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SURGICAL ENDOSCOPY
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Influence of different gases used for laparoscopy (helium, carbon dioxide, room air, xenon) on tumor volume, proliferation, and apoptosis

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Abstract
Background: Previous reports suggest that helium pneumoperitoneum used for laparoscopic surgery suppresses postoperative tumor growth. The present study was designed to determine the effects of gases used in laparoscopy on tumor volume, proliferation, and apoptosis in rats with implanted malignoma.
Methods: In 36 rats Morris hepatoma 3294A cells were implanted intrahepatically. Then, after 5 days, they underwent laparoscopy using helium ($n = 7$), CO_2 ($n = 7$), room air ($n = 7$), or xenon ($n = 8$). One group received anesthesia only ($n = 7$). Rats were killed 10 days after implantation to assess tumor volume, proliferation, and apoptosis.
Results: Helium pneumoperitoneum caused a significant smaller tumor volume compared to other groups (Kruskal-Wallis test: $p = 0.001$; median tumor volume: control: 44 mm^3; helium: 19 mm^3). There was no significant difference in tumor cell proliferation (PCNA) and apoptosis (TUNEL reaction) between the groups.
Conclusions: There was a significant decrease of tumor volume using helium pneumoperitoneum compared to the other gases, but no decreased tumor cell proliferation or increased tumor cell apoptosis.

Key words: Laparoscopy — Pneumoperitoneum — Helium — Tumor volume — Proliferation — Apoptosis

Minimally invasive (laparoscopic) surgery is an established procedure in therapy of benign diseases. Laparoscopy surgery leads, compared to open surgery, to reduced postoperative pain [7, 23], lessened trauma, and shortened stays in the hospital [5, 6]. Therefore in curative oncologic surgery the laparoscopic procedure is an interesting alternative to open surgery, but also has a plurality of risks and problems such as insufficient radical resection and tumor cell delay [3, 10]. However, previous experimental in vivo and in vitro studies have shown an increased tumor growth after laparotomy compared to laparoscopy [1, 2]. In recent years, based on this knowledge, there has been increased interest in the influence of the gases used for laparoscopy, especially on tumor growth.

Carbon dioxide is the most applied gas used for laparoscopy because of its positive qualities such as absence of color, nonexplosive nature, and good availability. Carbon dioxide is also easily absorbable. This property increases protection from gas embolism, which can be found in laparoscopy with room air [14, 21], but also can lead to metabolic acidosis at longer operation durations, which increases a risk for elderly patients with reduced pulmonary function [4, 19]. In the literature there are contrary opinions about the influence of carbon dioxide on tumor growth. Some authors describe increased tumor growth after carbon dioxide pneumoperitoneum [11, 16]; others perceive no effect on tumor growth [22]. Because of the above-mentioned problems, the use of inert gases such as helium for laparoscopic surgery is more and more often discussed. Helium has the same positive qualities as carbon dioxide but no metabolic effect and therefore no obvious influence on acid–base balance. Previous studies indicate that helium pneumoperitoneum used for laparoscopic surgery suppresses postoperative tumor growth and may lead to less mestastatic disease [12, 17]. However, the mechanism of decreased tumor growth by helium is unknown. The only known detriment of usage of helium is the increased risk of gas embolism [20] compared to carbon dioxide pneumoperitoneum. Xenon belongs, like helium, to the inert gases and has the same positive qualities. It is also supposed that xenon has a suppressive

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Table 1. Description of tumor volume distribution (in mm^3) in the different groups

	n	0%[a]	25%	50%	75%	100%
Helium	7	10	14.50	19	21.00	55
Room air	7	33	40.00	67	70.00	173
Carbon dioxide	7	38	56.50	67	81.00	94
Control group	7	22	37.50	44	59.00	73
Xenon	8	47	65.25	72	81.25	150
All	36	10	37.75	59	73.00	173

[a] Percentile, Kruskal-Wallis test

effect on tumor growth, because previous studies have shown a xenon-induced inhibition of cell cycle [18]. However, nowadays these gases are not generally used for laparoscopy because of poor availability and high primary costs. Room air, the gas with best availability, is no longer in use for pneumoperitoneum because of its negatives, for example the risk of gas embolism [14, 15]. In gasless laparoscopy, room air replaces the gas but without risk of gas embolism.

