

## Treatment with ozone/oxygen-pneumoperitoneum results in complete remission of rabbit squamous cell carcinomas

Siegfried Schulz<sup>1</sup>, Ulrich Häussler<sup>2</sup>, Robert Mandic<sup>2</sup>, Johannes T. Heverhagen<sup>3</sup>, Andreas Neubauer<sup>4</sup>, Anja A. Dünne<sup>2</sup>, Jochen A. Werner<sup>2</sup>, Eberhard Weihe<sup>5</sup> and Michael Bette<sup>5\*</sup>

<sup>1</sup>Veterinary Services and Laboratory Animal Medicine, Philipps University Marburg, Germany

<sup>2</sup>Department of Otolaryngology, Head and Neck Surgery, University Hospital Giessen and Marburg, Campus Marburg, Marburg, Germany

<sup>3</sup>Department of Diagnostic Radiology, University Hospital Giessen and Marburg, Campus Marburg, Marburg, Germany

<sup>4</sup>Department of Haematology, Oncology and Immunology, University Hospital Giessen and Marburg, Campus Marburg, Marburg, Germany

<sup>5</sup>Department of Molecular Neuroscience, Institute of Anatomy and Cell Biology, Philipps University Marburg, Germany

Head and neck squamous cell carcinomas (HNSCC) represent a group of metastasizing tumors with a high mortality rate in man and animals. Since the biomolecule ozone was found to inhibit growth of various carcinoma cells *in vitro* we here applied the highly aggressive and lethal VX2 carcinoma HNSCC tumor model of the New Zealand White rabbit to test whether ozone exerts antitumorous effects *in vivo*. Therapeutic insufflation of medical ozone/oxygen (O<sub>3</sub>/O<sub>2</sub>) gas mixture into the peritoneum (O<sub>3</sub>/O<sub>2</sub>-pneumoperitoneum) at an advanced stage of tumor disease led to a survival rate of 7/14 rabbits. Six of the seven surviving rabbits presented full tumor regression and the absence of local or distant lung metastases. Insufflation of pure oxygen (O<sub>2</sub>) resulted in a survival rate of 3/13 animals accompanied by full tumor remission in 2 of the 3 surviving animals. Of the 14 sham-treated animals only 1 had spontaneous tumor remission and survived. No adverse effects or changes in standard blood parameters were observed after repeated intraperitoneal insufflations of the O<sub>3</sub>/O<sub>2</sub> or O<sub>2</sub> gas. Animals with O<sub>3</sub>/O<sub>2</sub>-induced tumor eradication developed tolerance against reimplantation of the VX2 tumor. This could be reversed by immune suppression with a combination of dexamethasone and cyclosporin A suggesting an antitumorous effect of O<sub>3</sub>/O<sub>2</sub>-mediated activation of the body's own immunosurveillance. Although the exact mechanisms of action are still unclear the present data point to O<sub>3</sub>/O<sub>2</sub>-pneumoperitoneum as a promising new strategy in anticancer therapy.

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**Key words:** HNSCC; immunosurveillance; metastasis; O<sub>3</sub>/O<sub>2</sub>-pneumoperitoneum; VX2 carcinoma

Squamous cell carcinomas of the head and neck region (HNSCC) frequently metastasize and show a high mortality rate in man and animals. Being an accepted animal model for studying the progression and metastatic spread of HNSCC, the highly aggressive and lethal VX2 auricular carcinoma model of the New Zealand White (NZW) rabbit<sup>1,2</sup> was applied in our study. This tumor model was proven to be highly suitable to investigate new therapeutic approaches, since both the HNSCC and the VX2 carcinoma are similar in growth leading to early regional lymph node and subsequent distant metastatic spread.<sup>3–5</sup>

As is true for many cancers HNSCC tumor cells somehow evade the body's immune system. This immune escape can partly be explained by the frequently observed downregulation or loss of MHC Class I determinants<sup>6,7</sup> and an increase in CD4+CD25+ regulatory T cells (T<sub>reg</sub>) that are found responsible for depressed antitumor immunity.<sup>8–10</sup> Therefore, being able to activate the immunosurveillance toward HNSCC tumor cells should help in recognition and eradication of this tumor entity. Enhancing the immunosurveillance capacity is an emerging concept in recent cancer immunotherapies,<sup>11–13</sup> particularly those focusing on immunomodulators or upregulators of the immune response.<sup>14</sup> To augment the host's immune response against cancer cells, therapies with recombinant cytokines, dendritic cell immunization and tumor antigen vaccination as well as T cell-based immunotherapies are currently under investigation.<sup>15–17</sup>

Ozone, recently found to be produced endogenously by granulocytes,<sup>18</sup> is a gas with complex influence on free radical biology in man and animals.<sup>19</sup> Most research on the biological effects of ozone has focused on its lung toxicity due to inhalation of ambient ozone.<sup>20,21</sup> Therefore, airway application has limited *in vivo* studies of medical ozone in human diseases. Interestingly, ozone exhibited potent protective effects on polymicrobial-induced lethal sepsis without any detectable lung toxicity when applied as an O<sub>3</sub>/O<sub>2</sub> gas mixture into the peritoneum.<sup>22</sup> Additionally, evidence for antibody-catalyzed ozone formation in bacterial killing<sup>23</sup> suggests immune mediated effects of endogenously produced or exogenously applied ozone. Early *in vitro* studies described ozone as a radiomimetic gas,<sup>24</sup> able to selectively inhibit growth of isolated human alveolar, uterine, breast and endometrial carcinomas.<sup>25</sup>

This led us to hypothesize that intraperitoneal application of a medical O<sub>3</sub>/O<sub>2</sub> gas mixture stimulates the body's own antitumorous immunosurveillance.

