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Effect of Ozone/Oxygen-Pneumoperitoneum on Tumour Growth and Metastatic Spread of the Rabbit VX2 Head and Neck Cancer Model

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1 Introduction

1.1 Head and neck cancer (HNC)

HNC refers to a diverse group of neoplasms, including cancers of the oral cavity, the pharynx and the larynx. More than 90% of these cancers are squamous cell carcinomas (95). Other histologic types are relatively rare and include adenocarcinomas, adenoid cystic carcinomas, mucoepidermoid carcinomas, lymphomas and sarcomas.

1.1.1 Epidemiology and economics of HNC

Cancer is a major cause of morbidity and death in many countries of the world. The global cancer statistics reported approximately 10.9 million new cancer cases, 6.7 million cancer-related deaths and 24.6 million persons alive with cancer (within three years of diagnosis) worldwide in the year 2002 (64). About 6% of all new cases in 2002 were cancers of the oral cavity, the pharynx and the larynx, accounting for about 5% of the cancer-related deaths (64).

Age-standardized incidence rates (ASIR) for cancers are used to compare incidences of different regions or countries. The ASIR for cancers of the head and neck have shown great variance in 2002 between different world areas (64). The highest ASIR for cancers of the oral cavity and oropharynx were found in western Europe, southern Europe, south Asia, southern Africa and Australia/New Zealand. The highest ASIR for cancers of the larynx were found in southern Europe, eastern Europe, south America and western Asia. Nasopharyngeal cancers are endemic in some areas of China, southeast Asia, northwest India and northern Africa.

In the United States of America, a total of 559,312 cancer-related deaths were recorded in the year 2005 (23% of all deaths) (48). Only heart diseases caused more deaths in the same year (652,091 deaths). In men aged 60 to 79 years and in women aged 40 to 79 years cancer is the leading cause of death. In the time period from

1990 to 2004 the age-adjusted cancer death rates have decreased for cancers of the oral cavity, pharynx and larynx in both genders. Nevertheless, the 5-year relative survival rate for cancers of the larynx has not improved over the past 25 years in the United States (48). The American Cancer Society predicts 1.4 million new cancer cases in the United States of America for the year 2008 (48). Of these 1.4 million new cancer cases, 35,310 cases (2.5%) are expected to be cancers of the oral cavity and pharynx and 12,250 cases (0.9%) cancers of the larynx. The incidence in men is expected to be significantly higher than the incidence in women.

Since HNC is a common disease, the economic impact related to this disease is of great importance. Lee et al. performed a systematic review of the literature between 1990 and 2002 addressing the economics of HNC in developed countries of North America, Europe, Australia and Japan (57). The estimates for the nation's annual costs ranged from 1.2 billion Euros in Germany to 1.9 billion Euros in the United States. Included in these costs were direct as well as indirect costs. The estimated per-patient lifetime costs associated with the treatment of HNC ranged from 8,450 Euros in Greek oral cancer patients over 36,871 Euros in Dutch patients to 231,308 Euros in American lip, oral cavity and pharynx cancer patients. Although these data have to be interpreted carefully, it is clear that cancers of the head and neck cause enormous costs to the society.

Therefore, an effective and cheap anticancer therapy would not only be of high interest to the patient, but also for the global medical care as well as for the financial situation of state health insurance.

1.1.2 Treatment of HNC

Standard treatment

The standard modalities for the treatment of head and neck squamous cell carcinomas (HNSCC) are surgery, radiotherapy and chemotherapy, or combinations of these. The decision which treatment modality or which combination therapy is used depends on several factors, including tumour site, tumour stage, comorbidity, decision of the patient and aimed functional outcome.

Conventionally, surgery or radiotherapy is the treatment of choice for early stage disease (stages I and II) (95). Both are applied with curative intent. Depending on the stage and the site of the disease, prophylactic neck treatment or prophylactic neck dissection can be recommended. Two strategies of altered fractionation radio-

therapy are applied: hyperfractionation and accelerated fractionation (35). With both approaches, better local control rates could be achieved compared with standard fractionation radiotherapy. It is unclear if there is a survival benefit.

In general, the standard treatment of locoregionally advanced disease (stages III, IVa, IVb) is more complex. The traditional treatment approach for potentially resectable tumours is surgery with postoperative radiotherapy (95). Newer treatment strategies in the case of potentially resectable tumours add chemotherapy to surgery and/or radiotherapy (35). Induction chemotherapy with cisplatin and fluorouracil followed by definite radiotherapy was a standard treatment option for local and regionally advanced cancers of the larynx, with the often achievable goal of larynx preservation (35). The fact, that higher rates of larynx preservation could be achieved with concurrent chemoradiotherapy than with induction chemotherapy and subsequent radiotherapy, led to the assumption, that concomitant chemoradiotherapy is probably the best treatment option for locoregionally advanced laryngeal cancers (35). Concurrent chemoradiotherapy is the treatment of choice for local and regionally advanced cancers of the oropharynx and for locally advanced and unresectable tumours (35).

For patients with distant metastatic or recurrent disease, systemic chemotherapy is the standard treatment modality, applied with palliative intent. It remains unclear whether chemotherapy prolongs survival. Traditionally, single-agent chemotherapy was performed, with methotrexate or cisplatin as the most commonly used single agents (95). Other active agents are carboplatin, 5-fluorouracil, paclitaxel, docetaxel, doxorubicin and bleomycin. Treatment with combination chemotherapy resulted in better response rates than single-agent chemotherapy. The combination of cisplatin and fluorouracil is nowadays regarded as the standard chemotherapy regimen for metastatic or recurrent disease (35). However, in comparison with single-agent chemotherapy no improved survival could be shown in multiple studies. Newer regimens, for example the combination of cisplatin with a taxane (paclitaxel or docetaxel), are under investigation.

New treatment strategies

A novel approach in therapy of HNSCC is the treatment with epidermal growth factor receptor (EGFR) antagonists (35). The EGFR is a receptor tyrosine kinase which was found to be overexpressed in many head and neck cancers. Cetuximab

is a monoclonal antibody directed against the ligand binding site of the EGFR. Cetuximab was tested as a single-agent, in combination with radiotherapy and in combination with cytotoxic chemotherapy. An uncontrolled, multicenter phase II study enrolling 103 patients evaluated monotherapy with cetuximab in metastatic platinum-resistant disease (94). Partial response was seen in 13% of patients and stable disease was found in 33% of patients. A phase III randomized trial compared cisplatin alone with cisplatin and cetuximab in metastatic/recurrent head and neck cancer (22). No significant difference in overall survival and progression-free survival was found between these groups, but the cetuximab and cisplatin combination group showed significantly improved response rates. Another multicenter European study showed a significantly increased overall survival for the combination of cetuximab, cisplatin and fluorouracil compared with cisplatin and fluorouracil alone in patients with metastatic/recurrent disease (93). Cetuximab was also tested for locoregionally advanced disease in combination with radiotherapy (16). Compared with radiotherapy alone, progression-free survival and overall survival were significantly prolonged. Other anti-EGFR strategies are also under investigation, including the small molecule tyrosin kinase inhibitors erlotinib and gefitinib.

1.2 The VX2 carcinoma animal model

The VX2 auricle carcinoma is a highly suitable animal model for human HNSCC, since both are similar in growth leading to early regional lymph node metastases and subsequent distant metastatic spread.

1.2.1 History

In a first report in 1932, Shope discussed a tumour-like condition that was found in a wild cottontail rabbit shot in 1931 (82). These subcutaneous tumours were transmissible to both wild and domestic rabbits. A virus as the papilloma-producing agent was detected by Shope and Hurst in 1933 (83). A multistep transformation from benign papilloma to malignant carcinoma was observed in domestic rabbits bearing the papillomas for more than 4 months (70). The first successful transplantation of such a carcinoma in Dutch belted rabbits was reported in 1936, but a second transfer failed (51). Successful transplantation of the squamous cell carcinomas in Dutch belted rabbits with increasing take-rates, increasing anaplasia and frequent

occurrence of metastases until the 14th generation was reported in 1940 (52). This transplantable squamous cell carcinoma was called Carcinoma V2, while the name Carcinoma V1 was given to the previous cancer that was lost after the first successful transplantation. After World War II, the carcinoma was renamed Carcinoma VX2, due to the other meaning that V2 achieved during the war (71). During propagation to the 47th generation, the tumour had lost the power to immunize the host against the Shope papilloma virus, thus the Shope papilloma virus probably got lost during propagation (71). Heterologous transplantation to the brains of guinea pigs, hamsters, rats and mice and to the subcutaneous space of hamsters and mice succeeded (37).

Nowadays, the VX2 carcinoma is a well established and often used *in vivo* tumour model, which can be transplanted to a variety of anatomical sites in the rabbit.

1.2.2 The VX2 auricle carcinoma

The VX2 auricle carcinoma of the New Zealand White (NZW) rabbit is applied as an animal model for HNC in humans and was originally designed by van Es et al. (90). The tumour take-rate (with freshly prepared tumour cell suspensions) varies from 78% (89) to 99% - 100% (75; 32). One characteristic feature of the VX2 auricle carcinoma is that metastatic spread primarily occurs to the first draining lymph node and remains limited to the first draining lymph node for some time (sentinel lymph node) (32). Later, after breakdown of the physiologic integrity of the sentinel lymph node, further lymphogenic metastatic spread to a secondary lymph node station and finally haematogeneous metastatic spread to the lungs occurs. Dünne et al. investigated the lymphogenic metastatic spread (31; 32). About two weeks after the tumour induction, a high percentage of animals has lymph node metastases in the first draining lymph node (62.5% on day 14 (31) and 100% on day 18 (32)), but metastases to the second draining lymph node station do not occur until day 28 after tumour induction. In the course of the disease, lung metastases frequently occur. Van Es et al. found lung metastases in 47% of animals that were sacrificed at different time points more than 28 days after tumour induction (89).

Since 1999, the VX2 auricle cancer model was used in some experimental studies:

- intraarterial tumour embolisation studies, with assessment of the use of Dextran hydrogel microspheres for tumour chemo-embolisation and the use of Holmium-166 poly(L-lactic acid) microspheres for radio-embolisation (90; 92;

91)

- an immunotherapy study with local (peri-tumoural) interleukin-2 (IL-2) application (88)
- a systemic chemotherapy study with special regard to the metastatic lymph nodes, using intravenous applied cisplatin as the chemotherapeutic agent (30)
- a study investigating the effect of bipolar radiofrequency thermotherapy on lymph node metastases (33)
- a surgical intervention study comparing piecemeal laser resection with en bloc cold steel resection (75)
- a study evaluating the effect of translymphatic chemotherapy with cisplatin on lymph node metastases (29).

1.3 Ozone

1.3.1 Overview

Ozone is a triatomic molecule, composed of three oxygen atoms forming a cyclic structure. Martinus van Marum, a Dutch chemist, in 1785 first noticed the development of a distinct odour when using his electricity machine, named the “odour of electricity” (72). Christian Friedrich Schönbein, a German Professor of Chemistry at the University of Basel (Switzerland), in 1840 made the suggestion that this odour is caused by a distinct chemical substance and he proposed the name ozone for this substance, derived from the Greek word “ozein” (smell). The correct molecular formula of ozone, O_3 , was discovered by Jacques-Louis Soret in 1865 (72). In the atmosphere, ozone reaches the highest concentration in the stratosphere (ozone layer), where it is produced continually when ultraviolet radiation reacts with oxygen, a process known as the ozone-oxygen cycle. Thus, B and C ultraviolet radiation, known to be dangerous for humans when entering the troposphere in high concentrations, is absorbed in the stratosphere. On the other hand, tropospheric ozone, as it occurs in photochemical smog, is mainly regarded as a pollutant having harmful effects on human health.

Ozone is a highly reactive oxidant gas, that reacts immediately when it comes in contact with biomolecules. The primary target molecules for ozone in biological systems are unsaturated fatty acids (UFA), antioxidants like ascorbic and uric acid, thiol compounds like the amino acid cysteine, reduced glutathion (GSH), albumin, carbohydrates, enzymes, DNA and RNA (15). In the presence of water, the reaction of ozone with polyunsaturated fatty acids (PUFA) leads to the formation of hydrogen peroxide (H_2O_2) and lipid ozonation products (LOPs), which are regarded as important messengers mediating toxic as well as therapeutic effects of ozone in the human body (68; 15). Interestingly, ozone was recently found to be produced antibody-catalyzed in human neutrophils and therefore itself is considered a biomolecule (5).

1.3.2 Ozone and cancer

Toxicity and possible carcinogenicity of ozone

Research on the biological effects of ozone inhalation has predominantly been focused on pulmonary toxicity. In animal experiments, inhalation of ozone caused pulmonary inflammation, pulmonary oedema and epithelial cell damage in a concentration-dependent manner (55). Chronic exposure to elevated ambient ozone concentrations caused sustained bronchiolitis and the development of lung fibrosis in animal studies (55).

Much interest has been directed towards a possible carcinogenic effect of chronic exposure to elevated ambient ozone concentrations. The potential carcinogenicity of ozone has been studied in experimental animals. Some experiments using strain A/J mice provided evidence that chronic ozone exposure increases lung tumour development in mice (42; 56). In contrast, these results were not reproducible in a later experiment (98). Furthermore, chronic ozone exposure did not cause an increase of lung tumour development in Swiss Webster mice (56). The largest study on this topic was conducted by the United States National Toxicology Program (1). No evidence of a carcinogenic activity of chronic ozone exposure was found in male and female F344/N rats. On the other hand, the incidence of alveolar/bronchiolar adenoma was significantly increased in female B6C3F1 mice and was slightly increased in male B6C3F1 mice. Fewer studies exist which explore a possible carcinogenic effect of ozone in humans. The Adventist health study on Smog among Californian adults showed an increase in the incidence of lung cancers with elevated long-term

ambient concentrations of O₃ in men, but not in women (7).

Possible antitumoural effects of ozone

Interestingly, other reports suggest a possible antitumoural effect of ozone. Early research papers described ozone as a possible radiomimetic gas, leading to the development of distant signs (for example a sphering tendency of circulating erythrocytes) after inhalation in animals and men (19; 20). These distant signs were comparable to those seen after exposure to ionizing radiation. These observations inspired researchers to test, if ozone effects on cancer cells are comparable to those of ionizing radiation and if ionizing radiation and ozone can have an additive effect on cancer cells.

Comparing the effect on mouse ascites tumour cells in aqueous suspension, Sachsenmaier et al. only found small similarity between ozone and X-rays (73). There is some evidence from further *in vitro* studies, that exposure to ozonated air can selectively inhibit the proliferation of human cancer cells (lung adenocarcinoma, breast adenocarcinoma, uterine carcinosarcoma and endometrial carcinoma) at ozone concentrations that do not cause growth inhibition of human fibroblasts (87). The observation that growth inhibition of human cancer cells *in vitro* (endometrial cancer) after irradiation with a gamma emitter could be increased when the cancer cells were preincubated with an O₃/O₂ gas mixture, led to the proposal that ozone may have radiosensitizing properties (50).

Controlled randomized clinical trials that demonstrate a clear antitumoural effect of ozone therapy in human cancer patients do not exist. Two pilot studies by Clavo et al. which evaluated systemic ozone therapy for human cancer had encouraging results (26; 25). The method employed in these studies was the major autohaemotherapy, which will be described later. In the first study, they found that ozone therapy increased oxygenation in the most hypoxic metastatic lymph nodes in patients with advanced head and neck cancers (25). Regarding the fact that tumour hypoxia is a major cause of radioresistance, increased tumour oxygenation could be helpful to overcome radioresistance. In the second study, they compared the effect of ozone therapy and concurrent radiotherapy with chemoradiotherapy in patients with unresectable, locoregionally advanced and in some patients distant metastatic head and neck cancers (26). Despite the fact that the patients in the ozone therapy group were significantly older and had more advanced disease stages, the median

overall survival in both groups was not significantly different. There was even a tendency towards a longer median overall survival in the group which received the ozone therapy concurrently to the radiotherapy (8 months versus 6 months). These results implicate a possible additive effect of the ozone therapy in human patients with advanced cancer.

1.3.3 Ozone therapy today

Routes of ozone application and indications for ozone therapy

Several routes of ozone application have been described so far (15; 74). Ozone can be applied topically to external body surfaces (for example poorly healing wounds). For topical application ozonated oils are available too (78). Another principle of ozone application is insufflation via naturally existing body orifices, for example rectal, urethral, vaginal, auricular and nasal insufflation. Subcutaneous, intramuscular, intralesional and periarticular injection of ozone is possible as well. A further principle of ozone application is injection into preformed body cavities, for example intrapleural, intraarticular and intraperitoneal application, which was used in this study (59). The most commonly used method is the so called major autohaemotherapy (major AHT). A certain volume of fresh drawn blood is exposed to an equal volume of an O_3/O_2 gas mixture with a definite ozone concentration *ex vivo* and is afterwards reinfused intravenously (15). In minor autohaemotherapy (minor AHT) a much smaller blood volume is exposed *ex vivo* to an equal volume of an O_3/O_2 gas mixture with a higher ozone concentration than used in the major AHT, and the mixture is then injected intramuscularly into the gluteus muscle (15). Only of historical interest is the direct intravenous or intraarterial injection of an O_3/O_2 gas mixture. Due to the risk of pulmonary embolia, this technique was prohibited in Germany in 1984.

Indications for ozone therapy according to the German Medical Society for the Use of Ozone in Prevention and Therapy are summarized in Table 1.1 (6).

Ozone therapy and orthodox medicine

The therapeutical application of ozone is still a subject of controversy. In Germany, the G-BA (“Gemeinsamer Bundesausschuss der Ärzte und Krankenkassen”), which is formed by the national associations of doctors and dentists, the German Hospital

Table 1.1 – Indications for ozone therapy according to the German Medical Society for the Use of Ozone in Prevention and Therapy

Major AHT

- peripheral arterial circulatory disturbance
- cerebral circulatory disturbances (stroke, TIA)
- ocular circulatory disturbances (retinopathies)
- acute hearing loss, tinnitus
- virus-caused disease (herpes simplex)
- immune deficiency or weakness
- complementary therapy in geriatric and environmental medicine and oncology

Topical application

- external ulcers, skin lesions
- burns, superinfected
- local infections
- eye injuries and infections

Subcutaneous and intracutaneous injection

- herpes zoster
- neural therapy

Rectal Insufflation

- ulcerous colitis
- proctitis, stages I and II
- anal fistulae and fissures

Minor AHT

- acne vulgaris
- allergies
- as an adjuvant in cancer therapy
- immunoactivation in sports medicine

Intraarticular injection

- rheumatic diseases
- active gonarthrosis, shoulder stiffness
- chronic shoulder joint diseases

Federation and the federal associations of health insurance funds, is responsible for the assessment of new methods of medical diagnosis and treatment. In a statement of the G-BA published in the year 2001, ozone therapy was rejected for all indications with the rationale that benefit, necessity and economic efficiency of ozone therapy are not sufficiently documented (21). For supporters of ozone therapy, a lack of solid scientific data, excessive empiricism, ozone toxicity, the use of ozone therapy by charlatans and a disinterest of health authorities are possible reasons why ozone therapy has not been accepted by orthodox medicine, yet (15).

