Hyperbaric Oxygen Therapy

for the prevention of radiation-induced tissue injury in the head and neck region

An experimental mouse study

Linda Spiegelberg

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CONTENTS

Chapter 1

General Introduction

1

HEAD AND NECK CANCER

Each year, more than 500.000 cases of head and neck cancer (HNC) occur worldwide[1]. More than half of these are oral cavity cancers, while the rest comprises pharyngeal and laryngeal cancers (Figure 1). The vast majority (~90%)[2] of HNC diagnoses are squamous cell carcinomas, originating from the epithelium. HNC can be divided into three clinical stages: early, locoregionally advanced and metastatic, of which more than 50% belongs to the second category[3]. Metastases are seen in the lymph nodes of the neck, and are often the first sign of the disease. Morbidity rates are close to 25% but are highly dependent on staging. Early detection exponentially increases the chance of curing. Alcohol consumption, tobacco smoking and human papillomavirus infection are the most important risk factors (alcohol and tobacco accounting for 75% of HNCs)[4-6].

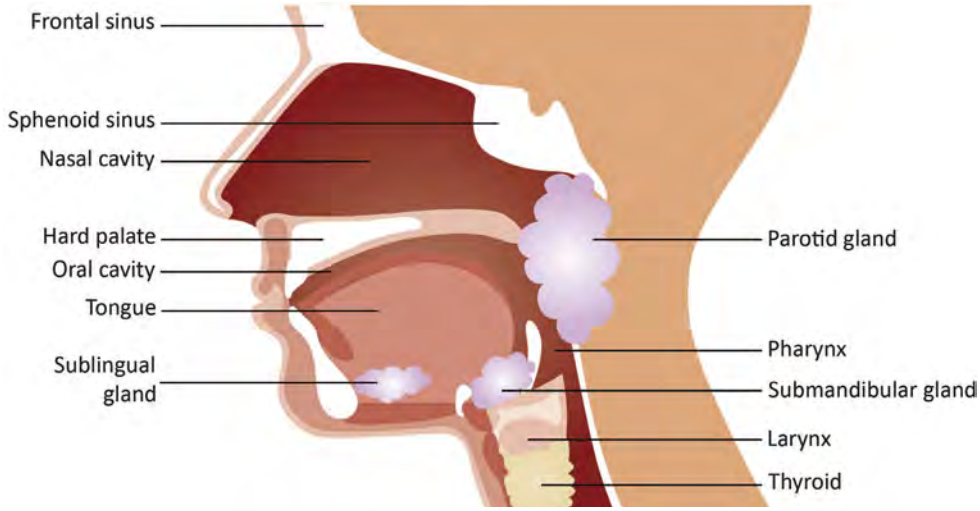

Figure 1 Schematic overview of the head and neck area.

RADIATION THERAPY FOR HEAD AND NECK CANCER

Treatment of HNC varies according to stage and location, but often surgery is used to resect the tumor, followed by radiation- and/or chemotherapy[7, 8]. Radiation therapy (RT) used in cancer treatment is based on its ionizing properties. Atoms, which are components of the molecules that form tissues, can be ionized by radiation, i.e. electrons are removed of these atoms, resulting in the breakage of chemical bonds and the molecule falling apart. This leads to damage to cell components including DNA, either directly or indirectly when water molecules in

the cell are ionized and form highly reactive free radicals (reactive oxygen species), that immediately react with any biomolecule in its surrounding. Damage to the DNA can acutely lead to cell death of tumor cells, or cause genomic instability transmitted through many generations via the progeny of surviving cells, which also ultimately leads to cell death[9]. Furthermore, radiation leads to vascular damage, creating a hypoxic, hypovascular and hypocellular environment with a compromised regenerative capacity.

RADIATION DAMAGE

Although radiation is targeted at the tumor, it is inevitable that normal surrounding tissue will also be affected. In general, rapidly dividing tissues, such as lymphoid organs, bone marrow and intestines, are highly radiosensitive, whereas slowly dividing tissues as brain and spinal cord have a low radiosensitivity[10].

In the head and neck region, relatively many different tissues are situated in close proximity of each other, responding differently to RT. RT can induce infection of oral mucosa (i.e. mucositis), while taste loss is caused by damaged taste buds and hyposalivation is due to salivary gland damage. Mucositis and taste loss are acute effects that can arise during RT, and are reversible[11, 12]. After RT, these complications will prolapse. Late effects are often irreversible and include hyposalivation, trismus (limited jaw opening due to increased muscle rigidity), radiation caries and osteoradionecrosis (ORN; RT-induced bone death)[13]. Salivary gland damage, leading to hyposalivation, is the most prominent side effect of RT in the head and neck region, while the risk of developing radiation caries and ORN poses a life-long threat[14]. This thesis focuses on RT- induced salivary gland and bone damage.

Salivary glands

Salivary glands are slowly dividing, highly differentiated tissues that have an unexpected high radiosensitivity[10]. Radiation affects salivary flow rate and composition, and thus salivary gland function, both acute and chronically.