### Material and methods

#### Animals

The study comprises 59 healthy adult Iffa Credo NZW outbreed rabbits in a body weight range from 2.0 to 3.0 kg, purchased from Charles River WIGA, Sulzfeld, Germany. All animals were kept in rooms with standardized air conditioning: at 20–22°C, 50–60% humidity and a 12 hr artificial day/night rhythm. Each rabbit was held in an individual steel cage, was fed with 100 g complete diet food pellets per day and had free access to acidified (hydrochloric acid, pH 2.7) tap water *ad libitum*. Animals could acclimatize for at least 5 days before the experimental procedure was started.

#### Experimental design and tumor transplantation

The study was in accordance with the guidelines of FELASA and was approved by the regional council Giessen [V 54-19 c 20-15(1) MR, No. 24/2005], Germany, according to the German Animal Protection Law. The rabbits were randomly divided into 3 experimental groups: (i) animals that received an O<sub>3</sub>/O<sub>2</sub> gas mixture therapy ( $n = 14$  animals), (ii) animals receiving O<sub>2</sub> gas therapy ( $n = 14$  animals), (iii) sham-treated animals that were treated similar to the other groups but receiving no gas insufflation ( $n = 14$  animals). Fourteen additional NZW rabbits were used as donors in which VX2 tumor cells were propagated by intramuscular pas-

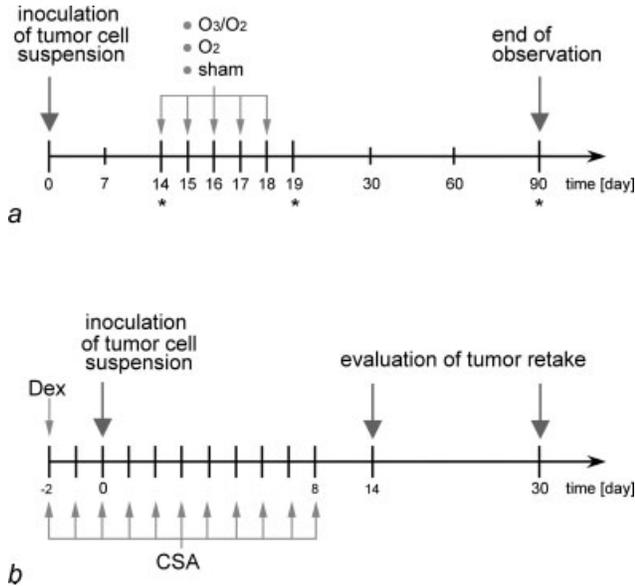
\*Correspondence to: Department of Molecular Neuroscience, Institute of Anatomy and Cell Biology, Philipps University, Robert-Koch Street 8, 35032 Marburg, Germany. Fax: +49-6421-2866780.

E-mail: bette@staff.uni-marburg.de

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**FIGURE 1** – Depicted is the experimental design of this study for (a) the treatment scheme and (b) the retake rate after immune suppression of the rabbits. \*Time points of blood withdrawal

sages. Fresh tumor cell suspension was derived from the hind leg tumor of donor rabbits as described previously.<sup>4</sup> The animals of all experimental groups were sedated with 5 mg/kg body weight xylazine intramuscularly (Rompun<sup>®</sup>, Bayer Vital, Leverkusen, Germany) and received a slow subcutaneous injection of  $1-2 \times 10^7$  vital tumor cells suspended in a volume of 0.3 ml into the area between the central auricular artery and the caudal margin at the dorsal middle-third of the right ear, as described in detail elsewhere.<sup>1,4,26</sup> Tumor growth was allowed for 14 days (for experimental design see Fig. 1a). On Day 14, the daily therapeutic treatment with O<sub>3</sub>/O<sub>2</sub> gas mixture or pure O<sub>2</sub> was initiated lasting for a period of 5 days. For this purpose, animals were premedicated with 0.1 ml/kg body weight glycopyrrolate (Robinul<sup>®</sup>, Riemser Arzneimittel AG, Greifswald, Germany) subcutaneously, followed by a short anesthesia by application of 0.3 ml/kg body weight medetomidine hydrochloride (Domitor<sup>®</sup>, Pfizer, Karlsruhe, Germany) intramuscularly plus 0.3–0.6 ml/kg body weight propofol (Propofol 1% Fresenius, Fresenius Kabi Deutschland, Germany) intravenously into the ear vein. After treatment, 0.3 ml/kg body weight of the antagonist atipamezole hydrochloride (Antisedan<sup>®</sup>, Pfizer, Karlsruhe, Germany) was applied intravenously. The sham group was treated likewise and served as a control. Animals of the O<sub>3</sub>/O<sub>2</sub> group received an insufflation of the O<sub>3</sub>/O<sub>2</sub> gas mixture and rabbits of the O<sub>2</sub> group received an insufflation of pure medical oxygen into the peritoneum. The ozone gas processor Medozon<sup>IP</sup> (Herrmann Apparatebau, Kleinwallstadt, Germany; license holder) was used for the generation and intraperitoneal insufflation of the O<sub>3</sub>/O<sub>2</sub> gas mixture and the O<sub>2</sub> gas volume. An integrated intra-abdominal pressure control (IAPC) in the Medozon<sup>IP</sup> gas processor avoids high abdominal pressures during the insufflation process. This novel Medozon<sup>IP</sup> gas processor has been invented by S. Schulz, the first author of this report and was recently patented (Az: DE102004017599.3; PCT/DR2005/000597; EP05740628.2-2310; patent holder Transmit GmbH Giessen, Germany). The freshly synthesized O<sub>3</sub>/O<sub>2</sub> gas mixture or pure medical O<sub>2</sub> gas was immediately insufflated from the Medozon<sup>IP</sup> O<sub>3</sub>/O<sub>2</sub> gas generator into the peritoneum of the rabbits via a sterile Ozon-Kit (Ozone-Set ip REF HAB no. 18052, Herrmann Apparatebau, Kleinwallstadt, Germany). The Ozon-Kit consists of a plastic tube of 150 mm length and a sterile filter at the side of which it is connected to the outlet of the Medozon<sup>IP</sup> generator. The tube of the Ozon-Kit was automatically flushed with approximately 10 ml of