2 Study objective

As described above, there is a lack of high quality animal and human studies evaluating ozone therapy for cancer treatment. The selective inhibition of the growth of human cancer cells *in vitro*, the encouraging results in human pilot studies and the proposed immune-modulatory effect of ozone, point interest on ozone as a possible therapeutic agent for cancer treatment. This study was performed to test the hypothesis that intraperitoneal application of a medical O₃/O₂ gas mixture (O₃/O₂-Pneumoperitoneum, O₃/O₂-PP) is an effective antitumour approach. Intraperitoneal application of the O₃/O₂ gas mixture was chosen because with this method, in comparison to the method of major autoheamotherapy, a high gas volume could be applied. The VX2 auricle carcinoma in NZW rabbits is a very reliable animal model for evaluation of intraperitoneal O₃/O₂ gas therapy, because the tumour is aggressive, easy accessible and the routes of metastatic dissemination are clearly delineated.

The O₃/O₂-PP method was evaluated for:

- overall survival of the rabbits
- effect on the growth of the primary auricular tumour
- effect on the development of regional and distant metastases
- adverse effects and safety

3 Material and methods

3.1 Animals and study protocol

The Animal Use Protocol (AUP) was approved by the Regierungspräsidium Giessen (government of Giessen), Germany, protocol number V 54-19 c 20-15(1) MR, No. 24/2005. This study was performed in accordance with the guidelines of the Declaration of Helsinki and the guidelines of FELASA.

Adult, male Iffa Credo NZW outbred rabbits (specific-pathogen free) were purchased from Charles River WIGA GmbH (Sulzfeld, Germany). In total, 61 rabbits weighing 2.0 to 3.0 kg were included in this study. 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 (hydrochloride acid, pH 2.7) tap water ad libitum. Animals could acclimatize for at least five days before the experimental procedure was started.

The study protocol is schematically presented in Figure 3.1. The purpose of this study was to test the effects of the intraperitoneal application of a medical O₃/O₂ gas mixture (O₃/O₂-PP) on tumour development and metastatic spread of the VX2 auricle carcinoma in NZW rabbits. The VX2 auricle carcinoma model is a highly suitable tumour model for human HNSCC (89). A VX2 tumour cell suspension was inoculated subcutaneously into the right auricle of 48 rabbits (day 0). Success of transplantation and tumour growth were monitored daily. Additionally, the body weight was measured daily and the draining lymph node stations of the auricle were palpated. On day 14 after tumour cell inoculation, the rabbits were randomly divided into three experimental groups (group A, group B, group C). Rabbits in group A received an intraperitoneal O₃/O₂ gas mixture therapy, rabbits in group B received an intraperitoneal O₂ gas therapy, rabbits in group C received a sham treatment. O₃/O₂ therapy, O₂ therapy or sham treatment were performed daily for five consecutive days, beginning on day 14 after tumour cell inoculation and ending

on day 18 after tumour cell inoculation. Thereafter, all animals were observed at regular intervals until day 90 after tumour cell inoculation. Observation included inspection with special regard to the macroscopic aspect of the primary tumour and to signs of animal distress, measurement of the body weight, measurement of the size of the primary auricular tumour, palpation and, if possible, measurement of the size of the draining regional lymph nodes. If severe infection (indicated by swelling, smelling, fever) or massive bleeding of the auricular tumour was observed, ear ablation was performed 1-2 cm proximal to the tumour margin to prevent animals from early death. After ablation of the tumour-bearing part of the ear, animals were observed as described above. If the animals showed signs of distress, pain or cachexia - defined as rapid or consistent body weight loss exceeding 15-20% of the maximal body weight of the animal - they were sacrificed as is recommended by the Canadian Council on Animal Care (2). On day 90 after inoculation of the tumour cell suspension the remaining tumour-bearing animals were sacrificed. All sacrificed animals were immediately dissected. Rabbits that were macroscopically tumour-free on day 90 after the tumour cell inoculation and exhibited no enlarged draining lymph nodes and no lung metastases, as determined by palpation and a computed tomography scan of the thorax, were defined as cured and included in a consecutive

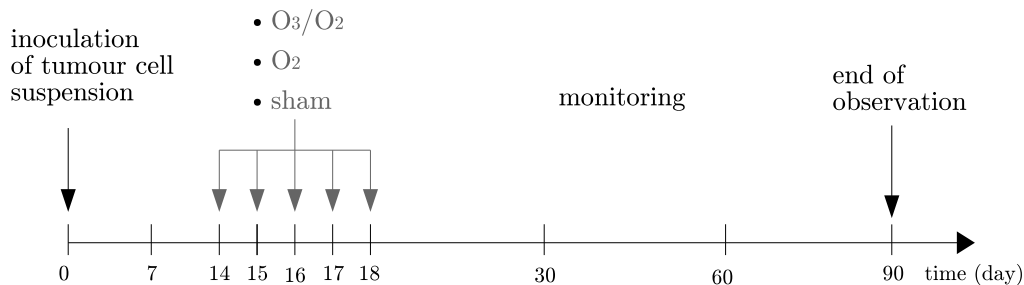


Figure 3.1 – Study protocol of the main study.

The VX2 tumour cell suspension was inoculated subcutaneously into the right auricle of 48 NZW rabbits on day 0 of the experiment and tumour growth was allowed for 14 days. On day 14 after the inoculation the rabbits were randomly divided into three experimental groups: A, B and C. Rabbits in group A received an intraperitoneal O_3/O_2 gas mixture therapy (O_3/O_2 -PP), rabbits in group B received an intraperitoneal O_2 gas therapy, rabbits in group C received a sham treatment. O_3/O_2 therapy, O_2 therapy or sham treatment were performed daily for five consecutive days, beginning on day 14 after tumour cell inoculation and ending on day 18 after tumour cell inoculation. Observation was done until day 90 after tumour cell inoculation.

study.

In this consecutive study, the six rabbits of the O₃/O₂ gas mixture therapy group with complete remission of the auricular tumour were randomly divided into two groups. One group was immune suppressed (n = 3) and the other group was sham-treated (n = 3). The tumour cell suspension was (re-)inoculated into both auricles of all animals and the tumour (re-)take-rate was evaluated on day 14 after the (re-)inoculation.

Thirteen rabbits were used as donors, in which VX2 tumour cells were propagated by intramuscular passage.

3.2 Induction of the VX2 carcinoma

3.2.1 VX2 tumour cell suspension

The *in vivo* VX2 tumour was used previously for several studies in the Department of Otolaryngology, Head and Neck surgery, University Hospital Giessen and Marburg, Campus Marburg, Marburg, Germany, and was originally provided by Prof. Dr. Robert J.J. van Es (Department of Oral and Maxillofacial Surgery, University Medical Center Utrecht, Utrecht, The Netherlands) (60; 30; 89).

The tumour cells were propagated *in vivo* by inoculation of a tumour cell suspension (as will be described below) into one or both upper hind limbs of altogether 13 NZW rabbits. On day 14 after inoculation of the tumour cell suspension into the hind limbs, the animals were sacrificed and the grown solid tumour was dissected, removed and cooled on dry ice. Necrotic tissue was removed and the tumour mass was cut with a scalpel into several small pieces. These were further fragmented with a razor blade. The resulting foamy mush was pressed through a nylon sieve (mesh size 300 μm) and collected in cooled Dulbecco's Modified Eagle Medium (DMEM, Biochrom AG, Berlin, Germany). The tumour cell and DMEM mixture was centrifuged twice. The thin fluid supernatant was removed and the remaining tumour cells were suspended with little DMEM, resulting in a viscous suspension. The number of cells in suspension was counted using a haemocytometer (Improved Neubauer Ruling). Viability of cells was estimated by the Trypan blue exclusion method. The suspension was standardized at a mean density of 3-6 x 10⁷ cells/ml and inoculated into the experimental rabbits and further donor rabbits within less than two hours.

3.2.2 Tumour cell inoculation

For tumour cell inoculation into the right auricle rabbits were sedated with 5 mg/kg body weight of the alpha2-agonist xylazine (Rompun[®], Bayer Vital, Leverkusen, Germany), injected intramuscularly into one hind limb. Experience showed that previous sedation facilitated the handling of nervous animals, reducing physical stress to the animal and resulting in better standardization of the injected suspension volume and thus, the resulting tumours.

To induce tumours, 0.3 ml of the tumour cell suspension, containing $1-2 \times 10^7$ vital tumour cells, were slowly injected subcutaneously with a 24G needle (Microlance 3, Becton Dickinson Medical Systems, Drogheda, Ireland, outer diameter 0.5 mm). Prior to the tumour cell suspension inoculation, a small amount of air was injected subcutaneously to produce a small subcutaneous pouch, which made it easier to inoculate the tumour cell suspension. Tumours were transplanted between the central auricular artery and the caudal margin at the dorsal middle-third of the right auricle. Sometimes regurgitation of the tumour cell suspension occurred, but the volume was very small and thus negligible.

3.3 Monitoring, blood withdrawal and photo documentation

3.3.1 Overview

Prior to encasement of the rabbits in this study, their general health condition was assessed by an experienced veterinarian. On day 0, prior to inoculation of the tumour cell suspension into the right auricle, the body weight and the basal body temperature were measured and an arterial blood sample was drawn from the central auricular artery of the left auricle. The sites of the draining lymph nodes (parotideal, submandibular, cervical) were located by palpation to rule out any enlarged lymph nodes before the tumour induction was performed.

The routine monitoring procedure consisted of:

- inspection with special regard to the macroscopic aspect of the primary tumour (bleeding, necrosis, ulceration) and to signs of animal distress (dyspnoea, cachexia)

- measurement of the body weight
- measurement of the primary auricular tumour in three dimensions (the largest diameter, one diameter perpendicular to the largest diameter and the complete thickness including the underlying auricle)
- palpation and, if possible, measurement of the parotid lymph node in one dimension (the largest diameter)
- the basal body temperature was measured in addition, if the primary tumour seemed to be infected or if the animal showed signs of distress.

In the time period from day 1 to day 19 this routine monitoring was done daily. In the time period from day 20 to day 90 this monitoring procedure was performed at least every second day. On day 14, prior to the first therapeutic session, and on day 19, 24 hours after the last therapeutic session, blood was taken from the central auricular artery of the left ear. The last arterial blood sample was taken on day 90 or on the day at which the animal was sacrificed.

3.3.2 Measurement of body weight, body temperature, tumour and lymph node size

The body weight was recorded with a Multina Plus baby scale (Soehnle Professional GmbH and Co. KG, Murrhardt, Germany) with “weight-lock-function”, that allows precise measurements with an accuracy of 0.01 kg even if the rabbit is agitated. The rectal body temperature was measured with a digital thermometer with an accuracy of 0.1°C. The size of the primary ear tumour and the largest diameter of the parotid lymph node were measured with a digital calliper (S Cal Work, Sylvac SA, Chrissier, Switzerland).

3.3.3 Blood withdrawal and blood parameters

Blood was taken from the central auricular artery of the left tumour-free ear. For the puncture, a 19G needle (Microlance 3, Becton Dickinson Medical Systems, Drogheda, Ireland, outer diameter 1.1 mm) was used.

EDTA whole blood samples were collected for haematological investigations with an autoanalyzer (Vet abcTM Animal blood counter, ABX Diagnostics, Goettingen,

Germany) that has been carefully adjusted and validated for the analysis of rabbit blood. The following parameters were determined by the autoanalyzer: total white blood cells (WBC) which were further differentiated into granulocytes (GRA), monocytes (MO) and lymphocytes (LYM); total red blood cells (RBC), haemoglobin (HGB) and haematocrit (HCT). Clinical chemistry investigations were done with a reflexion photometer (Reflovet[®] Plus, Roche Diagnostics, Mannheim, Germany). The following parameters were determined: creatinine (CREA), serum glutamate pyruvate transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT). The following blood samples were used: CREA - EDTA whole blood, SGOT/SGPT - heparin plasma. For arterial blood gas analysis a blood gas analyzer was used (ABL 500, Radiometer, Copenhagen, Denmark). The following parameters were measured: pH, pO₂ and pCO₂.

3.3.4 Photo documentation

A digital single-lens reflex camera model (Nikon D70, Nikon GmbH, Duesseldorf, Germany) was used for photo documentation. Photos of the tumour-bearing right auricle were taken on day 14 and then once a week up to day 90. The photos were edited with the Adobe Photoshop[®] CS program (Adobe Systems GmbH, Munich, Germany).

3.4 O₃/O₂ gas mixture therapy, O₂ gas therapy and sham treatment

3.4.1 O₃/O₂ gas mixture therapy (O₃/O₂-PP)

The O₃/O₂ gas mixture therapy was performed daily for five consecutive days (Figure 3.1). A short-lasting anaesthesia was given each day to sufficiently immobilize the rabbit during the short period of intraperitoneal O₃/O₂ gas mixture insufflations. Rabbits were premedicated with 0.02 mg/kg body weight glycopyrrolate (Robinul[®], Riemsler Arzneimittel AG, Greifswald, Germany) injected subcutaneously, followed by intramuscular application of 0.3 mg/kg body weight medetomidine hydrochloride (Domitor[®], Pfizer, Karlsruhe, Germany) plus 3-6 mg/kg body weight propofol (Propofol 1% Fresenius, Fresenius Kabi Deutschland, Germany) injected intravenously. After the treatment anaesthesia was finished by intravenous injection of

the medetomidine reversal agent atipamezole hydrochloride (Antisedan[®], Pfizer, Karlsruhe, Germany), 1.5 mg/kg body weight.

For generation and intraperitoneal insufflation of the medical O₃/O₂ gas mixture the Medozon^{IP} gas processor (Herrmann Apparatebau, Kleinwallstadt, Germany; certified ISO 13485:2003 Nr. Q1N 06 10 51287 005) was used (Figure 3.2). The gas mixture, which was generated from pure medical oxygen, was composed of 97.5% O₂ and 2.5% O₃. A standardized gas volume of 80 ml/kg body weight with a concentration of 50 µg O₃/ml gas was insufflated each day. This is an empirical dose with a medium concentration of ozone based on early observations in veterinary trials by Dr. S. Schulz (Veterinary Service and Laboratory Animal Medicine, Philipps University Marburg, Germany) (4).

The top of a 17G Vasofix[®] Braunuele[®] (Braun Melsungen AG, Melsungen, Germany) was carefully implanted into the right lower quadrant of the abdomen. Thereafter 2 ml of a sterile NaCl solution were slowly injected into the abdomen to control the correct intraperitoneal position of the Braunuele[®]. The end of the Braunuele[®] was connected to the Medozon^{IP} gas processor via a sterile Ozon-Kit (Ozone-Set ip REF HAB no. 18052, Herrmann Apparatebau, Kleinwallstadt, Germany). Beginning at the side where it is connected to the gas processor the Ozon-Kit consists of a sterile filter, followed by a plastic tube of 150 mm length with a stopcock where it is connected to the Braunuele[®]. The Medozon^{IP} gas processor has a special device to which the Ozon-Kit can be connected for removing the air from the plastic tube and refilling it with the recommended gas. To prevent inappropriate high abdominal pressure, the Medozon^{IP} gas processor has an integrated intraabdominal pressure control system (IAPC), which stops insufflation automatically when the measured intraabdominal pressure raises to a threshold value. During the insufflation process, the actually insufflated gas volume, the adjusted ozone concentration and the current intraabdominal pressure are presented on the monitor of the Medozon^{IP} gas processor. At the end of the insufflation process the Braunuele[®] was removed from the abdomen. In total, the rabbits received 400 ml/kg body weight of the O₃/O₂ gas mixture with an ozone concentration of 50 µg/ml over the five days.

3.4.2 O₂ gas therapy

The O₂ gas therapy was performed daily for five consecutive days. The animals were anaesthetized the same way as the animals in the O₃/O₂ group. For insuffla-

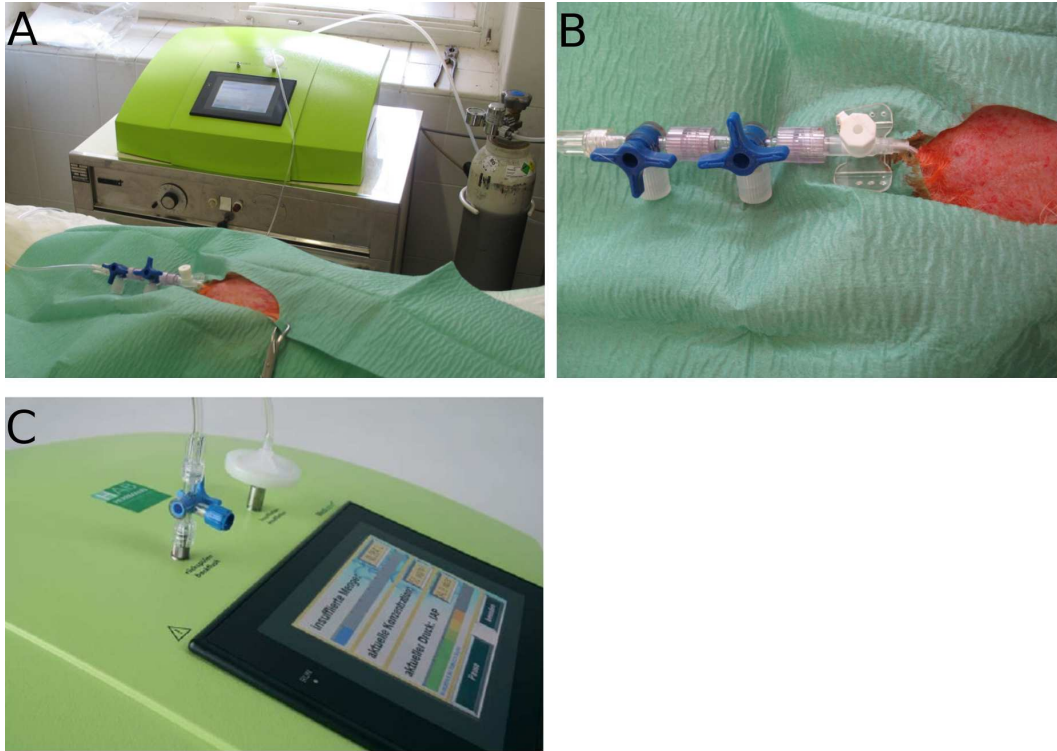


Figure 3.2 – Equipment for gas insufflations.