If the gas used for pneumoperitoneum has a suppressive effect on tumor growth and the pathogenic mechanism of the different gases is known, the aforesaid advantages of laparoscopic surgery could be added to with the pneumoperitoneum inducing agent also having a tumor suppression effect.

The purpose of this study is to evaluate the effect of the different gases (helium, carbon dioxide, room air, xenon) used for pneumoperitoneum in laparoscopy on intrahepatic tumor growth and the pathogenic mechanism in a well-described rat hepatoma model [24] measured by tumor volume, metastatic spread, proliferation, and apoptosis index. The idea was that suppression of tumor growth is based on decreased tumor cell proliferation or increased apoptosis rate of cancer cells; therefore proliferation rate and mitotic rate were evaluated.

Materials and methods

Tumor cells and animals

As tumor model a liver carcinoma Morris hepatoma 3924A that is well described by Yang et al. [24] was used. The Morris hepatoma 3924A is a moderately differentiated hepatocellular carcinoma and a syngenic carcinoma in ACI rats.

Even when hepatic tumors are not a focus for laparoscopic surgery, this tumor model was used because of its positive tumorbiological properties such as 100% growth rate and symmetrical augmentation.

In this study ACI rats weighting 240 to 275 g were used. The rats were delivered by Harlan Sprague-Dewless (Indianapolis, IN, USA). The animals were kept under specific pathogen-free and standardized conditions. The room temperature ranged between 22 and 24°C; the relative humidity of the air was between 50 and 60%. There were 12 h of light and 12 h of darkness. The rats were placed in standard cages and fed with a standard rat diet and tap water ad libitum.

The protocols were approved by the Animal Research Committee.

Anesthesia

The animals were anaesthetized with *S*-ketamine hydrochloride (S-Ketanest, 4 mg/kg body weight, intramuscular) and sodium pentobarbital (Nacoren, 20 mg/kg body weight, 1:5 with NaCl, intraperitoneal).

Operative procedures

Thirty-six male ACI rats were studied. After disinfection of the abdomen a small subxiphoid midline abdominal incision was made. A tumor cell suspension of Morris hepatoma 3924A at a concentration of 2×10^6 cells in culture solution was implanted intrahepatically in the left lateral liver lobe using a microliter syringe inserted at an angle of 20° to the liver surface to implant the cells subcapsulary. After implantation the abdominal wall was clothed with interrupted suture (3-0). As an analgesic, rats received 12.5 mg tramadol hydrochloride per 100 ml drinking water for 24 h a day until the animals were killed for evaluation.

Rats were randomized into five groups. Five days after implantation they underwent laparoscopy using helium ($n = 7$), carbon dioxide ($n = 7$), room air ($n = 7$), or xenon ($n = 8$). One group ($n = 7$) received anesthesia only and served as the control group. The anesthesia was similar to that noted above for tumor implantation. For laparoscopy a small midline abdominal incision to insert the trocar was made. An insufflator (Fa. Wolff, Germany) was used to produce a pneumoperitoneum of 8 mmHg. All animals were killed 10 days after tumor implantation by overdose of sodium pentobarbital to assess tumor volume, tumor cell proliferation, and apoptosis. The tumor size was measured by length (a), width (b), and thickness (c) with a caliper and tumor volume was approximated by $V = 1/6 \times \pi \times a \times b \times c$.

Immunohistochemistry

Tissues were fixed in 5% formalin, embedded in paraffin wax using standard techniques. Slides (3 μm) were cut and sections were dried on superfrost microscopic slides. Slides were deparaffinized and rehydrated.