the requested gas to remove air from the tube when the stopcock was opened to the “backflush” of the Medozon<sup>IP</sup> generator. The O<sub>3</sub>/O<sub>2</sub> gas mixture was applied in standardized volume of 80 ml/kg body weight containing 50 µg O<sub>3</sub> per milliliter gas mixture corresponding to a contingent of 2.5% O<sub>3</sub> and 97.5% medical O<sub>2</sub>. The stopcock connected to a Vasofix R Braunuele R (17G, Braun Melsungen AG, Melsungen, Germany) that was implanted into the right lower quadrant of the abdomen was used for IAPC during the insufflation process and allowed control of the actual IAP. Analogously, animals of the sham group were anesthetized and connected to the Medozon<sup>IP</sup> processor, but received no gas insufflation. All animals were observed until Day 90 post tumor cell transplantation. Their general health condition was monitored and the body weight, the size of the primary right ear tumor and the parotid lymph nodes were measured daily using the calliper Sylvac SA, Swiss system. To prevent animals from early death due to severe local infection at the tumor site or massive bleeding in the course of primary tumor development, ablation of the ear 1–2 cm proximal to the tumor was performed under anesthesia with 0.1 ml/kg body weight glycopyrrolate subcutaneously, followed by intramuscular injection with a combination of 5 mg/kg body weight xylazine and 70 mg/kg body weight ketamine. Signs of distress, pain or cachexy—defined as weight loss above 20%—were always criteria for euthanasia as recommended by the Canadian Council on Animal Care.<sup>27</sup> Furthermore, criteria for grading of adverse effects before and after O<sub>3</sub>/O<sub>2</sub> therapy were recorded according to the Cancer Therapy Evaluation Program<sup>28</sup> during the whole period of observation.

#### Immune suppression

The 6 rabbits of this report with complete remission of the VX2 tumor after insufflation of O<sub>3</sub>/O<sub>2</sub> gas mixture into the peritoneum (O<sub>3</sub>/O<sub>2</sub>-rem) were divided into 2 groups. Animals in one group were immune suppressed by a combination of dexamethasone (Dex; Dexamethasone, Jenapharm, Jena, Germany; 1.5 mg/kg body weight; subcutaneous) and cyclosporin A (CSA, Sandimmun<sup>®</sup>, Novartis Pharma, Nuremberg, Germany, 20 mg/kg body weight, subcutaneous) (O<sub>3</sub>/O<sub>2</sub>-rem + Dex/CSA,  $n = 3$ ), animals of the second group were sham-treated (O<sub>3</sub>/O<sub>2</sub>-rem + sham,  $n = 3$ ). A one-time dosage of dexamethasone was given on Day -2 subcutaneously together with the first CSA injection (Fig. 1b). Immune suppression was maintained by daily applications of 20 mg CSA/kg body weight given subcutaneously for 11 consecutive days. One additional rabbit that received the tumor suspension for the first time (control + sham) was used as control for the aggressiveness of the tumor suspension. Another rabbit that was immune suppressed by Dex/CSA (control + Dex/CSA) was used to monitor for possible effects of the immune suppressant agents on the take rate of the tumor. All rabbits used in this experimental part of the study received the same VX2 cell suspension derived from 1 donor rabbit. Two days after application of the first injection of the immune suppressant agents or sham treatment, all rabbits received an inoculation of the VX2 tumor cell suspension similar to the inoculation described earlier. To enhance the number of possible tumors, VX2 suspension was injected in both ears. The retake rate of the reimplanted tumor cell suspension was determined on Day 14 after inoculation.

#### Computed tomography

Rabbits were premedicated with 0.1 ml/kg body weight glycopyrrolate subcutaneously and anaesthetized with a combination of 5 mg/kg xylazine and 30 mg/kg ketamine by intramuscular injection. Computed tomography of the thorax was generated with the Siemens Somatom Plus 4 (Siemens, Erlangen, Germany).

#### Blood parameters

Arterial blood samples from the central auricular artery of the left tumor free ear were taken on Day 14 post inoculation (directly before the first gas insufflation/sham treatment) and on Day 19

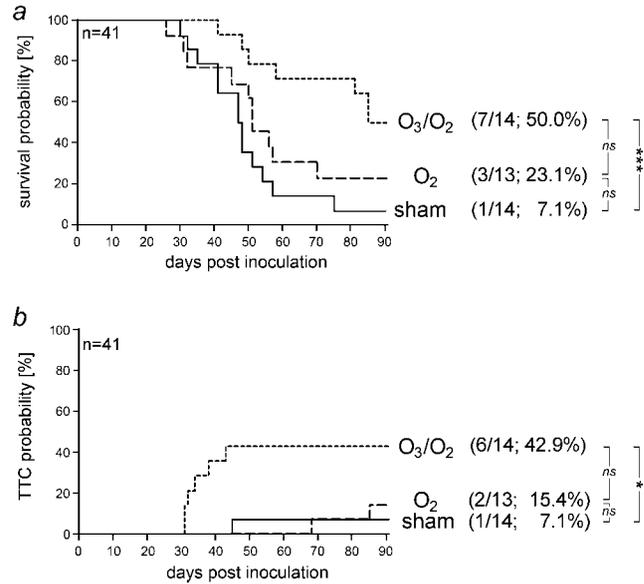
post inoculation (24 hr after the last gas insufflation/sham treatment). Additional blood samples were taken on Day 90 from the O<sub>3</sub>/O<sub>2</sub>-rem animals (Fig. 1a). For hematological investigations we used an autoanalyzer (Vet abc™ Animal Blood Counter, ABX Diagnostics, Goettingen, Germany), that has been carefully adjusted and validated for the analysis of rabbit blood. For differential blood counts the RBC count was determined together with the hematocrit (HCT) and hemoglobin (HGB). White blood cells were further differentiated into granulocytes, lymphocytes and monocytes. Data were expressed as mean values ± SEM. For clinical chemistry investigations creatinine, GOT, and GPT values were measured with the Reflovet® Plus system (Roche Diagnostics, Mannheim, Germany) to monitor for kidney and liver functions.

