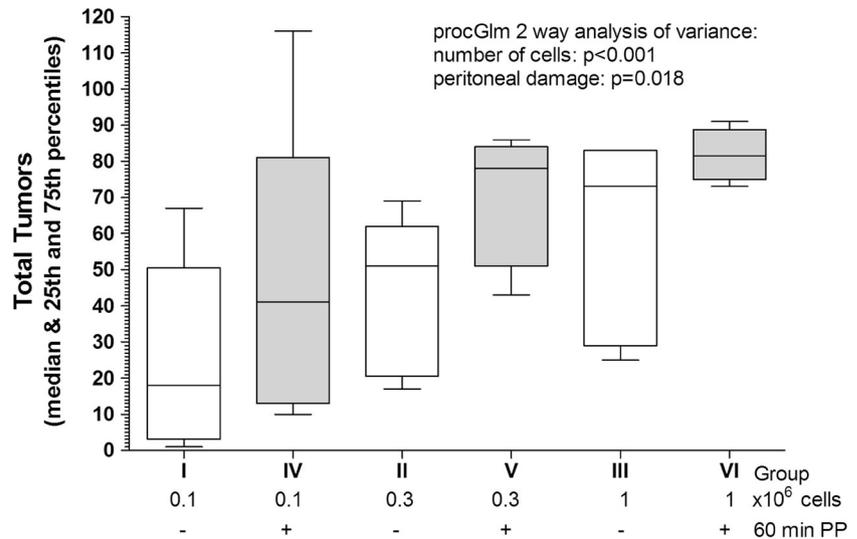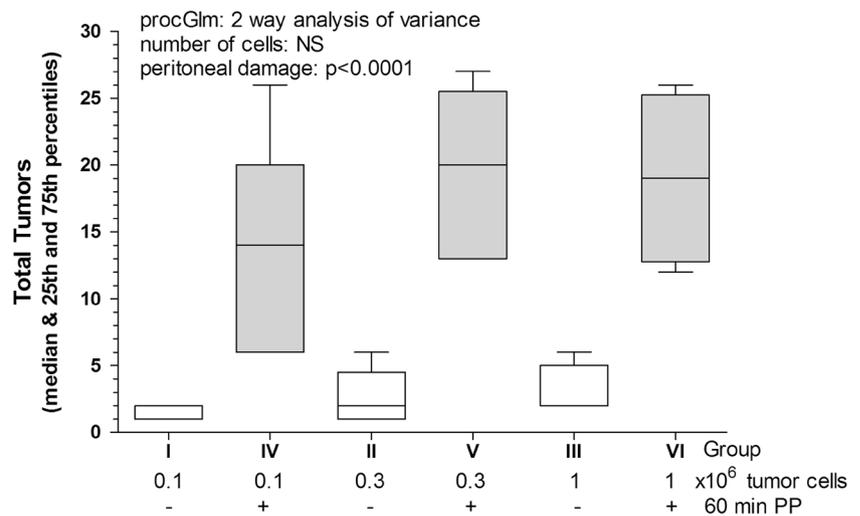


**Fig. 2** Tumor implantation in the abdominal wall after laparoscopic surgery using the CT-26 cell line. Implantation in the abdominal wall (*box plots*) of CT-26 tumor cells, injected i.p. as 0.1, 0.3 and $1 \times 10^6$, in control mice (no surgery and no pneumoperitoneum, groups I, II, and III, respectively; *open bars*) or after 60 min of pneumoperitoneum with non-humidified $CO_2$ (groups IV, V, and VI, respectively; *dark bars*). Tumor implantation increased with the peritoneal damage ($p < 0.0001$)



dry $CO_2$ PP and desiccation (23 ml/min). As expected, tumor implantation in the abdominal cavity increased with the number of cells injected ($p < 0.001$) and with peritoneal damage ($p = 0.018$; proc GLM two-way analysis of variance; Fig. 1). These effects of tumor implantation were observed equally in the abdominal wall (peritoneal damage $p < 0.0001$; number of cells, NS; Fig. 2), and in the bowel mesentery (peritoneal damage: NS; number of cells $p = 0.007$; data not shown), which were analyzed separately.