Picture A was made during the treatment process. A Braunuele[®] (Braun Melsungen AG, Melsungen, Germany) is implanted into the right lower abdominal quadrant of an anaesthetized rabbit. This Braunuele[®] is connected to a Medozon^{IP} gas processor (Herrmann Apparatebau, Kleinwallstadt, Germany) via a sterile Ozon-Kit (Ozone-Set ip REF HAB no. 18052, Herrmann Apparatebau, Kleinwallstadt, Germany). Picture B shows the Braunuele[®] connected to the Ozon-Kit which at that side consists of a plastic tube and a stopcock. Picture C shows the Medozon^{IP} gas processor and the other end of the Ozon-Kit with the sterile filter.

tion of pure oxygen the same Medozon^{IP} gas processor was used. A standardized gas volume of 80 ml/kg body weight of pure medical oxygen was insufflated intraperitoneally each time, resulting in an insufflated volume of 400 ml/kg body weight of pure medical oxygen over the five days.

3.4.3 Sham treatment

In the sham treatment group animals were anaesthetized as described above. A 17G Braunuele[®] was implanted into the abdominal cavity and connected via the Ozon-Kit to the Medozon^{IP} gas processor as described above, but no gas was insufflated.

3.5 Ear ablation

As mentioned above, to prevent animals from early death an ear ablation was performed. Ear ablation was indicated when massive bleeding of the primary tumour occurred or when redness, swelling and smelling of the tumour-bearing auricle combined with increased body temperature strongly suggested severe infection of the primary tumour and the underlying auricle.

Animals were premedicated with 0.02 mg/kg body weight glycopyrrolate (Robinul[®], Riemsler Arzneimittel AG, Greifswald, Germany) injected subcutaneously. General anaesthesia was given with 5 mg/kg body weight xylazine (Rompun[®], Bayer Vital, Leverkusen, Germany) and 70 mg/kg body weight ketamine (Ketavet[®], Pfizer, Karlsruhe, Germany), both injected intramuscularly. In some cases up to 10 mg/kg pentobarbital (Narcoren[®], Merial GmbH, Hallbergmoos, Germany) were additionally injected intravenously.

The resection line was placed 1-2 cm proximal to the tumour margin. The hair in this region was shaved and the skin was disinfected with Braunol[®] (Braun Melsungen AG, Melsungen, Germany). First, the central auricular artery was ligated proximal to the resection line. For resection a disposable scalpel was used. A 2-3 mm wide stripe of the cartilage along the resection line was additionally resected. Now the overlaying skin on both sides of the cartilage could easily be pulled and sutured together (2/0 Serapid, EP 3, Serag-Wiessner KG, Naila, Germany) with continuous suture. The sutures were removed on the 7th to 10th postoperative day.

The resected tumour was measured in three dimensions, as described above. For microbiological testing a smear was taken from the necrotic parts of the tumour and transferred into a Port-A-CulTM Tube (Becton Dickinson Medical Systems, Drogheda, Ireland) for transport. The microbial analysis was done by the Institute of Microbiology, University Hospital Giessen and Marburg, Campus Marburg, Marburg, Germany.

3.6 Sacrifice and dissection

3.6.1 Sacrifice

Endpoint criteria were defined according to the Canadian Council on Animal Care and the UK Co-ordinating Committee on Cancer Research (UKCCCR) (2; 3)

These endpoint criteria were:

- rapid or consistent body weight loss exceeding 15-20% of the maximal body weight of the animal
- interference with normal body functions due to the mass of tumour tissue at different locations (parotid lymph node enlargement - interference with chewing and eating; lung metastases - dyspnoe accompanied by cyanosis)
- somnolence and all further steps in the loss of consciousness

Ulceration, massive bleeding or infection of the primary tumour were criteria for ear ablation as described above. Animals were anaesthetized with 0.02 mg/kg body weight glycopyrrolate s.c., 5 mg/kg body weight xylazine i.m., 70 mg/kg body weight ketamine i.m. and up to 10 mg/kg body weight pentobarbital i.v. when necessary. Laparotomy was done. The aorta abdominalis was punctured and blood was taken for analysis. Sacrifice was induced by bleeding to death and by inducing a pneumothorax.

3.6.2 Dissection

The dissection was carried out in a standard manner for each rabbit. The size of the primary tumour was measured in three dimensions. The draining lymph node stations of both auricles had been identified and removed. In detail, these were the parotid lymph nodes, the caudal mandibular lymph nodes and the rostral mandibular lymph nodes (31). All lymph nodes had been weighed, a photo was made and the largest diameter of each lymph node was measured and documented. The next step was removal of the thorax organs. Therefore, the skin was detached from the underlying ribs and a sternocostal block was cut out and removed. The trachea was accessible and could be cut through in its proximal part. The whole thorax organs could be removed from the thorax by yanking the trachea. This block was weighed in toto. Then the lung was separated and both lobes were weighed. Metastases which were macroscopically visible were counted. To get a range of the size of visible metastases, the diameters of the largest and the smallest metastases were measured. The trachea was opened and observed for metastases. Photo documentation of the lung was done. The thoracic cavity was inspected for the presence of metastases. Afterwards the abdomen was inspected. Special regard was drawn to the presence of

metastases and to the presence of adverse effects of the intraperitoneal gas therapy (for example adhesions, scars, signs of inflammation).

3.7 Consecutive immune suppression study

3.7.1 Overview

Six rabbits of the O₃/O₂ gas mixture therapy group had no visible tumour or palpable enlarged lymph nodes at the end of the observation period on day 90. In other words, they were probably tumour-free and cured. To exclude metastatic spread to the lungs, a computed tomography of the thorax was performed in these animals. Thereafter, these six NZW rabbits were randomly divided into two groups. One group was immune suppressed (group A1; n = 3 rabbits), while the other group remained untreated (group A2; n = 3 rabbits). Two additional rabbits (not part of the previous study) were used. One was immune suppressed to monitor possible effects of the immune suppression on the tumour take-rate. The other one was only sham-treated and served as control for the aggressiveness of the tumour cell suspension. The tumour cell suspension was (re-)inoculated into both auricles of all rabbits to enhance the number of possible tumours. The technique of inoculation was the same as described above. Immune suppression was started 2 days (day -2) before the (re-)inoculation (day 0) and was maintained for 11 consecutive days. On day 14 after tumour cell inoculation, the retake-rate was assessed. Up to that day, the animals were monitored as described in the corresponding part of the main study. The study protocol is schematically presented in Figure 3.3.

3.7.2 Computed tomography

Rabbits were premedicated with 0.02 mg/kg body weight glycopyrrolate injected subcutaneously and then anaesthetized with a combination of 5 mg/kg body weight xylazine and 30 mg/kg body weight ketamine, both injected intramuscularly. The Siemens Somatom Plus 4 (Siemens, Erlangen, Germany) was used for this purpose. All images were regarded by an experienced radiologist of the Department of Diagnostic Radiology, University Hospital Giessen and Marburg, Campus Marburg, Marburg, Germany.

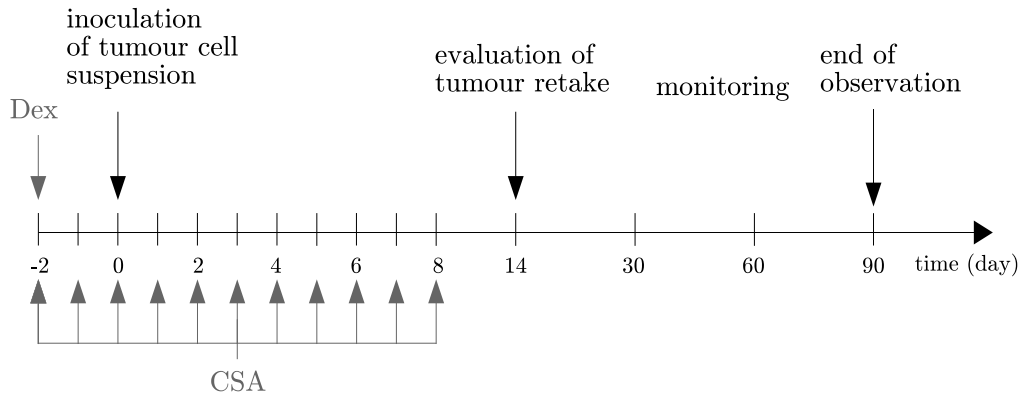


Figure 3.3 – Consecutive immune suppression study, experimental design.

The six rabbits of the main study with complete tumour remission (O_3/O_2 gas mixture therapy group) on day 90 were randomly divided into two groups. Group A1 ($n = 3$) received an immune suppression, group A2 ($n = 3$) was sham-treated. Immune suppression was started on day -2 with dexamethasone and cyclosporin A and was maintained by daily application of cyclosporin A up to day 10. On day 0 the tumour cell suspension was (re-)inoculated into both auricles of all animals. Two additional rabbits were used: one was immune suppressed likewise the rabbits in group A1, the other rabbit was sham-treated. Both rabbits received a tumour cell inoculation into both auricles on day 0. The tumour take-rate was assessed on day 14. Abbreviations: Dex, dexamethasone; CSA, cyclosporin A.

3.7.3 Immune suppression

Two days prior to tumour cell (re-)inoculation, the immune suppression was started. A single subcutaneous injection of 1.5 mg/kg body weight dexamethasone (Dex, Dexamethason®[®], Jenapharm, Jena, Germany) was applied together with a subcutaneous injection of 20 mg/kg body weight cyclosporin A (CSA, Sandimmun®[®], Novartis Pharma, Nuremberg, Germany). Immune suppression was maintained by daily applications of 20 mg/kg body weight CSA for 11 consecutive days.

3.8 Statistics

For comparison of survival rates of the three experimental groups the log rank test was performed considering $p < 0.05$ as significant. The survival probability of rabbits calculated from the time of tumour cell inoculation until day 90 was depicted according to the Kaplan-Meier method. The time to tumour clearance (TTC) probability was calculated from the time when the size of the solid auricular tumour dropped under 5% of the size measured on day 14 after tumour cell inoculation, a

time point when a solid auricular tumour had developed and gas insufflation therapies or sham treatment started. Statistical differences in mean tumour surface areas A between the three experimental groups were evaluated with the unpaired Student's t-test. To evaluate statistical differences of the mean body weight within each group the paired Student's t-test was used. Comparison of the blood parameters prior to the first gas insufflation or sham treatment (day 14) with those 24 hrs after the last gas insufflation or sham treatment (day 19) within each group was done with the paired Student's t-test. The difference of the mean weight of lymph nodes of the right and left neck side in all cured animals of the O₃/O₂ gas mixture therapy group was calculated with the unpaired Student's t-test. For all calculations with the Student's t-test $p < 0.05$ was considered as significant.

4 Results

From the total of 48 animals that received a tumour cell inoculation into the right auricle 7 were excluded from the study due to different reasons. Tumour induction failed in only one animal (2.1%). Other reasons for exclusion from the study were death due to anaesthesia complications, death in a transport box and death prior to treatment due to undefined reasons.

4.1 Clinical measurements

4.1.1 Survival

The observation period of the experiment ended on day 90 after inoculation of the tumour cell suspension. This time point was chosen since the highly malignant VX2 tumour model is characterized by a mortality rate of 100% within 90 days, independent of the localization of the tumour.

For survival analysis rabbits were assigned to two different groups: survival and non-survival. Survival corresponds to two situations. First situation: The animal survived until day 90, the auricle was not resected and macroscopically tumour-free, no enlarged lymph node could be palpated. These animals were defined as cured (O_3/O_2 cured, O_2 cured, sham cured). Second situation: The animal survived until day 90, the auricle was not resected but not tumour-free and/or enlarged lymph nodes could be palpated. These animals survived, but they were not cured (O_3/O_2 survived, O_2 survived, sham survived). Non-survival also corresponds to two situations. First situation: The tumour-bearing auricle was resected within 90 days after tumour induction, the rabbit thereafter survived until day 90. These animals could no longer be assigned to the survival group, because an earlier death due to infection or bleeding of the auricle would have been very likely without an ear ablation (O_3/O_2 ablation, O_2 ablation, sham ablation). Second situation: The animal was sacrificed before day 90 due to the defined end point criteria or died

spontaneously (O₃/O₂ dead, O₂ dead, sham dead).

O₃/O₂ group

Of the 14 rabbits that received intraperitoneal insufflations of the O₃/O₂ gas mixture, seven (50.0%) survived. On day 90, six (42.9%) could be defined as cured. The auricle of the seventh animal that survived, still beared a large mass of tissue with a brownish firm cap on day 90. Seven animals (50.0%) fulfilled the criteria for non-survival. Four of these seven survived until day 90, but had an ear ablation before. The ear resections were carried out on days 50, 81, 85 and 85. One animal died spontaneously on day 72 (ear ablation day 48), the remaining two were sacrificed according to the defined endpoint criteria on day 41 (no previous ear resection) and day 58 (ear resection day 48).

O₂ group

Of the 13 rabbits that received intraperitoneal insufflations of the O₂ gas, three (23.1%) survived. On day 90, two (15.4%) could be defined as cured. The auricle of the third animal that survived, still beared a large tumour mass. Ten animals (76.9%) fulfilled the criteria for non-survival. Four of these ten survived until day 90, but had an ear ablation before (days 31, 50, 51, 57). Three animals died spontaneously (days 26 (no previous ear ablation), 46 (ear ablation day 45), 70 (no previous ear ablation)) and three were sacrificed according to the defined endpoint criteria (days 39 (ear ablation day 32), 73 (ear ablation day 56), 80 (ear ablation day 51)).

Sham group

Of the 14 rabbits that received sham treatment, one (7.1%) survived. This rabbit was defined as cured. 13 animals (92.9%) fulfilled the criteria for non-survival. Two of these 13 survived until day 90, but had an ear ablation before (days 47, 54). Of the remaining 11 animals one died spontaneously on day 55 (ear ablation day 30), while ten were sacrificed according to the defined endpoint criteria (days 35 [no], 55 [47], 55 [32], 55 [41], 68 [51], 71 [48], 75 [no], 75 [41], 81 [57], 86 [48]); in [] is given the day after inoculation at which a previous ear resection was carried out.

Survival analysis

A Kaplan-Meier plot showing the 3-month survival probability is depicted in Figure 4.1. Calculated with the log rank test, the survival probability of the rabbits in

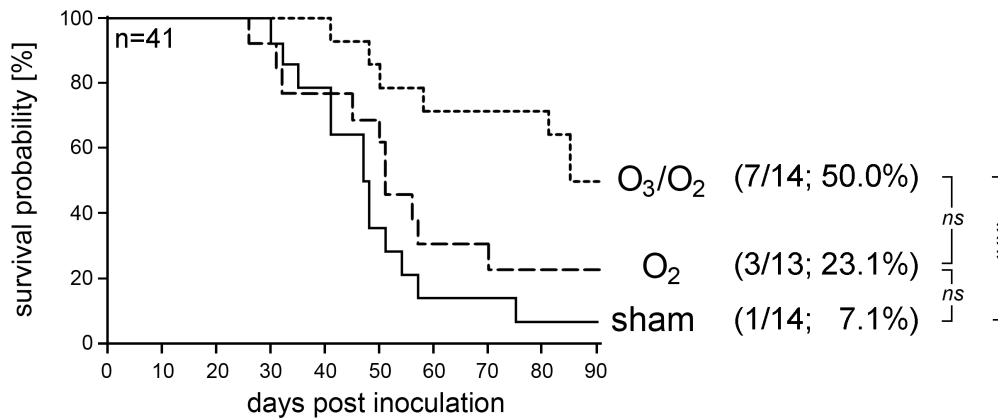


Figure 4.1 – Survival analysis. Kaplan-Meier survival curves for VX2 tumour-bearing NZW rabbits that received O₃/O₂ therapy (n = 14), O₂ therapy (n = 13) or sham treatment (n = 14) from day 14 to day 18 after tumour induction. In the O₃/O₂ therapy group 7/14 animals survived (50.0%), in the O₂ therapy group 3/13 animals survived (23.1%) and in the sham treatment group 1/14 (7.1%) survived until the end of the observation period 90 days after tumour cell inoculation into the right auricle. Statistical analysis was done with the log rank test. O₃/O₂ gas mixture therapy significantly increased the survival probability compared with the sham treatment ($p = 0.0006^{***}$), but did not increase the survival probability compared with the O₂ gas therapy ($p = 0.0559$). The difference of the survival probability between the O₂ gas therapy group and the sham group was not statistically significant ($p = 0.2448$). For calculation all animals with an ear resection were defined as “dead” from the time when the ear resection was carried out. Abbreviation: ns, not significant.

the O₃/O₂ gas mixture therapy group (50.0% survival) was significantly increased compared with the survival probability of the rabbits in the sham treatment group (7.1% survival) ($p = 0.0006$). The survival probability of the rabbits in the O₃/O₂ gas mixture therapy group was not significantly different from the survival probability of the rabbits in the O₂ gas therapy group (23.1% survival) ($p = 0.0559$), but this p value suggests a tendency towards an increased survival in the O₃/O₂ gas mixture therapy group. The difference of the survival probability between the O₂ gas therapy group and the sham group was calculated nonsignificant ($p = 0.2448$).