### Statistics

For comparison of survival rates of the 3 experimental groups the log rank test was performed considering  $p < 0.05$  as significant. The survival probability of rabbits calculated from the time of tumor cell inoculation until Day 90 was depicted according to the Kaplan–Meier method. The time to tumor clearance (TTC) probability was calculated from the time when the size of the solid auricular tumor dropped under 5% of the volume measured on Day 14 post tumor cell inoculation, a time point when a solid auricular tumor had developed and gas insufflation therapies or sham treatment started. Statistical differences in mean body weight between the experimental groups were evaluated at daily intervals with the unpaired Student's  $t$  test with 2 tail  $p$  values. To evaluate possible adverse effects due to the insufflation of O<sub>3</sub>/O<sub>2</sub>, pure O<sub>2</sub> or sham treatment, blood parameter were measured shortly before the first gas insufflation or sham treatment (Day 14) and 24 hr after the last gas insufflation or sham treatment (Day 19). Statistic differences within each experimental group were calculated by the paired Student's  $t$  test with 2 tailed  $p$ -values. The GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com) was used for all statistical calculations.

### Results

To test the hypothesis that intraperitoneal application of a medical O<sub>3</sub>/O<sub>2</sub> gas mixture is an effective antitumor approach, we subjected 42 NZW rabbits to unilateral inoculation with a VX2 tumor cell suspension into the subcutis of the right ear. All 42 animals developed a solid VX2 tumor at the site of inoculation. One animal died prior to the therapeutic session due to undefined reasons. On Day 14 after inoculation, when tumors had reached a mean size of  $6,082 \text{ mm}^3 \pm 515 \text{ mm}^3$ , rabbits were randomly divided into 3 groups of 14 or 13 animals each. The group, that received a daily intraperitoneal insufflation of the O<sub>3</sub>/O<sub>2</sub> gas mixture over 5 consecutive days, exhibited a survival rate of 50.0% (Fig. 2a). The probability of survival, calculated by the log rank test, demonstrated a significant increase ( $p = 0.0006$ ) in the O<sub>3</sub>/O<sub>2</sub>-treated group compared to the sham-treated group (7.1%) (Fig. 2a). Difference of the survival probability between the O<sub>3</sub>/O<sub>2</sub>-treated group and the O<sub>2</sub>-treated group were calculated as nonsignificant but the  $p$  value of 0.0559 suggest that the insufflation of the O<sub>3</sub>/O<sub>2</sub> gas mixture is more efficient than that of pure medical O<sub>2</sub> (Fig. 2a). The mean survival time for O<sub>3</sub>/O<sub>2</sub>-treated rabbits was 87.5 days, which is close to the end point of the total observation period (Day 90). In sharp contrast, the mean survival time for O<sub>2</sub>-treated rabbits was 51 days, which is comparable to that of the one survivor in the sham group (47.5 days) that exhibited a spontaneous remission of the tumor. Figure 2b depicts the TTC in the 3 experimental groups. The TTC in the O<sub>3</sub>/O<sub>2</sub>-treated group (42.9%) is significantly higher ( $p = 0.0243$ ) than that of the sham-treated group (7.1%). All 6 O<sub>3</sub>/O<sub>2</sub>-treated rabbits that had cleared the auricular tumor within 2–3 weeks after therapeutic intervention were completely free of cancer. The spontaneous remission of the tumor in one of the sham-treated rabbits on Day 45 and the tumor clearance observed in the O<sub>2</sub>-treated animals on Day 68 and 85



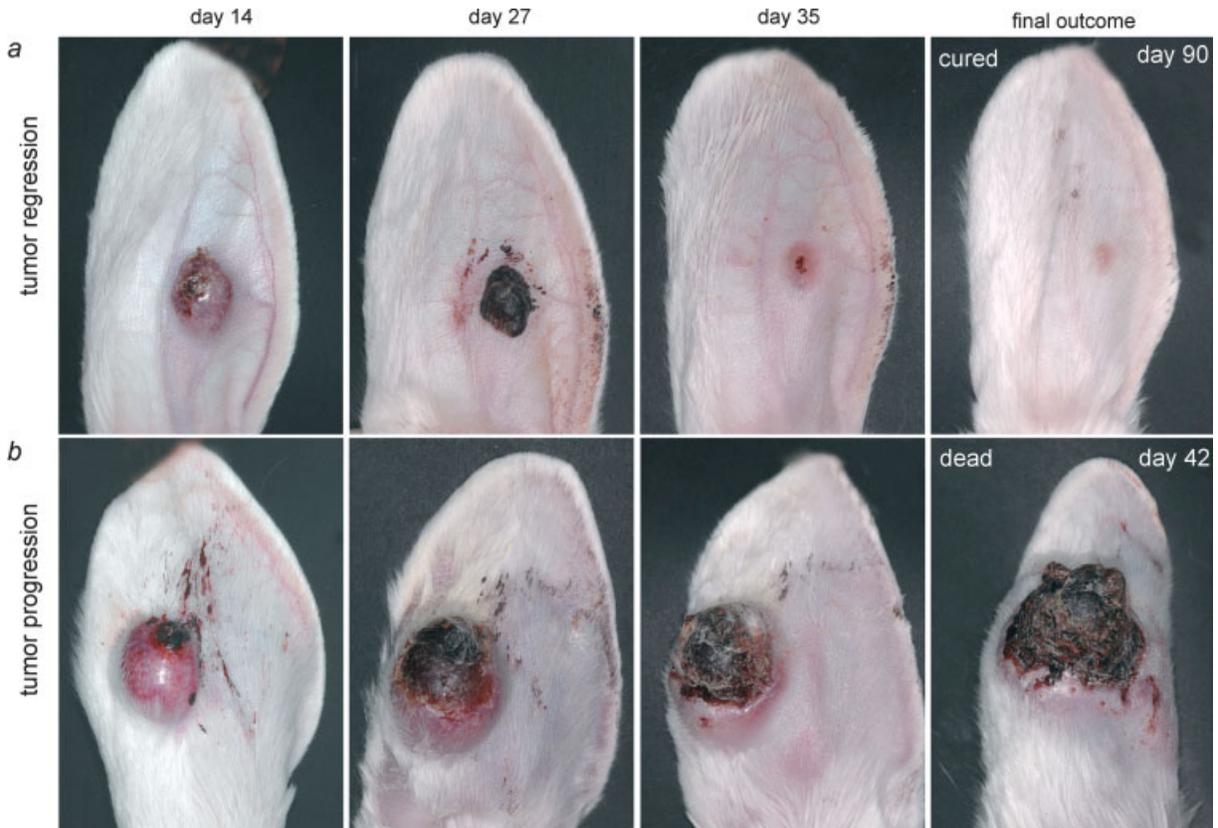
**FIGURE 2** – Kaplan–Meier plots showing the survival probability (a) and the time to tumor clearance probability [TTC, (b)] of rabbits ( $n = 41$ ) that have developed a solid auricular VX2 tumor on Day 14 after VX2 tumor inoculation. (a) The survival probability of rabbits receiving the O<sub>3</sub>/O<sub>2</sub> treatment was superior to sham-treated rabbits ( $p = 0.0006$ ) but was not significantly different from that of the O<sub>2</sub>-treated group ( $p = 0.0559$ ) as calculated by the log rank test. The survival probability of O<sub>2</sub>-treated rabbits did not differ significantly from that of sham-treated rabbits ( $p = 0.2448$ ). (b) The TTC probability significantly varied between O<sub>3</sub>/O<sub>2</sub>-treated and sham-treated rabbits ( $p = 0.0243$ ) but not between O<sub>3</sub>/O<sub>2</sub>- and O<sub>2</sub>-treated rabbits ( $p = 0.0665$ ). Also, there was no significant difference in the TTC probability between the O<sub>2</sub> and sham groups ( $p = 0.5781$ ). Statistically significant changes were marked with  $p < 0.05^*$ ;  $p < 0.001^{***}$ ; ns, not significant. Shown in brackets are the numbers and percentages of surviving animals on Day 90.