For detection of tumor proliferation, proliferation cell nuclear antigen (PCNA) imunhistochemical staining was performed. Tissues were prepared as described above. Endogenous peroxidase was inhibited by 30-min incubation in 3% hydrogen peroxide at 4°C. The slides were covered with goat serum for 20 min. This was after endogenous biotin was blocked by 10-min incubation with avidin solution and 10-min incubation with biotin solution (biotin blocking system, DAKO). Slides were incubated overnight at 37°C with PC 10 antibody against PCNA (1:200, DAKO) followed by sequential 10-min incubation with biotinylated link antibody and peroxidase-labeled streptavidin (LSAB 2 kit, DAKO), and staining was completed after incubation with substrate-chromogen solution (DAB, DAKO). PCNA nuclei of three microscopic fields from marginal areas of the tumor were counted.

Apoptosis of cells was analyzed using the TUNEL reaction (Roche Diagnostics). In the TUNEL technique terminal desoxynucleotidyl transferase (TdT), which catalyzes polymerization of labeled nucleotides to free 3′-OH DNA ends, is used to label DNA strand breaks in situ. Incorporated fluorescein was detected by anti-fluorescein antibody Fab fragments from sheep, conjugated with alkaline phosphatase. The localized substrate was shown by fast red naphthol (Sigma) staining. Apoptosis cells of three microscopic fields were counted.

Table 2. Tumor cell proliferation (cells per field) in the different groups

	n	0%[a]	25%	50%	75%	100%
Helium	7	6	23.5	89.0	121.5	125
Room air	7	7	33.0	97.0	132.0	198
Carbon dioxide	7	1	35.5	43.0	72.0	109
Control group	7	34	53.0	102.0	123.0	256
Xenon	8	77	99.0	107.5	157.0	262
All	36	1	42.0	96.5	122.5	262

[a] Percentile, Kruskal-Wallis test

Table 3. Tumor cell apoptosis (cells per field) in the different groups

	n	Missing	0%[a]	25%	50%	75%	100%
Helium	7	0	0	0.5	2.0	4.00	6
Room air	7	0	0	0.0	1.0	2.00	4
Carbon dioxide	7	0	0	1.0	2.0	3.00	7
Control group	5	2	3	4.0	4.0	4.00	9
Xenon	8	0	0	1.0	1.5	2.75	6
All	34	2	0	1.0	2.0	4.00	9

[a] Percentile, Kruskal-Wallis test

Metastatic spread

All rats were necropsied after death. The necropsy was performed according to a standardized protocol. All major lymph node regions, all body cavities, and all organs were thoroughly inspected. The liver was dissected to exclude further tumor areas. The primary tumor localization, the trocar sites on the abdominal wall, the lungs, and all nodules suspicious for malignancy were dissected and stored in formalin, stained with hematoxylin–eosin, and pathohistologically examined.

Statistics

Distribution of tumor volumes, proliferation rates and numbers of apoptotic cells were presented by their 0%, 25%, 50% (median), 75%, and 100% quantile. The Kruskall-Wallis test was used to compare five treatment groups. If this test showed a statistically significant result, pairwise comparisons of the five groups were also performed using the Mann-Whitney test. A result was considered as statistically significant if the p-value of its corresponding test statistic was $< 5\%$ ($p < 0.05$).

Results

In necropsy 10 days after tumor implantation a well-developed solitary hepatic tumor nodule was identified in all 36 rats, which corresponds with an implantation rate of 100%. There were no trocar site metastases and no metastatic tumor nodules found in lung, liver and lymph nodes of all rats. This confirms previous results with this tumor model [9, 24].

Examination of apoptosis rate in the control-group was only possible in five animals for technical reasons (preparation of tissues).

The establishment of helium pneumoperitoneum caused a significant reduction of tumor volume when compared to control or groups that received carbon dioxide, room air, or xenon for pneumoperitoneum (Kruskal-Wallis test: $p = 0.001$; 50% quantile: control group: 44 mm^3; helium: 19 mm^3; xenon: 72 mm^3; see Table 1). The hepatic tumor volume was not statistically significantly increased in the group receiving CO_2 for pneumoperitoneum (50% quantile: 67 mm^3) compared to control (Mann-Whitney test with control: $p = 0.06$). Xenon did not suppress tumor growth significantly; it even led to increased tumor growth compared to control group (Mann-Whitney test with control: $p = 0.01$).