4.1.2 Growth of the primary tumour

Mean tumour surface area A

The tumour surface area A is the product of the largest diameter of the tumour and the diameter perpendicular to that. The development of the mean tumour surface areas A of the three experimental groups is shown in Figure 4.2. The mean tumour

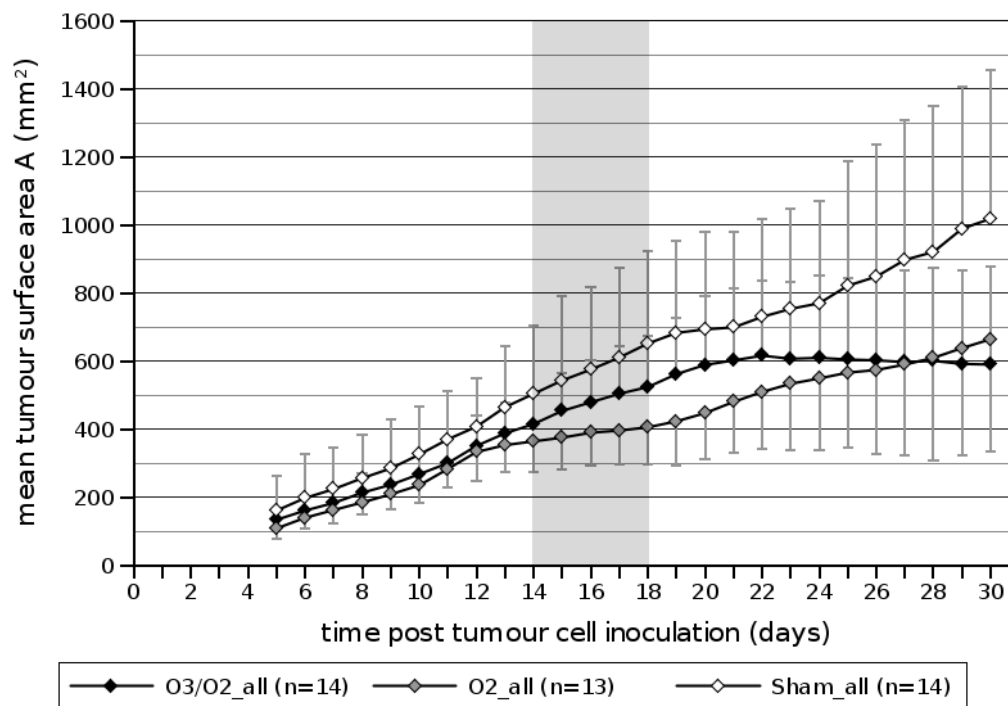


Figure 4.2 – Growth of the primary tumour. Development of the mean tumour surface areas A of the three experimental groups from day 5 (day 0 - inoculation of VX2 cell suspension) to day 30.

The tumour surface area A is the product of the largest diameter of the tumour and the diameter perpendicular to that and is denoted in mm^2 . The means of the three groups were compared for each day with the unpaired Student's t-test. Compared with the sham group, the mean tumour surface area of the O_3/O_2 group was significantly smaller from day 27 to day 30. Compared with the sham group, the mean tumour surface area of the O_2 group was significantly smaller on days 10 and 11 and from day 14 to day 30. Compared with the O_3/O_2 group, the mean tumour surface area of the O_2 group was significantly smaller from day 16 to day 20. The corresponding p -values are given in the main text. Note that on day 14 (just prior to the first insufflation of the O_3/O_2 gas mixture into the peritoneum) no statistically significant difference was observed comparing the mean tumour surface area of the O_3/O_2 group with the two control groups. For better optical clarity, the error bars (standard deviation) are plotted only in one direction. The treatment period is marked with a grey box.

surface area A on day 14 (directly before the first treatment was started) was $415.4 \pm 94.9 \text{ mm}^2$ for the O₃/O₂ group, $364.7 \pm 91.9 \text{ mm}^2$ for the O₂ group and $504.2 \pm 200.4 \text{ mm}^2$ for the sham group. The difference of the mean tumour surface areas between the O₃/O₂ group and the sham group was not statistically significant ($p = 0.146$, unpaired Student's t-test). The difference of the mean tumour surface areas of the O₃/O₂ group and the O₂ group was also not statistically significant ($p = 0.171$). The tumour surface areas of the animals in the O₂ group were significantly smaller than those of the animals in the sham group ($p = 0.031$).

Beginning on day 14 after inoculation of the tumour cell suspension into the right auricle, the gas insufflations (O₃/O₂ gas mixture or pure O₂ gas) or the sham treatments were performed on five consecutive days. On day 19, the first day after the last gas insufflation or sham treatment, the mean tumour surface area A of the O₃/O₂ group was $562.0 \pm 166.1 \text{ mm}^2$. The mean tumour surface area A of the O₂ group was $423.4 \pm 128.1 \text{ mm}^2$ and of the sham group was $683.2 \pm 270.0 \text{ mm}^2$. The difference of the mean tumour surface areas between the O₃/O₂ group and the sham group was not statistically significant ($p = 0.165$, unpaired Student's t-test). The mean tumour surface area of the animals in the O₂ group was significantly smaller than the mean tumour surface area of the animals in the O₃/O₂ group ($p = 0.023$) and than that of the animals in the sham group ($p = 0.004$).

As shown in Figure 4.2, the mean tumour surface area A of the O₃/O₂ group reached a maximum on day 22 and then decreased in size. Compared with the mean tumour surface area of the sham group, the mean tumour surface area of the O₃/O₂ group was significantly smaller from day 27 ($p = 0.031$) to day 30 ($p = 0.006$). As mentioned above, the mean tumour surface area of the O₂ group was significantly smaller than that of the sham group from day 14 to day 30. Comparing the mean tumour surface area of the O₂ group with that of the O₃/O₂ group, the mean tumour surface area of the O₂ group was significantly smaller only from day 16 to day 20 after inoculation of the tumour cell suspension. More importantly, after day 20 the difference was not statistically significant. Data analysis is shown up to day 30 after tumour induction. After day 30, the first tumours had regressed completely or the first ear resections were performed.

Tumour regression

As described previously by van Es et al. (88), hyperaemia of the auricle and a purple discoloration of the skin adjacent to the tumour normally come along with progressive growth of the tumour. The disappearance of these vascular signs was an important first clinical sign of beginning tumour regression after local IL-2 treatment. Their observation could be confirmed in this study. The hyperaemia disappeared and the skin adjacent to the tumour became more brightly, similar to the normal colour of the rabbits' auricular skin. Different stages of tumour regression in macroscopic view are shown exemplary in Figure 4.3. Pictures A-D demonstrate regression of the primary auricular tumour after O_3/O_2 gas mixture treatment until complete remission. In contrast, pictures E-H demonstrate progression of the tumour to the final stage in an animal of the sham group. Picture A shows the tumour 14 days after inoculation of the tumour cell suspension. At that time point, the tumour was growing fast and the tumour tissue had a viable aspect. A thin crust had developed at the top, probably a dried exsudate. The situation 13 days later (day 27 after tumour cell inoculation) in the same animal is shown in picture B. The surface area A had decreased in size. The former viable tumour tissue was now replaced by a dry, firm, brown to black necrotic mass. This macroscopical change from a viably looking tumour mass to a totally necrotic looking mass in some rabbits began one or two days after the first day of treatment. Typically, the described necrotic appearance was observed for some days without mentionable change. Finally, the necrotic mass dropped off, resulting in a situation shown in Picture C (day 35 after inoculation of the tumour cell suspension). A small, nearly round defect in the skin with a surrounding pink circle appeared at the site where the necrotic mass had dropped of. Macroscopically, no viable tumour tissue was visible. The end stage was a small scar at the site of the former auricular tumour. This scar is depicted in Picture D (day 90 after inoculation of the tumour cell suspension).

The exact days after tumour cell inoculation on which the primary tumours had disappeared completely are summarized in Table 4.1. Figure 4.4 depicts a Kaplan-Meier plot showing the time to tumour clearance (TTC) probability in the three experimental groups. The TTC probability was calculated from the time when the size of the solid auricular tumour dropped under 5% of the size measured on day 14 after tumour cell inoculation. The TTC probability, calculated with the log rank test, in the O_3/O_2 gas mixture therapy group (42.9% complete remission) was

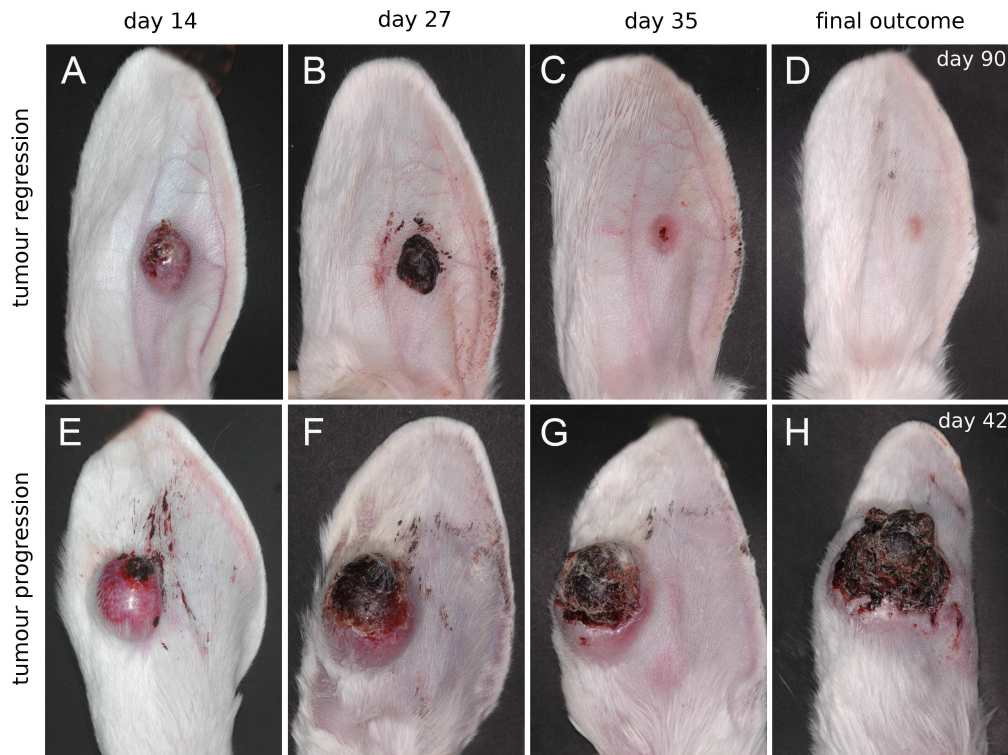


Figure 4.3 – Growth and development of the VX2 tumours after inoculation of the tumour cell suspension in the right ear of NZW rabbits.

Pictures A-D show representative macroscopic views of a solid VX2 tumour in the right ear of a rabbit on day 14 after tumour cell inoculation (A) and different stages of remission after O_3/O_2 gas mixture therapy (B-D). Note that only a small scar of the remitted auricular tumour remained on day 90 (D), the end point of our observation period. In sharp contrast, in rabbits that succumbed to tumour progression the auricular tumour continued growing, resulting in severe ulcerations associated with massive bleeding and onset of local infections, introducing the final stage of this tumour disease (representative stages of the same rabbit are shown in pictures E-H). Depicted are tumour stages on day 14, 27, 35, and at the end of the observation period (90 days (D) or 42 days in case of death (H))

significantly higher than that of the sham group (7.1% complete remission) ($p = 0.0243$). The difference between the TTC probability of the O_3/O_2 group and the O_2 group (15.4% complete remission) was not significant ($p = 0.0665$). The regression process of the two tumours in the O_2 group lasted considerably longer than that of the six animals in the O_3/O_2 group and that of the animal in the sham group with spontaneous tumour regression.

Table 4.1 – Complete remission of the primary auricular tumour

rabbit no.	treatment group	complete remission of primary tumour (day p.i.)
1	O ₃ /O ₂	34
6	O ₃ /O ₂	31
14	O ₃ /O ₂	38
15	O ₃ /O ₂	31
19	O ₃ /O ₂	32
33	O ₃ /O ₂	43
16	O ₂	68
48	O ₂	85
23	Sham	45

Abbreviations: no., number; p.i., post inoculation

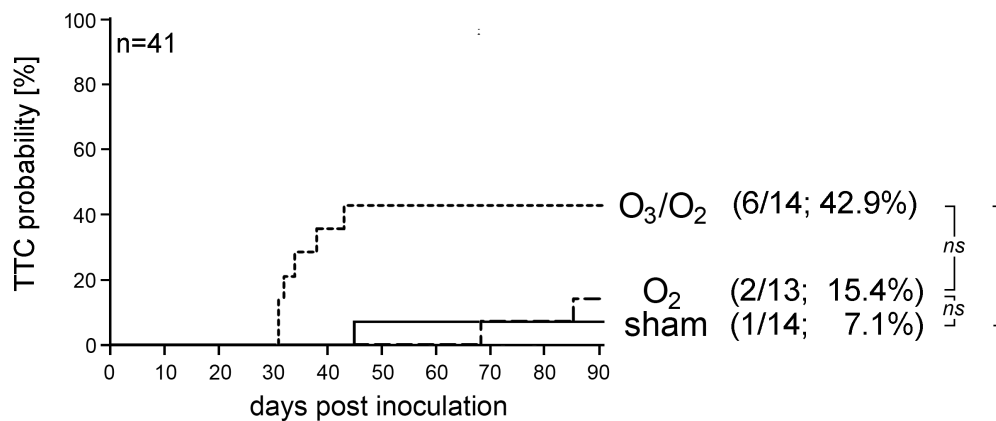


Figure 4.4 – Complete tumour remission. Kaplan-Meier plot showing the time to tumour clearance (TTC) probability in rabbits that have developed a solid auricular VX2 tumour on day 14 after VX2 tumour cell inoculation.

The TTC probability significantly varied between O₃/O₂-treated and sham-treated rabbits ($p = 0.0243^*$) but not between O₃/O₂- and O₂-treated rabbits ($p = 0.0665$). Furthermore, there was no significant difference in the TTC probability between the O₂ group and the sham group ($p = 0.5781$). Abbreviation: ns, not significant.

4.1.3 Lymph node palpation

Cured rabbits

Palpation of the necks of cured animals showed that all animals had an enlarged first echolon parotid lymph node on the right neck side at the beginning of the treatment period on day 14 after inoculation of the tumour cell suspension.

Table 4.2 summarizes the results found by lymph node palpation of all cured animals. At the beginning of the experiment all parotid lymph nodes of the cured

Table 4.2 – Initial growth and subsequent regression of parotid lymph nodes of the cured rabbits

rabbit no.	treatment group	enlarged lymph node day 14 p.i.	disappearance day p.i.
1	O ₃ /O ₂	yes	43
6	O ₃ /O ₂	yes	47
14	O ₃ /O ₂	yes	49
15	O ₃ /O ₂	yes	64
19	O ₃ /O ₂	yes	-
33	O ₃ /O ₂	yes	56
16	O ₂	yes	-
48	O ₂	yes	89
23	Sham	yes	84

Abbreviations: no., number; p.i., post inoculation

animals enlarged progressively until they reached a maximum size. The parotid lymph nodes of the two animals of the O₂ group grew larger (largest diameter up to 2.0 cm) than all other parotid lymph nodes of the cured animals. This finding is consistent with the finding that the primary tumours disappeared later than those of the animals of the O₃/O₂ group with complete tumour remission. Once having reached the maximum size, growth of the parotid lymph nodes stagnated. The same lymph node sizes could be observed for several days or few weeks. Then all parotid lymph nodes decreased in size. Finally, most of the lymph nodes could not be palpated any more. The days at which the parotid lymph nodes had disappeared completely are given in the right column of the table. In two animals with complete remission of the primary auricular tumour the parotid lymph node did not disappear completely. These two lymph nodes had a maximum size of 1.5 cm and 2.0

cm. At the end of the observation period only very small nodules could be palpated. Thus, the decrease in size of these two lymph nodes was remarkably as well.

All other rabbits

The parotid lymph nodes of all other animals were enlarged at the beginning of the treatment period. The lymph nodes were growing progressively after the end of the treatment period until the end of the observation period or until the animal was sacrificed. The weights of the lymph nodes at autopsy are given in Section 4.2, “Findings at autopsy”.

4.1.4 Body weight

The development of the body weight in the early phase of the experiment is depicted in Figure 4.5. Until day 14 after tumour cell inoculation the mean body weight of the rabbits in all treatment groups increased. This indicates that the developing tumour in the early phase had no substantial influence on the rabbits’ general health condition.

The daily treatment procedure, beginning on day 14 and ending on day 18, caused a statistically significant body weight loss of rabbits in the O₃/O₂ group and in the O₂ group, but not in the sham group (calculated with the paired Student’s t-test). Compared with the mean body weight before the first treatment on day 14, the weight loss was statistically significant from day 15-23 ($p < 0.01$) and from day 24-26 ($p < 0.05$) in the O₃/O₂ group. In the O₂ group the weight loss was statistically significant on day 15 and day 19 ($p < 0.05$) and from day 16-18 ($p < 0.01$). In the sham group, the weight loss was not statistically significant at all. The mean body weight, expressed in % of the mean body weight on day 14, reached its minimum in the O₃/O₂ group and in the O₂ group on day 18 after tumour induction, the last day of the daily therapeutic treatment. The maximum drop of the mean body weight was 8.3% in the O₃/O₂ group, 5.1% in the O₂ group and 2.2% in the sham group (on day 16 post tumour cell inoculation) compared with the mean body weight on day 14. Note that the mean body weight twelve days after the last treatment (day 30) was comparable in all three groups (2.99 kg O₃/O₂ group, 2.96 kg O₂ group, 3.01 kg sham group).