occurred at a much later time point than in the O<sub>3</sub>/O<sub>2</sub>-treated rabbits. This suggests a specific therapeutic effect of the O<sub>3</sub>/O<sub>2</sub> gas mixture on tumor clearance in this group, however, there was rather a trend than a significant difference between the TTCs of O<sub>3</sub>/O<sub>2</sub>-treated and O<sub>2</sub>-treated animals ( $p = 0.0665$ ).

Figure 3 shows a representative sequence of different tumor stages demonstrating primary tumor regression of the auricular tumor after O<sub>3</sub>/O<sub>2</sub> treatment until complete remission (Fig. 3a) or progression of the tumor to the final stage, which is characterized by strong ulceration and massive bleeding shortly before death (Fig. 3b).

To analyze, whether the treatment results in a regression and/or prevention of distant lung metastases in the thorax, which is typical for advanced VX2 tumor disease in rabbits,<sup>4,29</sup> we scanned the complete thorax of the animals with regression of the primary tumor for the presence of metastases using computed tomography (CT). In all 3 experimental groups lung metastases were absent in rabbits with remission of the primary auricular tumor (Fig. 4a). Contrary to the rabbits with tumor remission, all animals with persisting auricular tumors at Day 90 regularly exhibited numerous lung metastases (Fig. 4b).

The observation that reimplanted VX2 tumor cells were rejected in rabbits with tumor remission after perilesional treatment with IL-2<sup>30</sup> prompted us to test whether reimplantation of VX2 carcinoma cells will also fail in rabbits with tumor remission after O<sub>3</sub>/O<sub>2</sub> treatment. To test this, we divided the 6 O<sub>3</sub>/O<sub>2</sub>-rem animals from the present experiment into 2 groups. Each animal received a bi-auricular injection of the VX2 tumor cell suspension to raise the number of possible tumors. One group was immune



**FIGURE 3** – Growth and development of the VX2 tumor cells in the right ear of NZW rabbits after inoculation. Panel (a) shows representative macroscopic views of a solid auricular VX2 tumor in the right ear of a rabbit on Day 14 after tumor cell inoculation and different stages of remission after O<sub>3</sub>/O<sub>2</sub> therapy (O<sub>3</sub>/O<sub>2</sub>-pneumoperitoneum). The spontaneous tumor remission observed in one sham-treated rabbit and the 2 remissions after O<sub>2</sub> gas insufflation were similar (macroscopic views are not shown). Note, that only a small scar of the remitted auricular tumor remained on Day 90, the end point of our observation period. In sharp contrast, in rabbits that succumbed to tumor progression the auricular tumor continued growing resulting in severe ulcerations associated with massive bleeding and onset of local infections introducing the final stage of this tumor disease (b, representative stages of the same rabbit are shown). Depicted are tumor stages on Days 14, 27, 35, and at the end of the observation period (90 days or 42 days in case of death).

suppressed by Dex and CSA ( $n = 3$ ), the other group was sham-treated as described earlier ( $n = 3$ ). As expected, all sham-treated animals were protected for retake of the VX2 tumors, since no auricular tumors developed within the observation period of 90 days (Table I). In sharp contrast, immune suppressed animals developed tumors in 4 out of 6 tumor cell reinoculations (Table I). Tumor growth and size in these animals did not show any difference to the auricular tumors previously measured in immune competent rabbits of the sham group.