Experiment 2 confirmed that the mesothelial damage induced by 60 min of dry $CO_2$ PP and desiccation (23 ml/min) increased tumor implantation in the abdominal cavity (I vs II: $p = 0.0260$; Mann–Whitney test; Fig. 3). This implantation was decreased to a level comparable to that of the control group when mesothelial damage was prevented by conditioning, through humidification plus the addition of 10 % $N_2O$, 4 % of $O_2$ and cooling to 32 °C (II vs VI: $p = 0.03$). All other intermediary steps failed to reach

significance; that is, humidification (II vs III), humidification + 10 % $N_2O$ (II vs IV), and humidification + 10 % of $N_2O$ + 4 % of $O_2$ (II vs V).

When the number of tumors implanted in the abdominal wall was analyzed separately, similar conclusions were made (Fig. 4). Peritoneal damage increased tumor implantation (I vs II, $p = 0,0031$, Mann–Whitney test), whereas conditioning (humidified $CO_2$ plus 10 % $N_2O$ 4 % $O_2$ and cooling), decreased tumor implantation (II vs VI: $p = 0,026$) to levels comparable to those of the control group (I vs VI: NS). When analyzing the intermediary steps, humidified $CO_2$ reduced tumor implantation significantly (II vs III; 0.0318), whereas the effects of humidification + 10 % of $N_2O$ and humidification plus 10 % of $N_2O$ + 4 % of $O_2$ failed to reach significance (II vs IV and II vs V). Surprisingly, the number of tumors in the mesentery was not affected by mesothelial damage in either experiment 1 or 2.
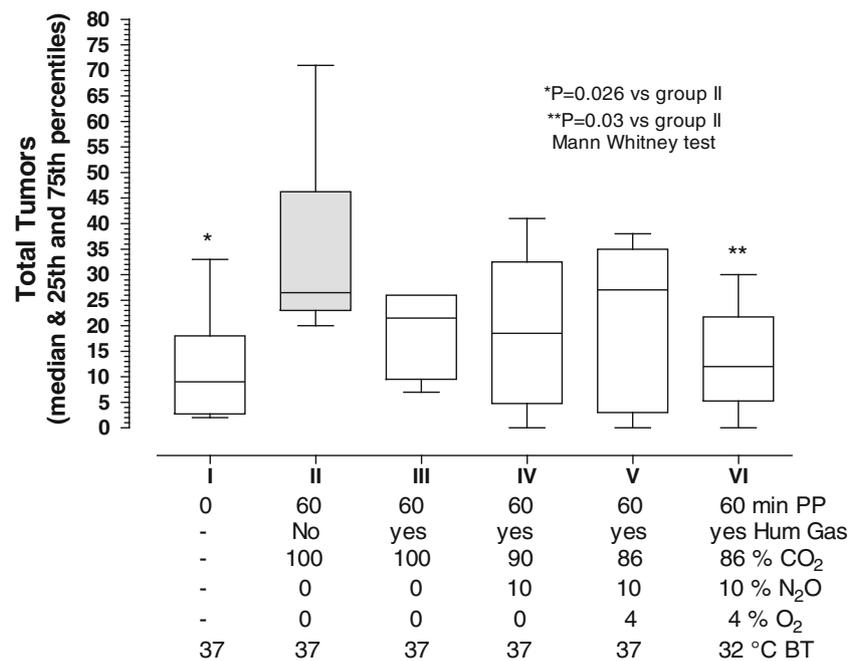
| | I | II | III | IV | V | VI |
|---|---|---|---|---|---|---|
| | 0 | 60 | 60 | 60 | 60 | 60 min PP |
| | - | No | yes | yes | yes | yes Hum Gas |
| | - | 100 | 100 | 90 | 86 | 86 % $CO_2$ |
| | - | 0 | 0 | 10 | 10 | 10 % $N_2O$ |
| | - | 0 | 0 | 0 | 4 | 4 % $O_2$ |
| | 37 | 37 | 37 | 37 | 37 | 32 °C BT |

**Fig. 3** Effect of peritoneal cavity conditioning on tumor implantation in the abdominal cavity. Effect of peritoneal cavity conditioning on the implantation of CT-26 tumor cells ($0.3 \times 10^6$) injected i.p. in mice not subjected to surgery or pneumoperitoneum (group I) or after 60 min of pneumoperitoneum with non-humidified $CO_2$ (group II; *dark bar*). To the latter group, we sequentially added beneficial factors such as humidification (group III), 10 % $N_2O$ (group IV), 4 % $O_2$ (group V), and cooling the body temperature to 32 °C (group VI). The body temperature was 37 °C in all other groups. Tumor implantation in the abdominal cavity increased with peritoneal damage ($p = 0.026$) and decreased with conditioning ($p = 0.03$)