There was no statistically significant difference in tumor cell proliferation between the groups using different gases for pneumoperitoneum and control groups (Kruskal-Wallis test: $p = 0.13$) (Table 2).

There was no statistically significant difference in the number of tumor cells undergoing apoptosis measured by the TUNEL reaction in the groups receiving helium, CO_2, air, xenon or control group, as shown in Table 3 (Kruskal-Wallis test: $p = 0.13$).

Discussion

Laparoscopic surgery is an established procedure in visceral surgery. Because of fears of insufficient radical resection and tumor cell delay, laparoscopy is not a customary technique in oncologic surgery [3, 10]. However, minimally invasive techniques in malignant diseases could also have several advantages: A variety of studies have shown a suppression of tumor volume after laparoscopy compared to laparotomy [1, 2], and it seems that the gas used for pneumoperitoneum has an effect on tumor growth, as helium suppresses postoperative tumor growth [8, 12, 17]. This knowledge has led to

increased interest in the influence of the gas used for laparoscopy on tumor growth and the pathogenic mechanism of tumor growth suppression.

In this study the effect of the different gases (helium, carbon dioxide, room air, xenon) used for pneumoperitoneum in laparoscopy on tumor growth and the pathogenic mechanism of tumor suppression was examined. The idea was that suppression of tumor growth—if it can be duplicated—is based on decreased tumor cell proliferation or increased apoptosis rates of cancer cells. Therefore proliferation rate and mitotic rate were evaluated.

In this study there was a statistically significant decrease of tumor volume using helium pneumoperitoneum for laparoscopy. So the results of Jacobi et al. [12] and Neuhaus et al. [17] about tumor cell suppression in a helium environment could be replicated. The in vitro results (cell culture) of Jacobi et al. [12] could also be confirmed by in vivo results (rats) of this presented study.

Gutt et al. [9] investigated tumor cell proliferation under CO_2 environment. They described an influence of the intraabdominal pressure on the tumor growth. This interesting aspect could be excluded in this study because we used the same pressure for all gases.

In contrast to our expectations, there was no relevant increase of tumor growth using carbon dioxide pneumoperitoneum for laparoscopy. Xenon, which belongs like helium to the inert gases and which leads to an inhibition of cell cycle [18], did not suppress tumor growth significantly. We could observe an increase in tumor growth in comparison to the control instead. It was expected that xenon, which has similar properties to helium, also leads to a suppression of tumor growth. But after the result of this study, other mechanisms of the gas seem to be more important for the effective tumor volume.

There was no significant difference in proliferation and apoptosis between the treatment groups using different gases and the control group.

There was no metastatic spread in any animal, which is a result of the short survival time of the animals. This is typical for the tumor model Morris hepatoma 3924A [8, 24].

In this study we could not find a correlation between suppression of tumor volume and tumor cell proliferation or apoptosis rate of tumor cells. Therefore the pathogenic mechanism of suppression of tumor growth after helium pneumoperitoneum is still unknown. It could be that the moment of examination, evaluation of proliferation and apoptosis, which was 5 days after laparoscopy, was too late. It can be imagined that the gases used for pneumoperitoneum have only a short-term effect and thereby the tumor volume is finally significantly different, but cell proliferation or cell setting is not significantly different between the studied groups. To prove this supposition, the chosen parameters would have to be examined in the first hours after laparoscopy. But in this case we would expect an approximation of the tumor volume after 5 days.

It is also conceivable that the gases have an effect on another step of cell cycle. Another reason for this effect could be that the tumor was too aggressive, which means too fast growing, to allow extraction of small differences in proliferation rate between the examined groups. To understand more about the pathogenic effect on tumor volume of different gases used for pneumoperitoneum in laparoscopy, which was shown in this and other studies, further investigations of pathogenic mechanism should be performed with special attention to the influence of helium on the cell cycle.

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