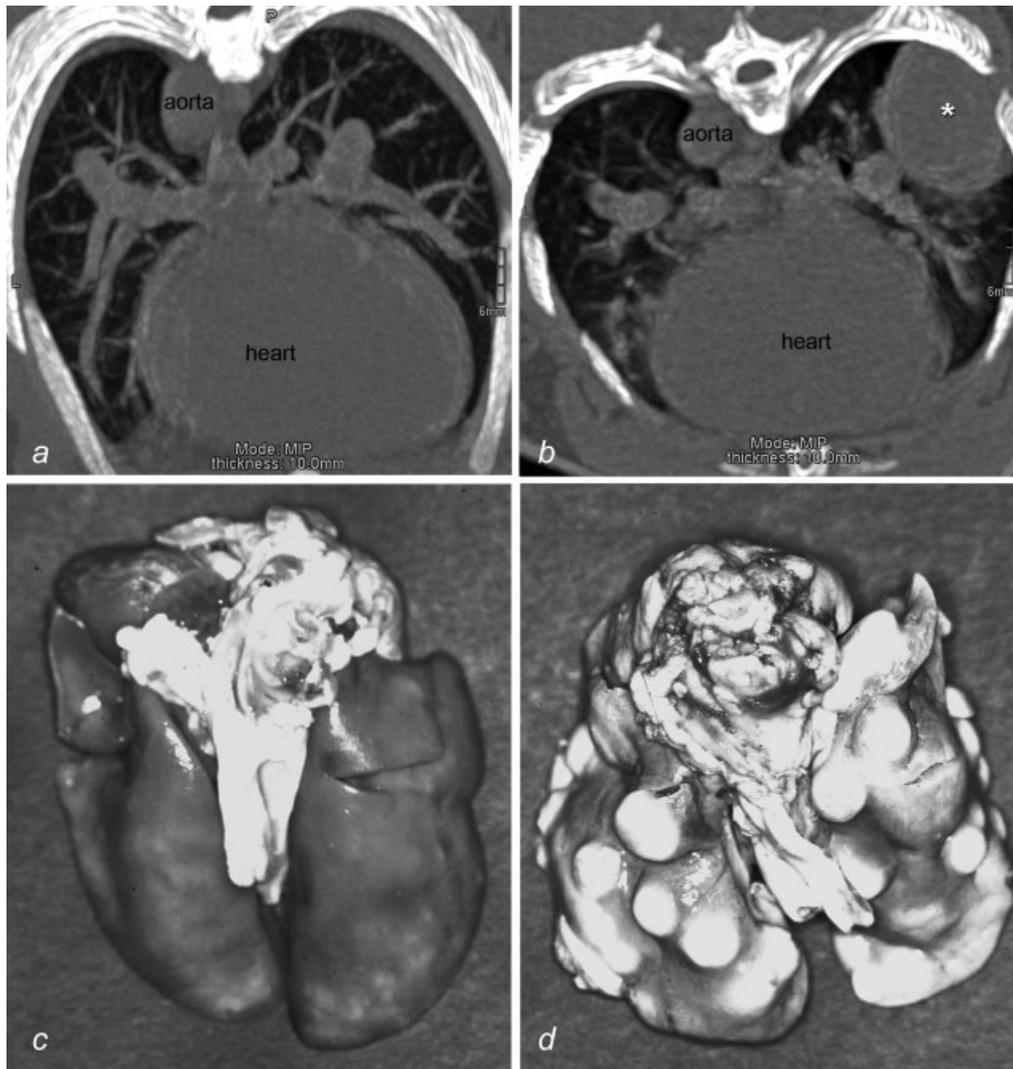
To test whether O<sub>3</sub>/O<sub>2</sub> therapy has any adverse effects, we measured the intraabdominal pressure (IAP) during the insufflation process and analyzed weight development and some of the major blood parameters that frequently serve as indicators for adverse effects by the common toxicity criteria.<sup>28</sup> Measurement of the IAP in our rabbits revealed no pressure above 5 mbar at any time. Body weight analysis revealed a maximal drop of about 8.1% ( $p < 0.001$ ) in the O<sub>3</sub>/O<sub>2</sub>-treated group compared to the mean body weight on Day 14 directly before starting the first therapeutic session (Fig. 5a). In comparison, the mean body weight dropped maximally 5.0% ( $p < 0.072$ ) in the O<sub>2</sub>-treated group and 1.5% ( $p < 0.299$ ) in the sham-treated group (Fig. 5a). After the end of the daily therapeutic gas insufflations or sham interventions, the body weight increased in all groups and reached comparable values on Day 42.

Measurement of blood parameters on Day 19 (24 hr after the last O<sub>3</sub>/O<sub>2</sub> gas insufflation or sham treatment) exhibited a slight increase in WBC count (Fig. 5b). Other values such as RBC count,

HGB, HCT, GOT and GPT remained within the physiological range (Fig. 5b).

## Discussion

We have shown for the first time that intraperitoneal application of a medical O<sub>3</sub>/O<sub>2</sub> gas mixture eradicates a highly metastasizing cancer at a high rate without exhibiting major adverse effects. The effectiveness of this new method is supported by the complete remission of the primary tumor along with prevention and/or remission of local and distant metastases, that is significantly higher than the observed spontaneous remission rate in sham-treated animals and in line with published observations in the VX2 tumor cell model of the NZW rabbit.<sup>4</sup> Previously we reported, that local metastases of the parotid lymph node were present with a probability of 62.5% on Day 14 and 100% on Day 32 after inoculation of the VX2 tumor cell suspension.<sup>1</sup> Thus, the presence of metastases during the therapeutical O<sub>3</sub>/O<sub>2</sub> session is highly likely. Interestingly, Hough *et al.* found VX2 tumor associated granulocytosis to be an indicator of metastatic spread to the lungs.<sup>32</sup> Similarly, on Day 19 after tumor cell inoculation we observed a mild granulocytosis in all animals possibly pointing to the presence of lung metastases. Our observation that all rabbits with remission of the primary tumor were macroscopically free of metastases, exhibiting no cicatrices in the lung indicates that O<sub>3</sub>/O<sub>2</sub> therapy is not only able to prevent but also to clear distant metastases. Therefore, we conclude that O<sub>3</sub>/O<sub>2</sub> therapy is effective in



**FIGURE 4** – VX2 tumor derived distant lung metastases. CT scans of the thorax showing no detectable lung metastases in a rabbit of the O<sub>3</sub>/O<sub>2</sub>-rem group (a) at the end of the observation period, but reveals a huge metastasis in the lung of a rabbit on Day 32 (b, asterisk) that succumbed to the VX2 tumor later on. (depicted are representative CT scans of the lungs amounting to the vena pulmonalis) The lower images show macroscopic views of the complete lungs of a healthy rabbit (c) and of an animal with multiple VX2 carcinoma derived lung metastases at the pleura visceralis (d).