The mean body weight of all animals of the survival group constantly increased from the end of the observation period until day 90, shown in Figure 4.6. In sharp

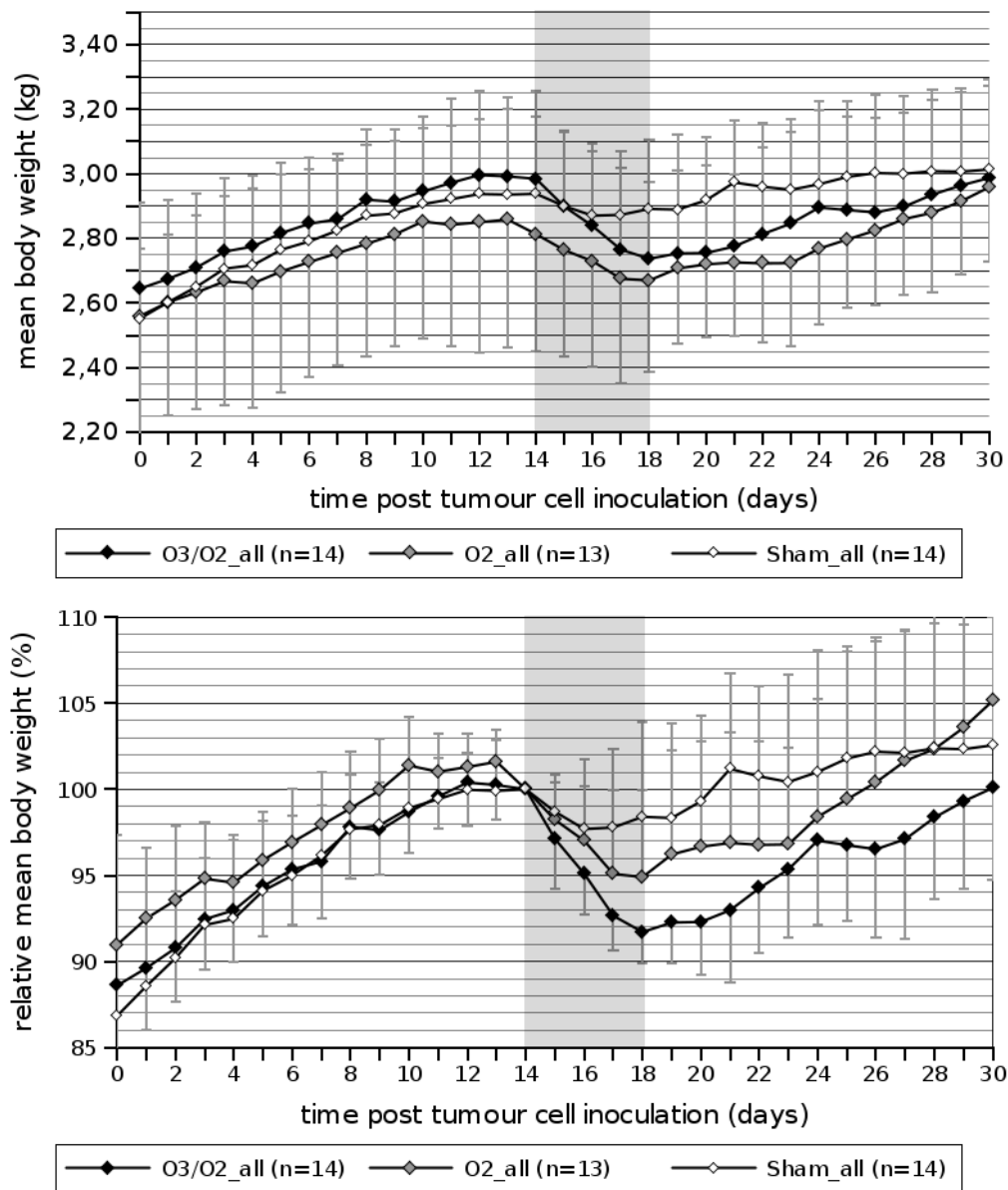


Figure 4.5 – Body weight. Development of the mean body weight from day 0 (at which VX2 cell suspension was inoculated) until day 30.

The mean body weights of the three experimental groups are drawn separately. The upper chart shows the absolute body weight in kg. For the lower chart, the mean body weight on day 14 was set 100% and changes were calculated relative to the body weight on day 14. Changes of the mean body weight within each group compared with the mean body weight on day 14 (the day when the treatment was started) were calculated with the paired Student's t-test. In the O₃/O₂ group, the body weight loss was significant from day 15 to day 26 ($p < 0.01$ day 15-23 and $p < 0.05$ day 24-26). In the O₂ group, the body weight loss was significant from day 15 to day 19 ($p < 0.05$ day 15 and day 19 and $p < 0.01$ day 16-18). The weight loss in the sham group was statistically not significant. On day 30, twelve days after the therapy was finished, the mean body weight of the rabbits in the different groups did not differ. For improved optical clarity, the error bars (standard deviation) are plotted only in one direction. The treatment period is marked with a grey box.

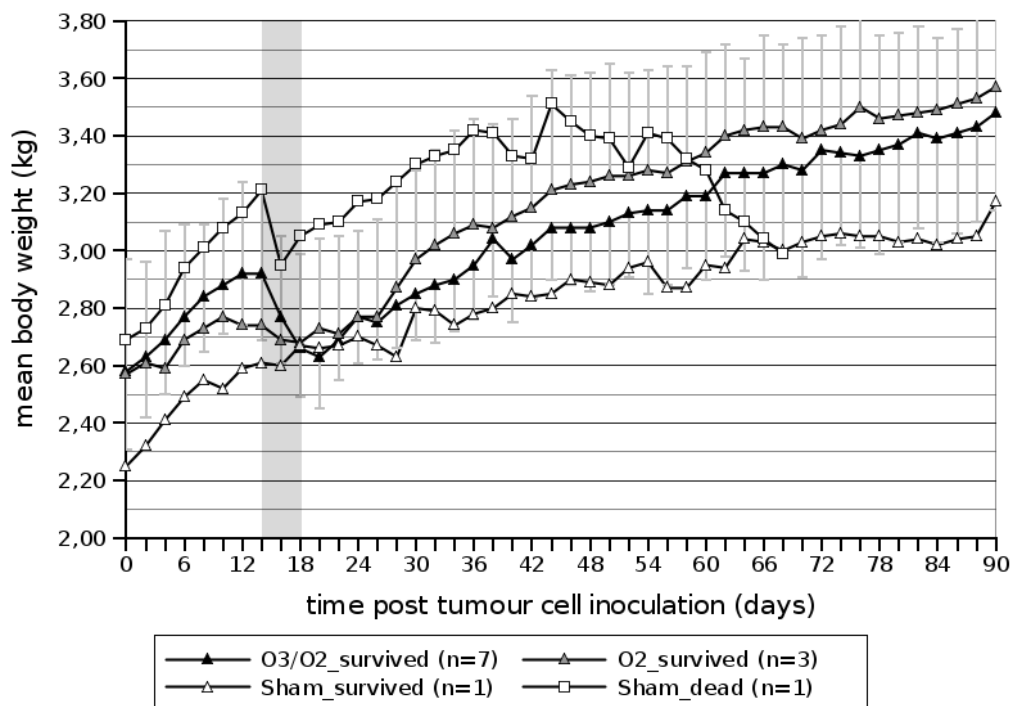


Figure 4.6 – Body weight. Development of the mean body weight of all animals that survived until day 90.

The mean body weight in all three treatment groups was constantly increasing from day 19 (the first day after the end of the treatment period) to day 90 after tumour induction, indicating that the animals were in good health condition. For improved optical clarity, the error bars (standard deviation) are plotted only in one direction. The treatment period is marked with a grey box. The development of the body weight of one sham-treated animal that was sacrificed on day 68 due to a body weight loss >15% is shown for comparison in this graph. At autopsy, numerous metastases in the lung were detected in that rabbit.

contrast, tumour-induced cachexia occurred in the late phase of the experiment in several animals of the non-survival group with progressive tumour growth. A rapid or consistent weight loss above 15-20% of the maximal body weight was a defined end point of the study. Van Es et al. found, that animals with a weight-loss of about 11.7% to 22.3% regularly had advanced pulmonary metastases (89). The development of the body weight of one sham-treated animal with severe disease is depicted in Figure 4.6.

4.1.5 Haematological and clinical chemistry blood parameters

To test for adverse effects of the O₃/O₂ therapy, arterial blood samples were taken on day 14 (before the beginning of the treatment period) and on day 19 (one day after the last treatment). To test for late adverse effects of the repetitive O₃/O₂-pneumoperitoneum, additional blood samples of the six animals with complete tumour remission were taken on day 90 after tumour cell inoculation (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 $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$ in Figure 4.7 and Table 4.3, which summarizes the results. Measurement of the blood

Table 4.3 – Effect of repetitive O₃/O₂-PP, O₂-PP and sham treatment on standard laboratory blood parameters.

	O ₃ /O ₂ (n=14)		O ₂ (n=13)		Sham (n=14)		O ₃ /O ₂	reference values (85)
	d14	d19	d14	d19	d14	d19	d90	
WBC	8.6	11.4 ^{***}	8.5	10.6 [*]	8.6	10.7	7.6	2.5-9.8 (10 ³ /mm ³)
GRA	3.4	5.5 ^{***}	3.1	4.8 [*]	3.5	4.9 [*]	1.8	1.6-3.7 (10 ³ /mm ³)
LYM	4.9	5.7	5.3	5.4	4.9	5.6 [*]	5.6	3.3-7.0 (10 ³ /mm ³)
MO	0.2	0.3 [*]	0.2	0.3	0.2	0.3	0.1	0.0-0.4 (10 ³ /mm ³)
RBC	5.85	5.55	5.68	5.31	5.64	5.59	5.91	5.20-6.80 (10 ⁶ /mm ³)
HGB	11.7	11.6	12.5	11.1	10.0	11.5	12.9	9.8-14.0 (g/dl)
HCT	38.4	36.4	39.0	38.9	35.8	36.1	40.2	36.0-47.0 (%)
CREA	0.736	0.863 ^{**}	0.743	0.935	0.787	0.800	0.848	0.5-2.6 (mg/dl)
GOT	17.39	13.73	14.20	8.94	15.15	13.72	29.72	8.0-56.0 (U/l)
GPT	34.7	27.3	34.7	21.6	22.9	21.0	74.9	18.0-123.0 (U/l)

Significant changes are marked with yellow colour.

parameters on day 19 (24 hrs after the last gas insufflation or sham treatment) revealed a statistically significant increase of the WBC count in the O₃/O₂ group ($p < 0.001^{***}$) and in the O₂ group ($p < 0.05^*$) compared with the WBC count on day 14. The increase of the WBC count in sham treated rabbits was statistically not significant. The increase of the WBC count was rather caused by an increase of the GRA count than by an increase of the LYM or MO count. The increase of the GRA count was statistically significant in all three treatment groups ($p < 0.001^{***}$ in the O₃/O₂ group and $p < 0.05^*$ in the O₂ group and the sham group). The O₃/O₂-PP

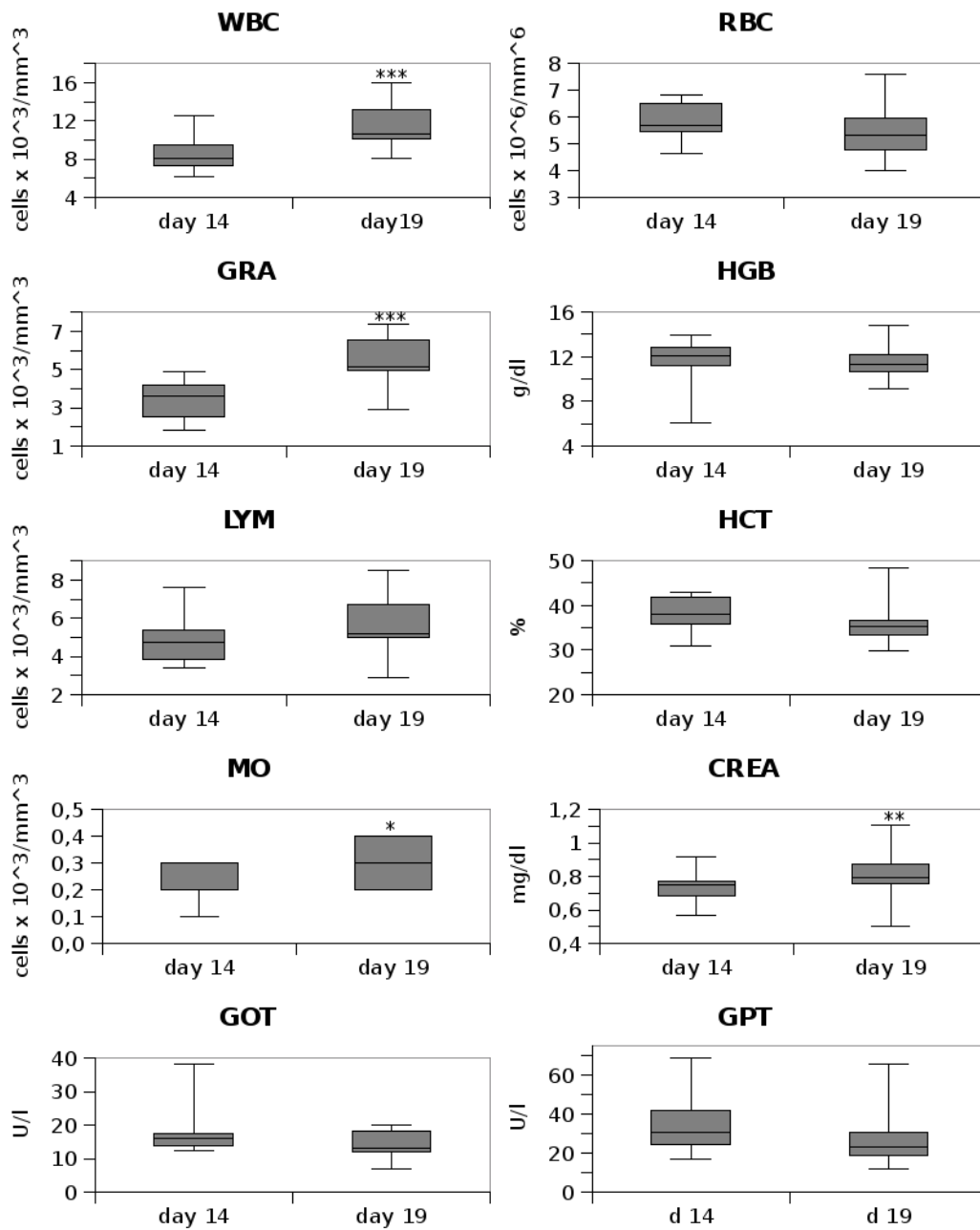


Figure 4.7 – Effect of repetitive O₃/O₂-PP on standard laboratory blood parameters. Shown are selectively the blood parameters of the O₃/O₂ group. Statistic differences between day 14 and day 19 were calculated with the paired Student's t-test and significant differences are marked with asterisks ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$). Abbreviations: WBC, white blood cells; GRA, granulocytes; LYM, lymphocytes; MO, monocytes; RBC, red blood cells; HGB, haemoglobin; HCT, haematocrit; CREA, creatinine; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase.

and the O₂-PP had no significant influence on the RBC count, HGB values and on HCT values. The values of the liver function parameters (GOT and GPT) and CREA values remained within the physiological range. Nevertheless, there was a statistically significant increase in the CREA values of the O₃/O₂ treated rabbits ($p < 0.01^{**}$) on day 19 compared with the values on day 14.

On day 90 after tumour cell inoculation, all measured arterial blood parameters of the six O₃/O₂ treated animals with complete tumour remission were within the physiological range. The slightly increased values of the WBC count and the GRA count observed on day 19, had fallen to lower values in the interim time.

4.1.6 Blood gas analysis

In some animals, arterial blood gas analysis was performed in addition to haematological and clinical chemistry investigations. Arterial blood gas analysis was done on day 14 (before the beginning of the treatment period) and on day 19 (24 hr after the last treatment). The results are summarized in Table 4.4 and Figure 4.8.

Table 4.4 – Effect of repetitive O₃/O₂-PP, O₂-PP and sham treatment on arterial pH, pO₂ and pCO₂ values

	O ₃ /O ₂ (n=9)		O ₂ (n=11)		Sham (n=11)	
	d14	d19	d14	d19	d14	d19
pH	7.462	7.478	7.460	7.500	7.451	7.464
pO ₂	79.0	72.3	77.8	75.7	80.6	77.3
pCO ₂	37.6	35.2	35.5	33.3	36.9	37.1

Repetitive O₃/O₂-PP, O₂-PP or sham treatment caused no statistically significant changes of arterial pH, pO₂ and pCO₂ values (calculated with the paired Student's t-test in each experimental group).

4.1.7 Microbiological analysis of infected primary tumours

To prevent rabbits from early death due to severe local infection at the primary tumour site or massive bleeding in the course of the primary tumour development ablation of the ear was performed under anaesthesia. The time points at which the ear resections were carried out have already been given in the subsection “survival analysis”. Clinically, signs of a local infection (redness, swelling, foul odour, heat to

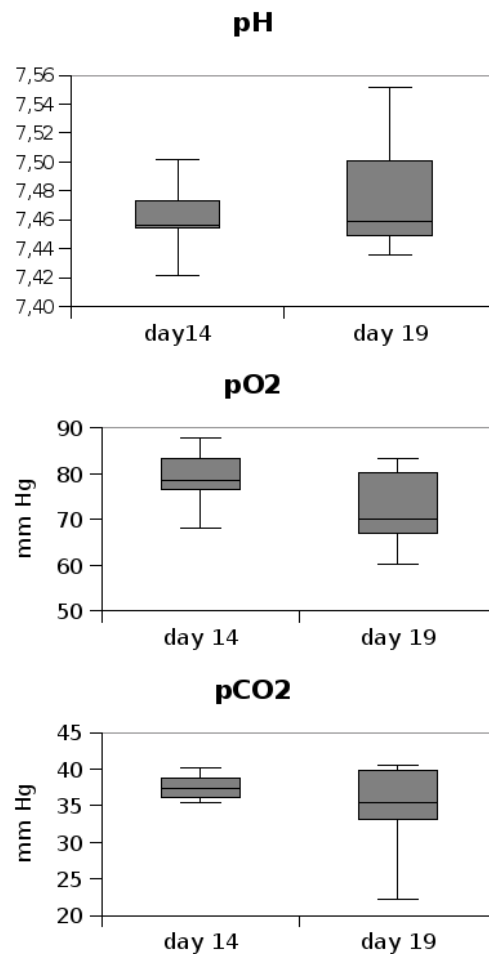


Figure 4.8 – Effect of repetitive O₃/O₂-PP on arterial blood gas parameters. Shown are selectively the blood gas parameters of the O₃/O₂ therapy group. Statistic differences between day 14 and day 19 were calculated with the paired Student's t-test. No significant differences were observed.

the site) of the tumour-bearing auricle were found in all animals before the resection was performed. The following microorganisms were found in smears of the infected necrotic tumour tissue: *Enterobacter cloacae*, *Enterococcus faecalis*, *Escherichia coli*, *Kluyvera ascorbata*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Except for *Kluyvera ascorbata*, all these bacteria are found in the normal intestinal flora of healthy rabbits and dogs (17). *Kluyvera ascorbata* is seen as an infrequent, probably opportunistic pathogen in humans, which was isolated in human sputum, urine and stool (34). *Pasteurella multocida*, the most common bacterial pathogen of rabbits, wasn't isolated at all (85).