## Discussion

These results contribute to our understanding of the role of the peritoneal cavity in adhesion formation. Adhesions are enhanced by factors causing acute inflammation, mesothelial damage, and cell retraction. The factors identified that prevent this acute inflammatory reaction and cell retraction are the addition of 10 % $N_2O$ and 4 % of $O_2$ to the $CO_2$ PP, together with cooling to 32 °C in the absence of desiccation. The combination of these factors is called peritoneal conditioning. We found that the same damaging factors increase $CO_2$ resorption [24], whereas conditioning can prevent this increase. In this study, as expected, the same damaging factors increased tumor cell implantation but this increase can be prevented by conditioning.

The increase in tumor cell implantation by $CO_2$ PP and desiccation, known to cause mesothelial cell retraction and bulging, confirms previous observations. In a study by Shen et al. [25], $10^6$ of CT-26 were inoculated into the lower abdominal cavity of mice, which were then randomized for PP with $CO_2$ or helium or to a control group. They demonstrated that PP enhances the implantation and growth of free intraperitoneal malignant cells, suggesting that insufflation during PP plays an important role in peritoneal dissemination when free tumor cells are in the abdominal cavity. Moreover, the effect achieved by $CO_2$

was greater than that achieved by helium. In another experiment using a rat model, mammary adenocarcinoma cells were injected into the lower right quadrant of the peritoneal cavity [26]. In comparison with a control group, the insertion of trocars with $CO_2$ PP resulted in greater tumor cell implantation than laparotomy incisions. In another rat model [27], following the i.p. injection of $10^5$ colon cancer cells, tumor growth was significantly increased by both cold and warm $CO_2$ PP in comparison with a control group.

These data demonstrate that the increase in tumor cell implantation by $CO_2$ PP and desiccation can be prevented by a combination of adding 10 % $N_2O$, 4 % $O_2$, cooling, and absence of desiccation, known as peritoneal conditioning. In fact, following conditioning, tumor implantation is comparable to that of control groups, both in the abdominal cavity and the wall. This observation is strikingly similar to that of adhesion formation, which is not surprising considering the underlying mechanism of a dose/trauma-dependent acute inflammation and mesothelial cell retraction, exposing the basal membrane. For adhesion formation, we demonstrated that the single most effective way of preventing mesothelial damage was to add $N_2O$ and that $O_2$ had little if any effect, although it was slightly effective when used alone. The second most important factor is cooling and avoiding desiccation; however, the
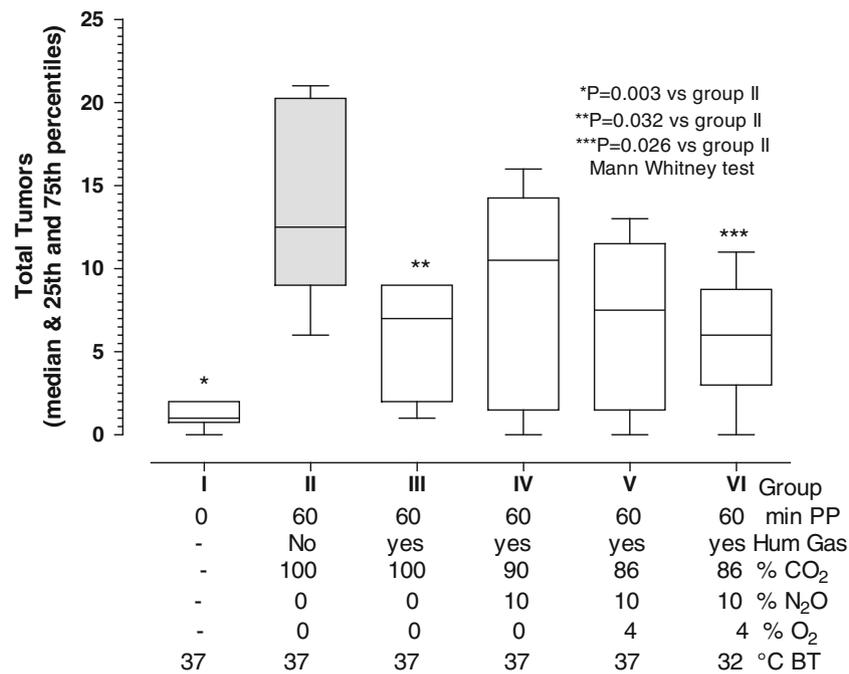
**Fig. 4** Effect of peritoneal cavity conditioning on tumor implantation in the abdominal wall. Effect of peritoneal cavity conditioning on the implantation of CT-26 tumor cells ($0.3 \times 10^6$) injected in mice not subjected to surgery or pneumoperitoneum (group I) or after 60 min of pneumoperitoneum with non-humidified $CO_2$ (group II; *dark bar*). To the latter group, we sequentially added beneficial factors such as humidification (group III), 10 % $N_2O$ (group IV), 4 % $O_2$ (group V), and cooling to a body temperature of 32 °C (group VI). The body temperature was 37 °C in all other groups. Tumor implantation in the abdominal wall increased with the peritoneal damage ($p = 0.003$) and decreased using humidified $CO_2$ ($p = 0.032$) and conditioning ($p = 0.026$)