**TABLE I** – RETAKE RATE OF AURICULAR VX2 TUMORS

Experimental group	Animals (n)	Tumors <sup>1</sup> (n)	Mean tumor volume (mm <sup>3</sup> )
O <sub>3</sub> /O <sub>2</sub> -rem + Dex/CSA	3	4/6	3,089
O <sub>3</sub> /O <sub>2</sub> -rem + sham	3	0/6	<200 <sup>2</sup>
Control + Dex/CSA	1	1/2	1,466
Control + sham	1	2/2	5,657

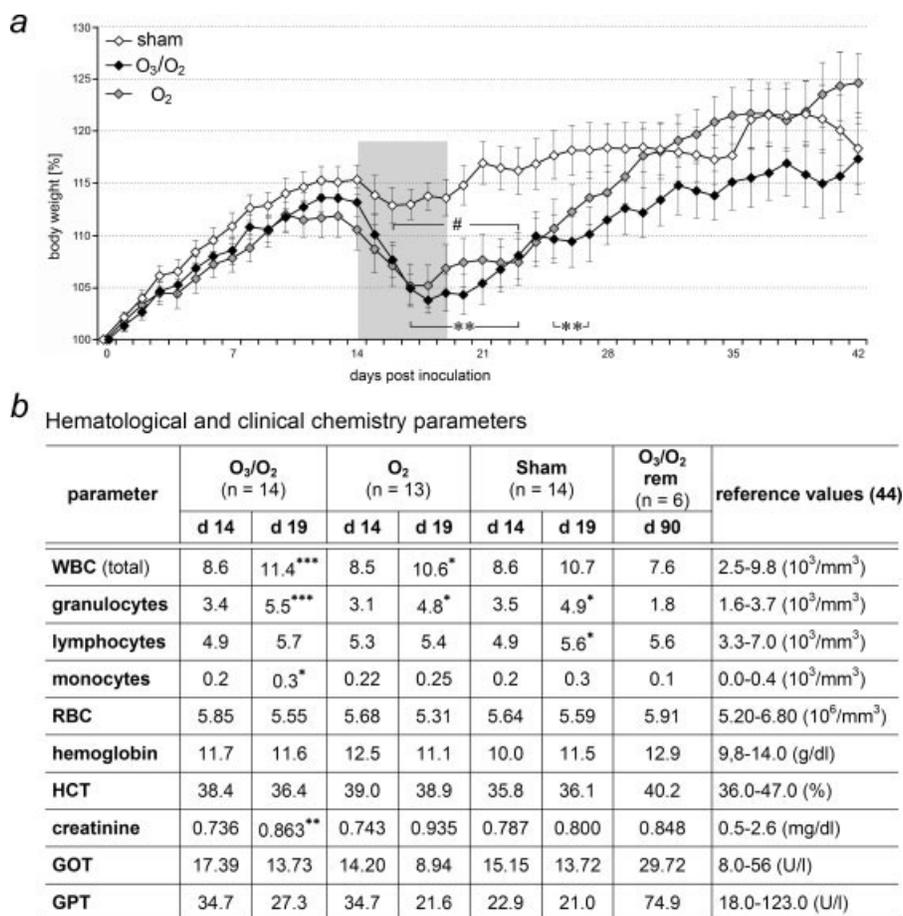
Bi-auricular reimplantation of VX2 tumor cells in rabbits with complete tumor regression (O<sub>3</sub>/O<sub>2</sub>-rem) and subsequent treatment with (O<sub>3</sub>/O<sub>2</sub>-rem + Dex/CSA) or without (O<sub>3</sub>/O<sub>2</sub>-rem + sham) dexamethasone and cyclosporin A. The take rate of tumors measured in 2 rabbits served as a baseline control: 1 rabbit (control + Dex/CSA) received the tumor suspension for the first time and was additionally immune suppressed, whereas the second rabbit (control + sham) was not immune suppressed.

<sup>1</sup>Because of the bi-auricular transplantation of VX2 cell suspension, 2 tumors per animal are possible. <sup>2</sup>The mean value of less than 200 mm<sup>3</sup> represents the basal swelling that occurs after implantation of the VX2 tumor cell suspension. Therefore volumes measuring less than 200 mm<sup>3</sup> are considered VX2 tumor negative.

eradicating the primary as well as to protect against possible regional and distant metastases.

Development of tolerance against reimplantation of VX2 tumors points to an involvement of the immune system in tumor clearance, particularly the adaptive immune system. This assumption is also supported by the observation that perilesional injection of IL-2 resulted in regression of auricular VX2 tumors in rabbits, although at a lower efficiency (25.0%)<sup>30</sup> than found in the O<sub>3</sub>/O<sub>2</sub>-pneumoperitoneum group (42.9%). The loss of tolerance against reimplantation of VX2 tumors after immune suppression further indicates that immunosurveillance is a key player in the eradication of VX2 tumor cells.