4.2 Findings at autopsy

4.2.1 Lymph nodes

Lymphogenic metastatic spread of the VX2 auricle carcinoma frequently occurs to the parotid lymph node and, in the course of the disease, to the caudal mandibular lymph nodes (31). Normally, no metastatic spread to the third draining lymph node station of the auricle (rostral mandibular lymph nodes) is found (31). For all animals that were sacrificed or died spontaneously, an autopsy was carried out in a standard manner, as described in the chapter “Material and methods”.

All animals of the O₃/O₂ gas therapy group with complete tumour remission at the end of the observation period were included in a consecutive immune suppression study. At the end of this study, they were sacrificed as well and an autopsy was carried out. As described later, in all three animals that received the tumour cell re-inoculation without concomitant immune suppression tumour induction failed. Therefore, development of lymph node metastases due to re-inoculation of the tumour cell suspension seems very unlikely. Comparison of the weight of the lymph nodes of the right (ipsilateral to the auricular tumour) and left (contralateral to the auricular tumour) neck side in these animals can thus give information on lymphogenic metastatic spread of the initial right auricular tumour. In those cured animals with tumour re-induction without concomitant immune suppression (n = 3), the mean weight of the parotid lymph nodes of the right neck side was 0.109 ± 0.042 g. The difference to the mean weight of the parotid lymph nodes of the left neck side (0.180 ± 0.069 g) was not statistically significant ($p = 0.203$, unpaired Student’s t-test). The mean weight of the caudal mandibular lymph nodes of the right neck side, 0.046 ± 0.018 g, was also not significantly different from that of the left neck side, 0.083 ± 0.015 g ($p = 0.053$). Thus, comparison of the mean weight of the draining lymph nodes of the initially tumour-bearing right side with the mean weight of the draining lymph nodes of the contralateral side, revealed no difference in animals of the O₃/O₂ gas therapy group with complete tumour remission.

All rabbits that were not cured, had an enlarged right parotid lymph node (ipsilateral to the tumour) compared with the left parotid lymph node (contralateral to the tumour) at autopsy. The exact weights of the parotid lymph nodes of both neck sides are given in Table 4.5. The weight of the enlarged right parotid lymph nodes ranged from 0.9 g in a surviving O₂-treated animal to 195.1 g in a sham-treated

animal that was sacrificed on day 86 after tumour induction. For comparison, the mean weight of all contralateral parotid lymph nodes listed in the table ($n = 30$) was 0.113 ± 0.071 g. Figure 4.9 shows representative macroscopic views of lymph nodes of the right neck side from an animal of the sham group which succumbed to the VX2 tumour.

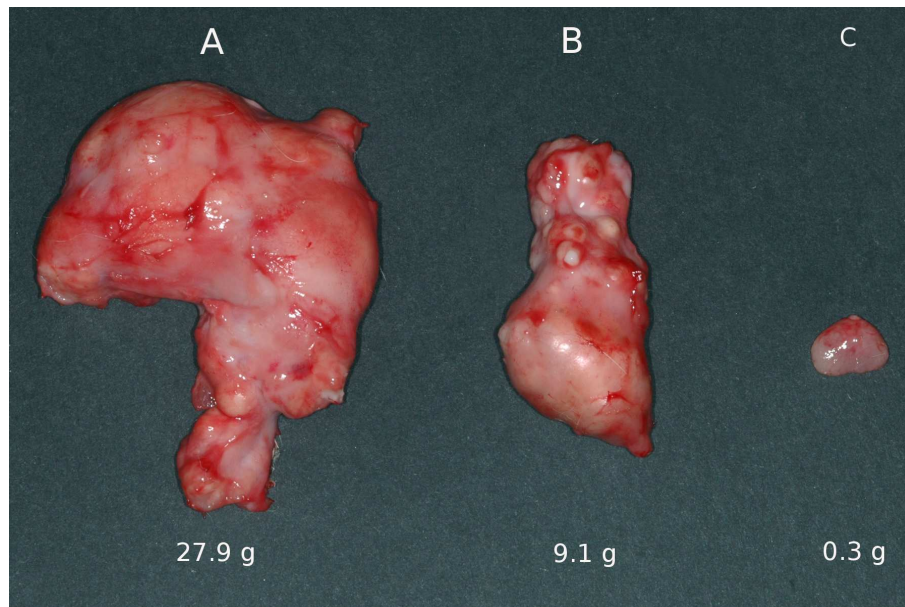


Figure 4.9 – VX2 tumour derived lymph node metastases.

Typical macroscopic views of enlarged and metastatic lymph nodes of the right neck side in an animal of the sham group. The parotid lymph nodes (A) often were remarkably enlarged, the caudal mandibular lymph nodes (B) sometimes were enlarged, the rostral mandibular lymph nodes (C) are normally not affected. Note, that the parotid lymph nodes and the caudal mandibular lymph nodes are normally only slightly larger than the rostral mandibular lymph nodes.

4.2.2 Lungs

Haematogeneous metastatic spread to the lungs represents a characteristic feature of the VX2 auricle cancer (32). Therefore, the thoracic cavity was examined for the presence of macroscopic metastases. Complete thorax scans using CT were performed for all rabbits with complete remission of the primary tumour at the end of the observation period (day 90 after tumour cell inoculation). Typical results of the macroscopic examination and of CT scans are shown in Figure 4.10.

Animals with complete tumour remission in all three treatment groups were free

Table 4.5 – Weight of parotid lymph nodes and lung metastases at autopsy

no.	autopsy (d)	Parotid LN ipsilateral (g)	Parotid LN contralateral (g)	Metastases lungs
<i>O₃/O₂ survived</i>				
10	90	92.5	–	no
<i>O₃/O₂ ablation</i>				
8	90	130.7	0.108	no
13	90	18.8	0.038	no
18	90	41.6	0.048	no
28	90	50.4	0.065	no
<i>O₃/O₂ dead</i>				
26	58	102.4	0.022	yes
30	41	2.9	0.201	no
31	72	68.3	0.100	yes
<i>O₂ survived</i>				
42	90	0.9	0.118	no
<i>O₂ ablation</i>				
35	90	190.4	0.177	yes
43	90	23.4	0.264	no
45	90	78.3	0.188	no
46	90	86.2	0.224	yes
<i>O₂ dead</i>				
21	39	8.1	0.065	yes
25	70	21.9	0.034	no
37	26	1.7	0.049	no
39	73	46.4	0.071	yes
40	46	43.1	0.253	no
44	80	43.5	0.209	no
<i>Sham ablation</i>				
17	90	67.3	0.067	yes
34	90	137.0	0.081	no
<i>Sham dead</i>				
2	55	27.9	0.143	yes
4	68	178.7	0.133	yes
9	86	195.1	–	yes
12	71	94.9	0.031	yes
20	55	46.1	0.174	no
22	55	87.2	0.066	yes
24	75	53.9	0.144	no
27	75	3.7	0.083	yes
29	55	60.1	0.061	yes
32	81	134.3	0.136	yes
36	35	2.4	0.028	no



Figure 4.10 – VX2 tumour derived distant lung metastases.

CT scans of the thorax showing no detectable lung metastases in a rabbit of the O_3/O_2 group with complete tumour remission (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 tumour 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).

of lung metastases on CT scans. Of the other animals, in 2/8 (25.0%) of the O_3/O_2 group, 4/11 (36.4%) of the O_2 group and 9/13 (69.2%) of the sham group pulmonary metastases were present on macroscopic examination (Table 4.5; no macroscopic

lung metastases - cell marked with green colour, macroscopic lung metastases - cell marked with red colour).

O₃/O₂ group

The one animal that survived but still beared a large tumour mass (O₃/O₂ survived), was free of lung metastases. Those four animals which received an ear resection and then survived until day 90 (O₃/O₂ ablation) were free of lung metastases as well. The animal that died spontaneously (O₃/O₂ dead) had multiple lung metastases at autopsy. Of the two animals that were sacrificed (O₃/O₂ dead) before day 90 after tumour cell inoculation, one had macroscopic lung metastases while the other showed a large lung abscess (*Staphylococcus aureus* was isolated) but no metastases.

O₂ group

The one animal that survived but still beared a tumour mass (O₂ survived) was free of lung metastases. Of those four animals that survived but had an ear ablation before(O₂ ablation), 2 had macroscopic lung metastases at autopsy. None of the three animals that died spontaneously (O₂ dead) had macroscopically visible lung metastases. In two of them infection of the lungs was found (in one animal *Klebsiella pneumoniae* and in the other *Staphylococcus aureus* was isolated). Of the three animals that were sacrificed before day 90 post tumour induction (O₂ dead), two had macroscopic lung metastases.

Sham group

Of those two animals that survived but had an ear ablation before (Sham ablation), one had macroscopic lung metastases, the other one was free of lung metastases. The one animal that died spontaneously (Sham dead) had no lung metastases. Of the ten animals that were sacrificed before day 90 post tumour induction (Sham dead), 8 had macroscopic lung metastases. One of the two other animals had a lung abscess due to infection with *Staphylococcus aureus*.

Comparison of some aspects of lung metastases

The number of unique tumour nodules per animal (both lungs) was 4 and 10 in the O₃/O₂ group, varied from 2 to 50 in the O₂ group and from 1 to 70 in the

sham group. To quantify the tumour mass, the weight of the lung of each animal was measured. The mean weight of all metastatic lungs was 20.15 ± 6.15 g in the O_3/O_2 group ($n = 2$), 54.43 ± 37.05 g in the O_2 group ($n = 4$) and 32.96 ± 31.62 g in the sham group ($n = 9$). For comparison, the mean weight of all lungs with no metastases was 12.34 ± 3.47 g.

4.2.3 Abdominal cavity

As described above, the O_3/O_2 gas mixture or the O_2 gas were insufflated in the abdominal cavity. Therefore, special regard was pointed to the inspection of the abdominal cavity to search for adverse effects of the pneumoperitoneum. In all cases, the abdominal organs were easy moveable and no adhesions, scars or signs of inflammation could be detected.

4.3 Consecutive immune suppression study

Van Es et al. made the observation that reimplanted VX2 tumour cells were rejected in rabbits with tumour remission after perilesional treatment with IL-2 (88). To test whether reimplanted VX2 tumour cells are also rejected in rabbits with tumour remission after O_3/O_2 -PP, we performed a consecutive study with the six cured animals of the O_3/O_2 group. The six animals were randomly divided into two groups. Rabbits in the first group ($n = 3$) were immune-suppressed with Dex and CSA as described above. Rabbits in the second group were sham-treated ($n = 3$), receiving no immunosuppressants. All animals received a bi-auricular injection of the VX2 tumour cell suspension. The development of the tumours was observed for 90 days. The results are summarized in Table 4.6. As expected, all sham-treated animals were protected for tumour retake. In sharp contrast, immune suppressed animals developed tumours in 4 out of 6 tumour cell reinoculations. Tumour growth and size in these animals did not show any difference to the auricular tumours previously measured in immune competent rabbits of the sham group.

Table 4.6 – Retake-rate of auricular VX2 tumours

group	animals (n)	tumours (n)
O ₂ /O ₃ -rem + Dex/CSA	3	4/6
O ₂ /O ₃ -rem + sham	3	0/6
Control + Dex/CSA	1	1/2
Control + sham	1	2/2

Abbreviations: rem, remission; Dex, dexamethasone; CSA, cyclosporin A

5 Discussion

5.1 General study results: survival, growth and regression of the primary tumour, metastatic spread

The aim of the study was to test if intraperitoneal application of a medical O₃/O₂ gas mixture (O₃/O₂-PP) could represent an effective antitumour approach as proposed after preliminary observations in NZW rabbits bearing the VX2 carcinoma (4). Survival analysis showed that the repetitive intraperitoneal application of the O₃/O₂ gas mixture significantly increased the 3-month survival rate of VX2 tumour-bearing NZW rabbits compared with the 3-month survival probability of sham-treated rabbits. Furthermore, it could be denoted that complete remission of the VX2 auricle carcinoma after repetitive O₃/O₂-PP occurred at a significantly higher rate than spontaneous complete remission in sham-treated animals.

It is of importance to distinguish true remissions from so-called “non-takes”. In some cases this can cause difficulties, since a reactive swelling at the inoculation site always occurs in the first week after tumour cell transplantation (89). Van Es et al. investigated this problem for the VX2 auricle carcinoma and reported the occurrence of spontaneous tumour regressions in 22% (16/72) of the transplantations, but they regarded all of them as “non-takes” (89). These tumours/reactive swellings reached a mean maximum size of 0.8 cm² ten days after tumour cell inoculation. Progressive tumour growth was observed for the remaining 78% (56/72) of transplantations and the occurrence of spontaneous true remissions at a later point in time was not reported. These data suggest that the occurrence of spontaneous true remissions of the VX2 auricle carcinoma is a rare event. In our study the maximum size of the six tumours in the O₃/O₂ group that went into remission ranged from 3.0 cm² to 8.6 cm², the mean time after tumour induction at which they reached the maximum size was 21 days (range 16 - 26 days). Thus, the size of these tumours by far exceeded

the size that was found for the reactive swellings in “non-takes” and all tumour remissions in O₃/O₂-treated animals can be regarded as true remissions. The same is true for the tumour of the sham-treated animal that reached a maximum size of 4.1 cm² at day 23 after tumour cell inoculation.

The literature does not contain much information about the frequency of spontaneous true remissions of the VX2 auricle carcinoma of untreated NZW rabbits. A spontaneous remission rate of 41% (9/22) was found, if tumour pieces instead of tumour cell suspensions were used for tumour induction (89). These tumours had a mean maximum surface area of 2.4 cm² 21 days after the tumour induction. Despite the fact that the point in time at which these tumours went into regression was similar to that found in our experiment, comparison of remission rates makes little sense, since the tumours in our study were remarkably larger before they went into regression. Thus, as described previously, it can be expected that spontaneous true remissions occur infrequent and that the high remission rate of the primary auricular tumour must be explained by a specific therapeutic effect of the intraperitoneally insufflated medical O₃/O₂ gas mixture.

The VX2 auricle carcinoma is characterized by initial lymphogenic metastatic spread, similar to human HNSCC (32). At the end of the observation period on day 90 after tumour cell inoculation the parotid lymph node (sentinel lymph node) was not palpable in five of the six animals of the O₃/O₂ group with complete regression of the primary tumour. The sixth animal still had a very small nodule, which could be palpated at the anatomic site of the right parotid lymph node. These results raise the question, whether the O₃/O₂ therapy prevented metastatic spread of the VX2 auricle carcinoma or, whether already existing metastases at the time of the treatment were cleared by the O₃/O₂ therapy. Clinically, palpation at day 14 after tumour induction prior to the first gas insufflation revealed an enlarged ipsilateral parotid lymph node in all rabbits. This enlargement might be due to metastatic spread to the lymph node or to reactive processes. Dünne et al. found a single solid metastasis in 62.5% of histologically investigated ipsilateral parotid lymph nodes on day 14 after tumour cell inoculation into the subcutis of the auricle (31). In an other experiment conducted by the same authors, 100% of the rabbits had an ipsilateral parotid lymph node metastasis on day 18 after inoculation of the tumour cell suspension (32). Metastatic spread to the ipsilateral caudal mandibular lymph nodes, which represent the secondary lymph node station, was found 28 days after tumour induction (31; 32). Thus, the existence of ipsilateral parotid lymph node

metastases in at least some of the animals during the therapeutical O₃/O₂ sessions from day 14 to day 18 is highly likely. Haematogeneous metastatic spread to the lungs generally occurs later in this tumour model (32). All six animals of the O₃/O₂ group with complete remission of the auricular tumour were free of detectable lung metastases on CT scans, which were performed after the end of the observation period. Previous experiments with the VX2 auricle carcinoma found tumour cells in pulmonary vessels 32 days after tumour induction without detection of solid pulmonary metastases (32). Van Es et al. found lung metastases in 31% (5 of 16) of rabbits that were sacrificed at different time points before day 28 after tumour induction (89). A small metastasis was found in the lung of one rabbit that was sacrificed 10 days after tumour induction. Early pulmonary metastases are often due to seeding of the tumour cells at implantation (89). Thus, the presence of early lung metastases in few rabbits during the O₃/O₂ sessions is possible, but it can be expected that the majority of animals at that time point was free of lung metastases. In summary, these observations indicate that the O₃/O₂ therapy is not only able to prevent but also to clear lymph node and possibly distant metastases.

Interestingly, despite the fact that the ipsilateral parotid lymph node at autopsy was enlarged in all O₃/O₂-treated animals that were not cured, only a relatively small percentage of them (2 out of 8, 25.0%) compared with a high percentage of sham treated animals (9 out of 13, 69.2%) also had macroscopically detectable lung metastases. Since 90% of human cancer deaths are caused by distant metastases it would be of high benefit, if O₃/O₂-PP could inhibit haematogeneous metastatic spread of cancer cells (84). Whether this is really the case cannot be reliably answered by our experiments, because we did not carry out a histologic evaluation of enlarged parotid lymph nodes.

The insufflated O₃/O₂ gas mixture was composed of 97.5% oxygen and 2.5% ozone. Therefore, we used a second control group of rabbits which received intraperitoneal insufflations of pure oxygen. As expected, the insufflation of pure oxygen did not significantly increase the 3-month survival probability and the complete remission rate compared with sham treatment. Unlike our expectations, the difference of the 3-month survival probability and the complete remission rates between the O₃/O₂-treated group and the O₂-treated group was also not statistically significant. The corresponding *p*-values however suggest that the insufflation of the medical O₃/O₂ gas mixture is more efficient than that of pure medical O₂. The two tumours of O₂-treated animals that remitted completely had a maximum size of 5.2 cm² (day

36) and 6.5 cm^2 (day 24) and then decreased in size more slowly than those tumours of the O_3/O_2 -treated animals. Full remission was observed at day 68 and day 85 after tumour cell inoculation, which is by far later than the observed full remission in O_3/O_2 -treated animals. A possible explanation for these findings might be that pure oxygen has some antitumourous effects, but only the combination with ozone is potent enough to cause a statistically significant effect. Thus, the small O_3 fraction seems to be a crucial factor. In this context it might be of importance that the mean tumour surface area A of the O_2 -treated animals was significantly smaller than that of the two other groups during the treatment period. Many factors influence the initial tumour growth, for example aggressiveness of tumour cells, quantity of inoculated tumour cells and immune status of the host. The exact cause of this observation in our experiment remains unclear.