effectiveness of cooling and desiccation on adhesion formation is difficult to demonstrate in our mouse model after the addition of $N_2O$, since so few adhesions remain. These tumor experiments were therefore designed to demonstrate the eventual effect of adding $N_2O$ after humidification. We also demonstrated that humidifying the insufflation gas decreased tumor implantation when compared with the control group subjected to dry $CO_2$ PP, although the effect of humidification was not as great as that of full conditioning since it reduced tumor implantation in the wall but not in the whole abdominal cavity. These results underline the importance of preventing desiccation in the context of preventing damage to the mesothelial cells. It is not surprising that the effects are more pronounced in the abdominal wall, which is directly exposed to the PP, than in the mesentery, which is only partially exposed.

The experiment was not powered to detect subtle differences, especially since variability in tumor implantation was much higher than the variability previously observed for adhesion formation. Searching the literature for each individual factor did not reveal data concerning tumor implantation following the addition of oxygen or humidification. Most experiments investigate port site metastasis and have observed that helium is associated with less port site metastasis than pure $CO_2$, $N_2O$ or air [25, 26, 28].

However, Hopkins et al. found no difference in tumor spread when using $CO_2$, $N_2O$, or helium [29]. When studying the effect of warm vs. cold $CO_2$ gas on tumor spread, it was found to be less when warm gas was used [27]. This was in contrast to our findings, but we can only speculate about this discrepancy. As described in our "Methods", we controlled the temperature of the mice, the time between the induction of anesthesia and manipulations, and the ventilation, strictly. We have data only on cooling mice to 26 °C without desiccation, but not on what happens when mice are cooled further. Thus, the apparent conflicting data could be explained by differences between mice and rats, or by the fact that when cold gas was used, the rats were cooled much more, as a result of desiccation if trocar insertion was not leak free. The role of immunology in this will also need investigation. Moreover, our search of the literature revealed no evidence of the use of humidified gas, which could explain the discrepancy.

The findings of this study obviously raise questions about such surgery in humans. Translation to human surgery was started by investigating cooling and desiccation in detail and the results can be summarized as follows [30]: first, to avoid desiccation, cooling has to be done with a third means. If cold humidified gas is used for insufflation, this gas will warm up in the abdomen at 37 °C, making

desiccation unavoidable. Second, cooling of the abdominal cavity to 28 °C will not affect core body temperature unless desiccation occurs. We do not know the consequences of cooling to lower temperatures, although data from the mouse model show that the relationship between temperature and adhesions is exponential so that cooling to less than 26 °C would be unlikely to affect the results further. The choice to use 10 % $N_2O$ and 4 % $O_2$ was based on mouse experiments [18]. We also recently finalized a randomized control trial in which it was demonstrated in humans that conditioning decreases $CO_2$ resorption and decreases postoperative pain, while being the single most effective means of preventing postoperative adhesions [24].

In conclusion, as described for adhesion formation in mice, laparoscopic surgery with dry $CO_2$ PP increases tumor implantation in the abdominal cavity and wall. Moreover, full conditioning of the abdominal cavity, achieved by humidified $CO_2$ with 10 % $N_2O$ and 4 % $O_2$ together with a lower local temperature, reduces tumor implantation in the abdomen and its wall. The clinical implication that full conditioning might inhibit tumor spread in oncologic surgery is suggested by the recent discovery that full conditioning decreases adhesion formation in humans [24].

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