Numerous effector cells from the adaptive and from the innate immune system might be responsible for tumor regression. In an animal model with a mice strain that exhibits spontaneous regression/complete resistance (SR/CR) to multiple transplantable cancer cell lines,<sup>33</sup> leukocytes infiltrating the tumor site consisted of a mixture of multiple subsets of effector cells composed primarily of macrophages, polymorphonuclear cells (PMN), NK cells, and cytotoxic T lymphocytes.<sup>34</sup> Depletion or transfer of specific leuko-



**FIGURE 5** – (a) Course of mean body weight from Day 0 (at which VX2 cell suspension was inoculated) until Day 42. On Day 0 the initial body weight of each rabbit was set to 100%; changes were expressed in percentage of initial body weight. The period of the therapeutic O<sub>3</sub>/O<sub>2</sub> gas mixture insufflation is marked by a gray box. Changes in the body weight within an experimental group were calculated for each day by the Student's *t* test. Significant loss of body weight was found in the O<sub>3</sub>/O<sub>2</sub>-treated group (\**p* < 0.05; \*\**p* < 0.01) and in the O<sub>2</sub>-treated group (<sup>#</sup>*p* < 0.05) compared to the mean body weight of each group on Day 14 before initiation of treatment. The body weight change in the sham group was not significant. (b) Effect of repetitive O<sub>3</sub>/O<sub>2</sub>-pneumoperitoneum on standard laboratory blood parameters.<sup>31</sup> Arterial blood samples were taken on Day 14 postinoculation (directly before the first O<sub>3</sub>/O<sub>2</sub> insufflation, O<sub>2</sub> insufflation or sham treatment) and on Day 19 post inoculation (24 hr after the last gas insufflation or sham treatment). To consider possible long-term effects of O<sub>3</sub>/O<sub>2</sub>-pneumoperitoneum, blood parameters of all O<sub>3</sub>/O<sub>2</sub>-rem animals were also measured on Day 90 representing the end of the observation period. Statistic differences between Day 14 and Day 19 in each experimental group were calculated with the paired Student's *t* test and statistically significant changes were marked with *p* < 0.05\*; *p* < 0.01\*\*; *p* < 0.001\*\*\*. Abbreviations: WBC, white blood cells; RBC, red blood cells, HCT, hematocrit; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase.

cyte populations of the adaptive or the innate immune system showed, that primarily the innate immune system is involved in successful tumor regression and complete resistance against reimplantation of the tumors in SR/CR mice.<sup>34</sup> Each leukocyte subpopulation exhibits individual tumor cell killing mechanisms by the secretion of different effector molecules such as perforins, granzymes or reactive oxygen species (ROS). In the SR/CR mouse model the release of ROS by macrophages was identified as one major effector mechanism of the anticancer immune response.<sup>35</sup> Therefore, production of ROS by activated macrophages and/or granulocytes could be a possible mechanism of anticancer effects induced by the insufflation of O<sub>3</sub>/O<sub>2</sub> gas mixture in our study. In this context, the observed mild leukocytosis including granulocytosis after the therapeutical sessions with O<sub>3</sub>/O<sub>2</sub> might represent activated leukocytes as potent anticancer effector cells also in the VX2 model.

A local effect of insufflated free ozone in the peritoneal cavity on auricular VX2 tumor cells seems unlikely because of its high and quick reactivity with membranes and numerous cellular biomolecules such as nucleic acids, proteins and unsaturated fatty

acids.<sup>36,37</sup> Regarding the reactivity of ozone with unsaturated fatty acids that are present in cellular membranes and the chemical half life of ozone, it is of interest that free ozone in the lung is unable to penetrate a biological fluid layer thicker than 0.1 μm.<sup>38</sup> Additionally, transport of free ozone via the blood route and thus the generation of ROS by free ozone at the tumor site appears not likely, because any remaining free ozone, which did not interact with unsaturated fatty acids or proteins in the serosa and penetrates the peritoneum, will at last completely react with proteins and membranes of local cells of the adventitia attached to the peritoneum. Therefore, lipid ozonation products originated from the reaction of ozone with several biomolecules<sup>39</sup> at the serosa are most likely the mediators responsible for triggering the observed antitumorigenic effects in the VX2 model. In this context, intraperitoneal insufflated O<sub>3</sub>/O<sub>2</sub> gas mixture might react on the arachidonic acid metabolism in the mesothelium consisting of different mesothelial cell types, which is reminiscent to the situation in the eicosanoid metabolism of human airway epithelial cells after exposure to ozone.<sup>40</sup> Several components of the prostanoid biosynthesis pathway were found to exhibit oncolytic and antimetastatic

effects on tumor cells and tumor progression.<sup>41,42</sup> Encouraging studies are currently under way to analyze changes in the plasma levels of different arachidonic acid metabolites during the course of O<sub>3</sub>/O<sub>2</sub> insufflation in rabbits (unpublished data).

Since O<sub>3</sub>/O<sub>2</sub>-pneumoperitoneum is a new therapeutic approach it is of high importance to analyze possible adverse side effects. Acute side effects due to high IAP that might occur during the period of insufflation are unlikely, since the measured peak pressure was below 5 mbar, classified as low IAP in laparoscopic surgery.<sup>43</sup> The slight and transient reduction in body weight during and shortly after the therapeutic treatment might represent a mild adverse affect (weight loss 5–10%, Grade I) comparable to that observed in mice (weight loss up to 14%, Grade II) that had inhaled ozone.<sup>44</sup> Since no severe side effects such as sustained reduction in body weight and gas embolism, fever, diarrhea or peritonitis were observed, it appears that the O<sub>3</sub>/O<sub>2</sub> therapy is relatively safe. Late adverse effects of O<sub>3</sub>/O<sub>2</sub>-pneumoperitoneum treatment are rather unlikely, since 2 rabbits from a previous pilot study developed no clinical signs after more than 5 years of tumor remission (not shown).

Taken together, insufflation of medical O<sub>3</sub>/O<sub>2</sub> gas mixture into the peritoneal cavity appears to be a highly promising new tool in the treatment of cancer. The exact mechanisms underlying the proposed enhancement of the O<sub>3</sub>/O<sub>2</sub>-pneumoperitoneum in immunosurveillance are still unknown. Clearly, more work is required to clarify the basal biochemical, physiological and immunological mechanisms underlying the antitumorigenic and antimetastatic effect of the O<sub>3</sub>/O<sub>2</sub>-pneumoperitoneum on VX2 tumors. Certainly, the effectiveness of this novel therapeutic approach—including possible yet unknown side effects—has to be proven on other tumor animal models of different tumor types before eventually entering clinical trials in humans.

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