5.2 Safety of the O_3/O_2 therapy

Since the O_3/O_2 -PP method is a new therapeutic approach it is of high importance to analyze possible adverse effects. The here used Medozon^{IP} gas processor has an integrated intraabdominal pressure control system (IAPC), which allows measurement of the IAP during and after the insufflation process. 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 (63).

Each insufflation process lasted approximately two to three minutes. Possible acute hyperalgesic effects of the intraperitoneally insufflated O_3/O_2 gas mixture could not be observed since the insufflation was performed in deep short narcosis. Nevertheless it must be emphasized that Zorraquin et al. described acute pain reactions after intraperitoneal insufflation of an O_3/O_2 gas mixture in humans when no analgesics are given (101). The rabbits awoke from narcosis after six to eight minutes and thereafter showed no detectable signs of pain.

We found a slight but significant reduction in body weight during and shortly after the therapeutic treatment, which was a little more pronounced in the O_3/O_2 -treated group than in the O_2 -treated group. Similarly, a significant immediate weight loss up to 14% was found in mice and rats after inhalation of ozone (54). One day after the last treatment the mean body weight already began to increase, reaching its initial value eleven days later. Thus, the body weight loss might represent a mild transient

adverse effect. The observed transient weight loss could be the result of a decreased food uptake, an influence on the fat metabolism or some unnoticed intraabdominal pain reactions caused by the insufflated ozone and/or oxygen. Probably the O₃/O₂-PP had no late adverse effects on the general health condition of NZW rabbits, since the body weight of all cured rabbits constantly increased until the end of the experiment.

The blood tests were unsuggestive of severe side effects of the O₃/O₂ therapy. A mild leukocytosis, predominantly due to granulocytosis, was found one day after the last treatment in all three treatment groups, but it was a little more pronounced in the O₃/O₂ group. These results are in line with findings by Bette et al. who found an increase in the number of circulating WBC (predominantly GRA and LYM) in rats after repetitive O₃/O₂-PP (8). In cured animals WBC counts had fallen to normal values prior to the end of the experiment. Thus, leukocytosis also was a transient event after the O₃/O₂ gas insufflation. O₃/O₂-PP had no detectable effect on the RBC count and on liver enzymes. Significantly increased CREA values on day 19 were still within the physiological range and possibly caused by hypovolaemia due to decreased fluid intake. An effect on blood gas parameters could not be detected. Clearly, the measurements cannot be used to describe very early occurring changes of blood gas parameters after O₃/O₂ therapy, since they were performed not earlier than 24 hours after the last gas insufflation. All measured blood parameters of cured animals of the O₃/O₂ group were within the physiological range after the end of the observation period.

Since ozone is toxic to the lungs in a concentration-dependent manner, one could expect that ozone is also toxic to the membranes within the abdominal cavity (55). However, at inspection of the abdominal cavity no abnormalities were detected.

Taken together, since no severe side effects such as sustained reduction in body weight, gas embolism, fever, diarrhoea, peritonitis or obvious pain reactions were observed, it appears that the O₃/O₂ therapy is relatively safe. Late adverse effects of the O₃/O₂-PP are rather unlikely, since two rabbits from a previous pilot study by Dr. S. Schulz (Veterinary Service and Laboratory Animal Medicine, Philipps University Marburg, Germany) developed no clinical signs after more than 6 years of tumour remission (data not shown).

5.3 Former studies evaluating an effect of ozone on cancer cells *in vitro*, in animal studies and in humans

There are few studies that evaluate an effect of ozone on isolated cancer cells or on cancers in animals and humans. Sachsenmaier et al. compared the effects of an O_3/O_2 gas mixture and of X-rays on mouse ascites tumour cells in aqueous suspension (73). Depending on the exposure time and thus, on the ozone dosage, mouse ascites tumour cells in suspension were destroyed and lost their tumour inducing capacity, when they were brought in direct contact to the O_3/O_2 gas mixture. The main attack point was the cell membrane. The effect of irradiation with X-rays had only small similarity with the ozone effect. The destruction of the cell membrane appeared much later and seemed to be less important. Intracellular effects seem to play a key role in the cellular damage caused by X-rays. There is some evidence from further *in vitro* studies that exposure to ozonated air can selectively inhibit the proliferation of human cancer cells (87). Sweet et al. found a dose-dependent growth inhibition of cancer cells of human lung adenocarcinoma, breast adenocarcinoma, uterine carcinosarcoma and endometrial carcinoma after incubation of the cell cultures with ozonated ambient air at ozone concentrations of 0.3, 0.5 and 0.8 ppm for eight days, while the growth of human fibroblasts was not (0.3 and 0.5 ppm) or only moderately inhibited (0.8 ppm) (87). These authors concluded that cancer cells must be more sensitive to oxidative stress than normal cells. These results were confirmed by Karlic et al. (50). They incubated human cancer cell cultures (in liquid medium) of an ovarian adenocarcinoma and an endometrial adenocarcinoma and human fibroblasts for two hours with an O_3/O_2 gas mixture at ozone concentrations of 0.03, 0.1 and 0.3 ppm. Only the growth of the ovarian cancer cells was selectively inhibited (at 0.03 ppm) or they were destroyed (at 0.1 and 0.3 ppm) after incubation with the O_3/O_2 gas mixture. Furthermore, they observed a possible additive effect of ozone and ionizing radiation on tumour growth inhibition. Pre-incubation of the endometrial cancer cells with the O_3/O_2 gas mixture at an ozone concentration of 0.1 ppm for 2 hours one week prior to irradiation with the gamma emitter Ra^{226} resulted in growth inhibition of the tumour cells after irradiation, while exposure to the O_3/O_2 gas mixture or irradiation alone had no inhibitory effect on the growth of these cells. These authors concluded that ozone can selectively inhibit the

growth of some cancer cells and that ozone must have radiosensitizing properties. This issue was evaluated before by Grundner et al. (38). They incubated different amounts of an O₃/O₂ gas mixture with an ozone concentration of 140 µg/ml with Ehrlich-ascites cancer cells in an aqueous suspension. Irradiation with X-rays (2040 R) was performed directly before, during or after the ozone-incubation. In all three situations, the exposure to the O₃/O₂ gas mixture had a dose-dependent negative effect on the reproductive integrity of the cancer cells. Exposure to the O₃/O₂ gas mixture after irradiation had a stronger inhibitory effect on the reproductive potential of mouse ascites tumour cells than ozone pre-treatment followed by irradiation. These authors concluded that the co-operation of two mechanisms of which each alone has toxic effects must have caused these results. Recently, Cannizzaro et al. gave some insight in the mechanism by which ozone possibly causes inhibition of cell growth or cell death in two human neuroblastoma cell lines.(23). In SK-N-SH cells (human neuroblastoma cell line), they observed an arrest of the cells in the G2-phase of the cell cycle, probably due to an inactivation of the cyclin B1/cdk1 complex which is critical for the cell cycle progression. In the SK-N-DZ cells (human neuroblastoma cell line) increased apoptosis via caspase 3 activation was found. Furthermore, they found that exposure to an O₃/O₂ gas mixture combined with the application of chemotherapeutic agents cisplatin and etoposide had an additive effect to chemotherapy resulting in stronger cell growth inhibition *in vitro*. Zänker et al. had already demonstrated a synergistic effect of ozone and 5-fluorouracil on human cancer cells *in vitro*. In this study 5-fluorouracil resistant human cancer cell lines became susceptible to the chemotherapeutic agent after incubation with ozone at a concentration of less than 1 ppm (102). These results from *in vitro* studies are hardly comparable with the results of our study, since a direct contact of ozone and the tumour cells is highly unlikely with our experimental design (as will be described later).

Up to now, only few animal studies evaluating a possible antitumoural effect of ozone exist. Grundner et al. carried out intravenous injections of a small amount of an O₃/O₂ gas mixture in Naval Medical Research Institute (NMRI) albino mice bearing Crocker-sarcoma 180 or Ehrlich-ascites tumour (39). After 5 intravenous injections in a time period of 9 days (each time 0.05 ml O₃/O₂ gas mixture with an ozone concentration of 140 µg/ml), there was a tendency towards a higher cure rate in Crocker-sarcoma bearing mice but this was statistically not significant. There also was a tendency towards higher cure rates in Crocker-sarcoma 180 bearing mice

when the i.v. injections of the O₃/O₂ gas mixture were performed prior to or after irradiation with X-rays compared with irradiation alone, but again this was statistically not significant. The Crocker-sarcomas of the animals treated with the O₃/O₂ gas mixture alone were significantly smaller than the tumours in control animals one and seven days after the end of the treatment period. However, in a subsequent experiment the results were not reproducible. Only a tendency towards (not statistically significant) smaller tumour sizes was seen in animals receiving the O₃/O₂ gas mixture injections after irradiation compared with animals that were only irradiated. In the experiments with the Ehrlich-ascites tumour no effect of O₃/O₂ gas mixture alone or with concomitant irradiation was observed. These authors concluded that the lack of a clear antitumoural and radiosensitizing effect in these experiments might be explained by the fact that the injected gas volume was very small. The mice in these experiments received a single dosage of about 0.3 µg ozone/g body weight (25 g body weight, 0.05 ml O₃/O₂ gas mixture per animal, 140 µg/ml ozone concentration) while rabbits in our experiment received a single dosage of 4 µg ozone/g body weight (80 ml/kg body weight O₃/O₂ gas mixture, ozone concentration 50 µg/ml). The same researchers conducted a consecutive animal experiment in which they brought the O₃/O₂ gas mixture in direct contact to the tumour tissue *in vivo* (40). NMRI mice bearing an intraperitoneal Ehrlich-ascites tumour were treated with intraperitoneal injections of 3.0 ml of an O₃/O₂ gas mixture with an ozone concentration of 140 µg/ml for 4 consecutive days. When the tumour take-rate after transplantation of the ascites at the end of the treatment to other mice was assessed, a small but statistically not significant delay of tumour growth was noted. A further delay and a lower take-rate was observed, when these animals were irradiated prior to insufflation of the O₃/O₂ gas mixture, but compared with the corresponding control group it was not statistically significant. The greatest delay and the lowest take-rate could be observed for tumour ascites of animals, that were irradiated after the injection of the O₃/O₂ gas mixture, but there was no statistic significance compared with the group receiving pure oxygen before the irradiation. In another group of mice the tumour cells were implanted in a subcutaneous air bag and 0.5 ml of an O₃/O₂ gas mixture with an ozone concentration of 140 µg/ml was injected directly into the air bag daily on five consecutive days. When these tumours grew they developed a small solid and a fluid phase. The amount of the produced fluid was measured. Injection of the O₃/O₂ gas mixture reduced the amount of fluid significantly compared with injection of pure oxygen. When the O₃/O₂ gas mixture

was given after local irradiation, the fluid volume was reduced significantly compared with animals receiving oxygen after irradiation. A statistically non-significant reduction in the measured fluid volume also was observed, when the O₃/O₂ gas mixture was given prior to irradiation, compared with the animals in which oxygen was given prior to irradiation. The results suggest a direct and radiosensitizing or radiomimetic effect of the O₃/O₂ gas mixture, but the authors are cautious, since the fluid volume produced by the solid tumour, that is free of tumour cells, is a very imprecise measurement for the activity of the tumour cells. Again, these observations are hardly comparable to our study, since direct contact of ozone and the cancer cells is highly unlikely to occur with our experimental design.

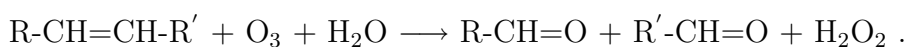
Controlled randomized clinical trials that demonstrate a clear antitumoural effect of ozone therapy in human cancer patients do not exist. Two pilot studies by Clavo et al. evaluated ozone therapy for human cancer treatment (26; 25). With the fact in mind that tumour hypoxia is a relevant cause of radioresistance, they evaluated the effect of ozone therapy on tumour oxygenation in patients with advanced head and neck cancers (25). Patients were treated three times with the major AHT. A volume of 200 ml fresh drawn whole blood was *ex vivo* exposed to an equal volume of an O₃/O₂ gas mixture with an ozone concentration of 60 µg/ml and then reinfused to the patient. The pO₂ value was measured in metastatic lymph nodes. Interestingly, metastatic lymph nodes which had initially the lowest pO₂ value showed the largest increase in oxygenation after the AHT sessions, while metastatic lymph nodes with initially high pO₂ values showed a decrease in oxygenation. The same researchers also compared the effect of ozone therapy and concurrent radiotherapy with chemoradiotherapy in patients with unresectable, locoregionally advanced and in some patients distant metastatic head and neck cancers (26). Ozone therapy was performed concurrently with radiotherapy, either with the above described technique of major AHT or by rectal insufflation of 300 ml of an O₃/O₂ gas mixture with an ozone concentration of 60 µg/ml. The patients in the chemoradiotherapy group received either induction chemotherapy with a combination of drugs (taxane, platinum, 5-FU) or concurrent chemoradiotherapy with taxane. Despite the fact that the patients in the ozone therapy group were significantly older and had more advanced disease stages, the median overall survival in both groups was not significantly different and there was even a tendency towards a longer median overall survival in the group which received the ozone therapy concurrently to the radiotherapy (8 months versus 6 months). These results indicate a possible additive effect

of the ozone therapy in human patients with advanced cancer.

5.4 Considerations on the effects of ozone therapy in general and possible antitumoural effects in particular

Ozone is a highly reactive oxidant gas, that reacts immediately when it comes in contact with biomolecules. The primary target molecules for ozone in biological systems are UFA, antioxidants like ascorbic and uric acid, thiol compounds like the amino acid cysteine, GSH, albumin, carbohydrates, enzymes, DNA and RNA (15).

Pryor et al. have discussed a cascade mechanism for the toxic action of ozone. They proposed that the reaction of ozone with primary target molecules produces secondary more stable products that react with secondary targets to produce tertiary products, and so on (67). In this view, the reaction of ozone with (P)UFA as primary target molecules in the lung lining fluid, the blood plasma or the cell membranes is central. The net reaction in the presence of water (reflecting the situation in human blood plasma, lung lining fluid, urine and so on) is given by



Thus, the reaction of one mole of (P)UFA with one mole of ozone formally leads to the formation of two moles of aldehydes (R-CH=O) and one mole of hydrogen peroxide (H₂O₂) (68). Other peroxidic substances like the Crigee ozonides and hydroxy hydroperoxides are only formed in very small amounts (less than 5%) under these conditions (68). Regarding the proposed cascade mechanism of ozone action, aldehydes and hydrogen peroxide are the secondary products that may react with secondary targets.

It is well known that surface-active phospholipid (SAPL) lines the peritoneum, providing a slippery and non-adhesive surface for smooth movement of the abdominal organs. Phosphatidylcholine (PC) is the major SAPL. Interestingly, the PC profile of the peritoneal surfactant is different from that of the pulmonary surfactant in humans and rats (24; 36). In the peritoneal surfactant of humans only unsaturated phosphatidylcholine (USPC) was found (e.g. palmitoyl-linoleoyl-phosphatidylcholine (PLPC), palmitoyl-oleoyl-phosphatidylcholine (POPC)). USPC means that the PC contains one or two UFA. In contrast, the major PC of the pul-

monary surfactant is a saturated phosphatidylcholine (SPC) containing no UFA. Regarding the high reactivity of ozone with UFA and PUFA, these USPC species which are among the first biomolecules that come into contact with the intraperitoneally insufflated ozone, are probably the main primary target molecules that ozone reacts with.

Pryor discussed the issue, how far ozone can penetrate into tissues without being consumed by reactive absorption (67). He used a calculational method (the Einstein-Smoluchowski equation) to estimate the amount of ozone that could pass through a lipid bilayer or the lung lining fluid layer (mucus and surfactant). According to his calculations, little or none of the ozone would penetrate the lung lining fluid layer without reacting with target molecules in this lung lining fluid layer (GSH, ascorbate, uric acid, UFA). This model leads to similar results, when applied for the ozone passage through a lipid bilayer. Only a small part or none of the ozone would reach the cytoplasm, a substantial fraction or all of the ozone would react with target molecules (UFA, thiols, tocopherol) in the bilayer. Transport of free ozone via the blood route and thus, the generation of reactive oxygen species (ROS) by free ozone at the distant tumour site appears not likely. Therefore, LOPs originated from the reaction of ozone with biomolecules at the peritoneal surface are most likely the messengers responsible for triggering the observed antitumoural effect in the VX2 model. Bocci, who extensively studied the effect of exposure of human whole blood *ex vivo* to an ozone/oxygen gas mixture, states that the major ozone messengers can be divided into two groups. On the one hand, immediately and short-acting messengers called ROS (including hydrogen peroxide) and on the other hand late and long-lasting messengers called LOPs (including aldehydes) (15). While the ROS have a lifetime of less than one second and cannot be transported via the blood route to distant sites, the half-life of LOPs is much longer and distribution to distant tissues is possible. Therefore, it is logical to regard more detailed what is known about biological effects of ROS (hydrogen peroxide) and LOPs (aldehydes).

H₂O₂ is well known as an inter- and intracellular signaling molecule (41). When produced extracellular from the ozonolysis of PUFA, H₂O₂ can easily diffuse into the cytoplasm of cells. H₂O₂ was found as a messenger in the activation of transcription factor NF κ B in Jurkat T-cells (77). Activated NF κ B moves to the nucleus and is able to switch on genes for cytokine production. The activation of NF κ B via the second messenger H₂O₂ was proven for some other cell types (murine fibroblasts, pre-B cells, HeLa cells), but this effect seems to be cell-specific, since many cells have proven

insensitive to H_2O_2 mediated $NF\kappa B$ activation (including monocytes, astrocytoma cells, J. Jahn lymphoblastoid T cells, KB epidermal cells) (18). Bocci and colleagues found an induction of interferon gamma, $TNF-\alpha$, transforming growth factor- β , IL-2 and IL-8 after *in vitro* incubation of human blood or human mononuclear cells with ozone (12; 10; 11; 14; 13; 15; 65). Thus, activation or modulation of the immune system via the second messenger H_2O_2 may be a mechanism of the effect of ozone therapy. Interestingly, O_3/O_2 -PP exhibited potent protective effects on polymicrobial-induced lethal sepsis in rats, probably by altering the immune status (79).

Pryor et al. have studied the biological effects of LOPs, mainly in an attempt to clear the toxic pulmonary effects of ozone inhalation (69). Former *in vitro* studies suggested a dose-dependent increase of phospholipase (PLA_2 , PLC, PLD) activity in guinea pig tracheal epithelial cells after incubation with ozone (99). Incubation of a human bronchial epithelial cells with HHP-C7 (the hydroxyhydroperoxide formed from the ozonation of palmitoleic acid) resulted in increased arachidonic acid release due to activation of PLA_2 (69). In a consecutive study, Kafoury et al. could show that exposure of human bronchial epithelial cells in culture to 1-palmitoyl-2-(9-oxononanoyl)-sn-glycero-3-phosphocholine (PC-ALD), a specific PLA_2 stimulatory LOP, significantly increased the release of platelet-activating factor (PAF) and prostaglandin E_2 (PGE_2) and exposure to 1-hydroxy-1-hydroperoxynonane (HHP-C9), a specific PLC stimulatory LOP, significantly increased the release of IL-6, IL-8 and PAF (49). These authors concluded that LOP-dependent release of proinflammatory mediators may play an important role in the early inflammatory response seen during exposure to ozone.

LOPs can furthermore elicit an upregulation of endogenous antioxidants, such as glutathion or superoxid dismutase (66). LOPs were also shown to induce oxidative stress proteins like haeme-oxygenase 1, which after activation degrades haemoglobin leading to the formation of the potent antioxidant bilirubin (9). It is therefore believed that ozone therapy, as an acute oxidative stress, is capable of inducing endogenous antioxidant responses, which help to overcome chronic oxidative stresses to which the human body is often exposed (15).

We could show that rabbits with complete remission of the VX2 tumour after O_3/O_2 therapy were resistant to reinoculation of the VX2 tumour cell suspension into both auricles. This observation points to an involvement of the immune system in tumour clearance, particularly the adaptive immune system. The loss of

tolerance against reimplantation of VX2 tumours after immune suppression supports this hypothesis. Van Es et al. already showed that stimulation of the immune system with the immune modulating agent IL-2 resulted in regression of the auricular VX2 tumours in rabbits, although at a lower efficiency (25.0%) than found in the O₃/O₂-PP group (42.9%) (88). Numerous effector cells from the adaptive and from the innate immune system might be responsible for tumour regression. In an animal model with a mice strain that exhibits spontaneous regression/complete resistance (SR/CR) to multiple transplantable cancer cell lines, leukocytes infiltrating the tumour site consisted of a mixture of multiple subsets of effector cells composed primarily of macrophages, polymorphonuclear cells, NK cells and cytotoxic T lymphocytes (28; 44). Depletion or transfer of specific leukocyte populations of the adaptive or the innate immune system showed, that primarily the innate immune system is involved in successful tumour regression and complete resistance against reimplantation of the tumours in SR/CR mice. Each leukocyte subpopulation exhibits individual tumour cell killing mechanisms by the secretion of different effector molecules such as perforins, granzymes and ROS. In SR/CR mouse model the release of ROS by macrophages was identified as one major effector mechanism of the anticancer immune response (45). Therefore, production of ROS by activated macrophages and/or granulocytes could be a possible mechanism of anticancer effects induced by the insufflation of O₃/O₂ gas mixture in our study. In this context, the observed mild leukocytosis including granulocytosis after the therapeutical sessions with O₃/O₂ might represent activated leukocytes as potent anticancer effector cells also in the VX2 model.

Assuming that the hypothesis of leukocyte activation as the key antitumoural effect of O₃/O₂-PP is true, the next question would be where this activation takes place. On the one hand it seems to be possible that leukocytes are activated within the peritoneal cavity or the peritoneum and that these activated leukocytes or products of activated leukocytes are distributed via the circulation. On the other hand it might be possible that ozone reaction products, most likely the LOPs, are distributed via the circulation and that leukocyte activation takes place at sites distant from the peritoneum. The peritoneum is mainly protected by the innate immune system, the adaptive immune system is said to function as a secondary amplification system (43). The greater omentum harbours the so-called “milky spots”, which probably play a key role in the immunity of the peritoneum (27). The milky spots in humans and laboratory animals are accumulations of leukocytes. Macrophages

represent the major cell type, followed by B-lymphocytes, T-lymphocytes and to a small percent mast cells (81). Activated macrophages migrate from the omental milky spots into the peritoneal cavity, a process which is mediated by the expression of cell adhesion molecules (27; 80). Interestingly, the intraperitoneal administration of granulocyte/macrophage-colony-stimulating factor (GM-CSF) increased the tumouricidal activity of milky spot macrophages against intraperitoneal tumours in rats (53).

Intraperitoneally insufflated ozone might also 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 (58). Several components of the prostanoid biosynthesis pathway were found to exhibit oncolytic and antimetastatic effects on tumour cells and tumour progression (46; 76). Encouraging studies are currently under way to analyze changes in the plasma levels of different arachidonic acid metabolites during the course of O₃/O₂ insufflation in rabbits.

5.5 Ozone therapy and HNSCC

As is true for many cancers, HNSCC tumour cells somehow evade the body's immune system. Many mechanisms have been identified that contribute to the diminished immune surveillance of HNSCC tumour cells (100; 47). By the production of immune inhibitory factors cancer cells directly suppress the immune reaction of the host. Immune inhibitory factors that were found to be produced by HNSCC are PGE₂, TGF β , IL-6 and IL-10 (100). In addition, induction and mobilisation of immune inhibitory CD34+ cells and blockade of dendritic cell maturation were observed in patients with HNSCC (47). Tumours not only suppress the immune system of the host, they also have developed mechanisms for immune escape. This can partly be explained by the frequently observed downregulation or loss of MHC class I determinants (96; 61). Therefore, being able to activate the immune surveillance capacity is an emerging concept in recent cancer immunotherapies, particularly those focusing on immunomodulators or upregulators of the immune response (62; 86; 97). To augment the host's immune response against cancer cells, therapies with recombinant cytokines, dendritic cell immunization and tumour antigen vaccination as well as T cell-based immunotherapies are currently under investigation (100; 47). In this context, the intraperitoneal O₃/O₂ gas mixture therapy seems to be a

promising new tool in cancer treatment.

6 Summary

About 6% of all newly diagnosed malignancies worldwide are cancers of the head and neck. They account for nearly 5% of all cancer-related deaths. Surgery, radiation therapy and chemotherapy as well as combinations of these three are currently regarded as the standard treatment. While high cure rates can be achieved for the early stage disease, cure and survival rates for locoregionally advanced and distant metastatic disease are still disappointing. Thus, the development of new treatment strategies is essential, particularly for the treatment of cancers with metastatic involvement of the lymph nodes.

The VX2 auricle carcinoma of New Zealand White rabbits (NZWR) is an accepted animal model for human head and neck cancers, since both are similar in growth leading to early regional lymph node metastases and subsequent distant metastatic spread. Ozone, 2002 recognized as a biomolecule since it was found to be endogenously produced by human neutrophils, exhibited protective effects in a rat sepsis model when insufflated intraperitoneally as a gas mixture with oxygen. This finding suggests that ozone might have immunomodulatory properties. *In vitro*, ozone was found to inhibit selectively the growth of human cancer cells. However, until now there are no randomized controlled trials that show a clear therapeutic effect of ozone on cancer disease.

The aim of this study was to test the effects of an intraperitoneally insufflated O₃/O₂ gas mixture (O₃/O₂-pneumoperitoneum, O₃/O₂-PP) on growth of the primary tumour and metastatic spread of the VX2 auricle cancer in NZWR. Fourteen days after inoculation of a VX2 tumour cell suspension into the subcutis of the right auricle rabbits were randomized to three experimental groups. The treatment was performed daily for five consecutive days, beginning on day 14 after tumour cell inoculation. Rabbits in Group A (n = 14, O₃/O₂ group) each day received an intraperitoneally insufflated volume of 80 ml/kg body weight of an O₃/O₂ gas mixture with an ozone concentration of 50 µg/ml gas mixture. Rabbits in Group B (n = 13, O₂ group) received the same amount of pure oxygen and rabbits in Group

C (n = 14, Sham group) were sham-treated. Arterial blood samples were taken on day 14 (prior to initiation of treatment) and on day 19 (after the last treatment) and standard haematological and clinical chemistry blood parameters were measured.

O₃/O₂-PP significantly increased the 3-month survival probability (50.0%) and the complete remission rate (42.9%) compared with sham treatment (7.1% 3-month survival probability and complete remission rate). The 3-month survival probability (23.1%) and complete remission rate (15.4%) of the rabbits that received pure medical oxygen were neither significantly different from the sham group nor from the O₃/O₂ group. All animals with complete remission of the primary tumour had an enlarged sentinel lymph node (parotid lymph node) at the beginning of the treatment period that regressed simultaneously. None of these rabbits had detectable lung metastases at day 90 on computed tomography scans of the thorax. No severe side effects could be detected except for a mild transient body weight loss and a mild leukocytosis pointing to O₃/O₂-PP as a relatively safe method. Animals of the O₃/O₂ group with complete tumour remission were resistant to the tumour cells after reinoculation of the tumour cell suspension. However, when treating these animals with dexamethasone and cyclosporin A, rabbits became susceptible to reinoculation of the tumour again. This indicates a crucial role of the immune system in tumour eradication with subsequent resistance to reimplantation.

However, prior to a potential clinical use of the O₃/O₂-PP method, further research is essential to clear up the exact mechanisms by which ozone exerts this antitumoural effects. Particularly, further clarification is needed regarding the dose-response relationship, evaluation of yet undetected adverse effects and reproducibility of the results of the VX2 tumour model in other tumour model systems.

7 Zusammenfassung

Weltweit machen bösartige Tumoren des Kopf-Hals-Bereichs etwa 6% aller Krebsneuerkrankungen aus, mit einem Anteil an den Krebssterbefällen von circa 5%. Operation, Radiotherapie und Chemotherapie sowie deren Kombinationen gelten heute als anerkannte Therapieverfahren. Während bei lokal und regional begrenzten Tumoren hohe Heilungsraten erzielt werden, sind die Heilungs- und Überlebensraten beim Vorliegen von Lymphknoten- und Fernmetastasen nach wie vor deutlich geringer. Die Entwicklung neuer Therapieverfahren, insbesondere zur Therapie von bereits metastasierten Tumoren, ist deshalb unbedingt notwendig.

Das aurikuläre VX2-Karzinom beim weißen Neuseelandkaninchen ist ein akzeptiertes Tumormodell für humane neoplastische Erkrankungen des Kopf- und Halsbereichs, da es in seinem Wachstumsverhalten mit früher lymphogener und im fortgeschrittenen Krankheitsverlauf auftretender Fernmetastasierung große Ähnlichkeit zu den beim Menschen vorkommenden Kopf-Hals-Tumoren aufweist. Ozon, welches 2002 auch als endogen von neutrophilen Granulozyten gebildetes Biomolekül entdeckt wurde, zeigte bei intraperitonealer Applikation als Gasgemisch mit Sauerstoff einen protektiven Effekt im Sepsismodell der Ratte, was auf einen bereits zuvor postulierten immunmodulatorischen Effekt hindeutet. *In vitro* hemmte Ozon selektiv das Wachstum von Zellkulturen humaner Karzinome, jedoch gab es bisher keine randomisierten kontrollierten Studien mit ausreichend großen Fallzahlen, die einen klaren therapeutischen Effekt von Ozon bei Krebserkrankungen zeigen konnten.

Es war daher das Ziel dieser Studie, den Effekt eines intraperitoneal applizierten Ozon-Sauerstoff-Gasgemischs (O_3/O_2 -Pneumoperitoneum, O_3/O_2 -PP) auf die Primärtumorentwicklung und Metastasierung des VX2-Karzinoms beim weißen Neuseelandkaninchen zu untersuchen. Dazu wurden die Kaninchen 14 Tage nach subkutaner Inokulation einer VX2 Tumorzellsuspension in das rechte Ohr randomisiert drei Versuchsgruppen zugeordnet. Die Behandlung, beginnend am Tag 14 nach Tumorzellinokulation, wurde täglich an fünf aufeinander folgenden Tagen durchgeführt. Den Kaninchen in Gruppe A ($n = 14$, O_3/O_2 Gruppe) wurden pro Be-

handlung 80 ml/kg Körpergewicht eines Ozon-Sauerstoff-Gasgemischs mit einer Ozonkonzentration von 50 $\mu\text{g/ml}$ Gas intraperitoneal insuffliert, den Kaninchen in Gruppe B ($n = 13$, O_2 Gruppe) dieselbe Menge puren Sauerstoffs. Die Kaninchen in Gruppe C ($n = 14$, Sham Gruppe) erhielten eine Sham-Behandlung. Arterielle Blutproben wurden am Tag 14 (vor Behandlung) und am Tag 19 (nach Behandlung) entnommen und auf hämatologische und klinisch-chemische Standardparameter untersucht.

Die Behandlung mittels O_3/O_2 -PP erhöhte signifikant die 3-Monats-Überlebensrate (50.0%) und die komplette Remissionsrate (42.9%) der behandelten Tiere verglichen mit der Sham-Behandlung (7.1% 3-Monats-Überlebensrate und komplette Remissionsrate). Die 3-Monats-Überlebensrate (23.1%) und die komplette Remissionsrate (15.4%) der mit purem Sauerstoff behandelten Tiere war weder gegenüber der Sham Gruppe noch gegenüber der O_3/O_2 Gruppe signifikant unterschiedlich. Alle Tiere mit kompletter Remission des Primärtumors zeigten parallel auch eine Remission des zum Zeitpunkt der Therapie vergrößert tastbaren Wächterlymphknotens (Parotislymphknoten). Bei diesen Tieren wurden bei einer Computertomographie des Thorax am Tag 90 keine Lungenmetastasen gefunden. Abgesehen von einem leichten transienten Gewichtsverlust und einer milden Leukozytose konnten keine wesentlichen Nebenwirkungen gefunden werden. Daher scheint die Behandlung mittels O_3/O_2 -PP eine relativ sichere Methode zu sein. Die Tiere aus der O_3/O_2 Gruppe mit kompletter Tumorremission zeigten sich bei Reinokulation der Tumorzellsuspension resistent gegen die Tumorzellen. Durch Suppression des Immunsystems mit Dexamethason und Ciclosporin A konnte die Resistenz jedoch durchbrochen werden und die Kaninchen entwickelten wieder Tumoren nach Reinokulation der Tumorzellsuspension. Dies deutet auf eine wichtige Rolle des Immunsystems bei der Tumoreradikation mit nachfolgender Resistenz hin.

Bevor die O_3/O_2 -PP Methode eines Tages vielleicht beim Menschen eingesetzt werden kann, ist zunächst eine weiterführende Forschung notwendig um den exakten Mechanismus der beobachteten Ozonwirkungen zu klären. Im Speziellen sollte eine Dosis-Wirkungs-Beziehung erstellt, nach bislang nicht entdeckten Nebenwirkungen gesucht und die Reproduzierbarkeit der Ergebnisse vom VX2-Karzinom an anderen Tumormodellen getestet werden.

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List of abbreviations

AHT	Autohaemotherapy
ASIR	Age-standardized incidence rates
AUP	Animal Use Protocol
CREA	Creatinine
CSA	Cyclosporin A
CT	Computed tomography
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle Medium
EGFR	Epidermal growth factor receptor
EDTA	Ethylenediaminetetraacetic acid
G-BA	Gemeinsamer Bundesausschuss der Ärzte und Krankenkassen
GOT	Glutamic oxaloacetic transaminase
GPT	Glutamate pyruvate transaminase
GRA	Granulocytes
GSH	Reduced glutathion
HCT	Haematocrit
HGB	Haemoglobin
HNC	Head and neck cancer
HNSCC	Head and neck squamous cell carcinoma
IAPC	Intraabdominal Pressure Control
IL	Interleukin
LOP	Lipid ozonation (oxidation) product
LYM	Lymphocytes
MO	Monocytes
NMRI	Naval Medical Research Institute
ns	not significant
NZW	New Zealand White
PAF	Platelet-activating factor
PC	Phosphatidylcholine
PGE	Prostaglandin E
PL	Phospholipase
PP	Pneumoperitoneum
(P)UFA	(Poly)unsaturated fatty acid

RBC	Red blood cells
ROS	Reactive oxygen species
SAPL	Surface-active phospholipid
SPC	Saturated phosphatidylcholine
TTC	Time to tumour clearance
USPC	Unsaturated phosphatidylcholine
WBC	White blood cells

Publications

SCHULZ, S., **HÄUSSLER, U.**, MANDIC, R., HEVERHAGEN, J.T., NEUBAUER, A., DÜNNE, A.A., WERNER, J.A., WEIHE, E., BETTE, M. Treatment with ozone/oxygen-pneumoperitoneum results in complete remission of rabbit squamous cell carcinomas. *Int J Cancer* 122, 10 (May 2008), 2360-2367.

Verzeichnis der akademischen Lehrer

Meine akademischen Lehrer an der Philipps-Universität Marburg waren die Damen und Herren Professoren und Dozenten:

Alter, Arnold, Aumüller, Barth, Basler, Baum, Berger, Bertallanfy, Bette, Bien, Cetin, Christiansen, Czubayco, Daut, Dodel, Dominguez, Donner-Banzhoff, Dünne, Eberhart, Eilers, Feuser, Fuchs-Winkelmann, Gerdes, Görg, Gotzen, Grimm, Griss, Gudermann, Hamer, Hasilik, Hellwig, Herrmann-Lingen, Hertl, Herzum, Hofmann, Höglinger, Hoyer, Jungclas, Kalinowski, Kann, Klenk, Klose, Knake, Koch, Köhler, König, Koolmann, Kretschmer, Krieg, Kroh, Kroll, Kuhlmann, Kuhn, Langer, Leonhardt, Lill, Lohoff, Löffler, Maier, Maisch, Mandic, Max, Moll, Möller, Moosdorf, Mueller, Mutters, Neubauer, Oertel, Olbert, Printz, Remschmidt, Renz, Richter, Roeper, Röhm, Rosenow, Rothmund, Schäfer, Schmidt, Schnabel, Schneider, Schradler, Schulz, Schultz, Seitz, Steiniger, Stiasny-Kolster, Stiletto, Sundermeyer, Tebbe, Torossian, Vogelmeier, Vogt, Voigt, Wagner, Waldegger, Weihe, Westermann, Werner, Wulf.

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