Anatomy and morphology

Major and minor salivary glands together are responsible for the production of saliva, which has many different functions ranging from the lubrication of the throat and mouth, initiating digestion to maintaining oral health[15]. Minor salivary glands can be found in the lower lip, tongue, palate, cheeks and pharynx. The major salivary glands consist of the parotid-, submandibular-, and sublingual glands. The latter two are situated in the floor of the mouth, while the parotid gland is located in front of the ear and extends to the lower borders of the jawbones (Figure 1). The submandibular glands produce approximately 65% of saliva in an unstimulated

state, while upon stimulation the parotid glands are the main contributors of saliva secretion. The production of saliva takes place in the serous and mucous acinar cells of the glands. Parotid glands mainly have serous acinar cells, and produce a serous, more watery, secretion, while sublingual glands consist predominantly of mucous acinar cells and the submandibular glands contain a mixture of these two. Besides acinar cells, salivary glands contain ducts that modify the ionic composition of the acinar secretions as it is transported to the oral cavity, and myoepithelial cells which are wrapped around acinar cells and regulate secretion from acinar cells into ducts by contracting upon nervous stimulation (Figure 2).

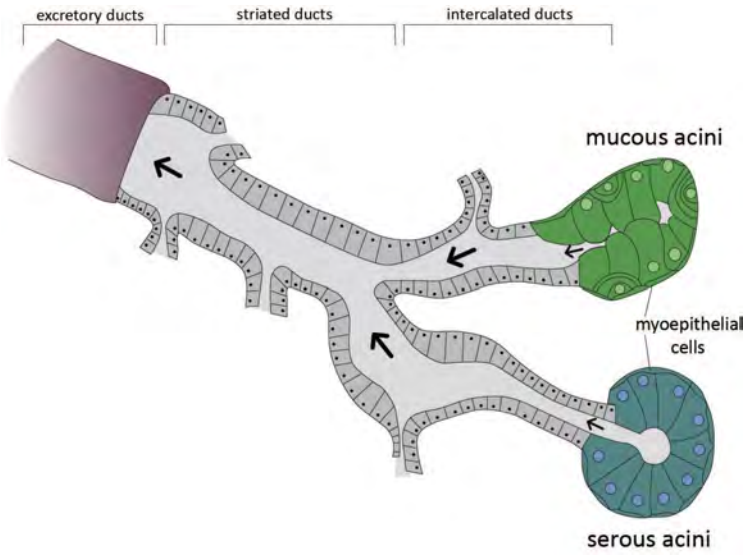

Figure 2 Schematic overview of the salivary gland. Arrows depict direction of salivary flow.

Radiation damage

Early radiation damage is thought to target the plasma membrane of acinar cells, since water excretion is severely inhibited directly after irradiation while cell number remains intact. Takagi et al showed irreversible radiation-induced damage to water channels in acinar cells and a significnant loss of plasma membrane-bound aquaporin 5[16]. Based on various experiments, Coppes et al distinguished four phases of radiation-induced damage to the submandibular glands of rats[17]. Phase 1 (0-10 days) is the acute phase in which water excretion is quickly impaired with no cell loss visible. In phase 2 (10-60 days), the compromised acinar cells disappear, with no compensation of cells. There are no major changes observed in terms of cell number and salivary flow in phase 3 (60-120 days). Phase 4 represents the expression of late radiation damage with a lack of functional acinar cells, caused by the killing of progenitor- and stem cells and replacement by connective tissue

(i.e. fibrosis). Generation of acinar cells does take place, however flow rate is still deteriorated, suggesting that the new cells cannot function properly due to damage of ducts, blood vessels and nerves.

In HNC patients, radiation damage to the salivary glands leads to chronic hyposalivation, which is subjectively experienced by patients as a dry mouth and called xerostomia. Xerostomia is the most common complication of radiation therapy for head and neck cancer[18]. Patients have trouble with speech, mastication, swallowing and suffer from impairment of taste and disturbance of sleep patterns, which greatly affects quality of life[13, 19, 20]. In addition, xerostomia may result in compromised protection of teeth resulting in progressive dental decay.

Bone

Radiation can lead to necrosis of bone, so called osteoradionecrosis (ORN). In the head and neck area, the mandible is prone to develop ORN since it is often in the radiation field and has a relatively poor blood supply[21].

Anatomy and morphology

The mandible is the largest and strongest bone of the face and consists mainly of cortical, or dense, bone of which the majority is composed of inorganic hydroxyapatite and organic collagen. Only a small part consists of cells, i.e osteoblasts, osteocytes and osteoclasts. Osteoblasts and osteoclasts play an important role in bone remodeling (Figure 3), in which old bone is continuously resorbed and new bone formed, allowing the bone to adapt to mechanical load and strain. Osteoclasts derive from a monocyte stem cell lineage and resorb bone upon activation. In the reversal phase that takes place after resorption, mononuclear cells appear on the bone surface and provide signals for osteoblast differentiation and migration. Subsequently, osteoblasts that derive from osteoprogenitor cells produce osteoid, which is primarily composed of collagen type 1 and eventually becomes mineralized. Osteoblasts then become surrounded with the bone matrix they have produced and differentiate into osteocytes, the mature bone cells that are responsible for matrix maintenance and regulate the bone's response to stress and mechanical load[22]. In normal homeostasis, bone resorption and formation are balanced, but a number of clinical diseases can cause an imbalance resulting bone loss or excessive bone formation[23].

Radiation damage

Radiation induces damage to small arteries leading to a reduced circulation, which influences the viability of osteogenic cells[24]. Whether radiation also directly targets osteogenic cells remains a matter of debate. Subsequently the bone has a reduced ability to heal, which especially poses a risk when the jaw is infected.

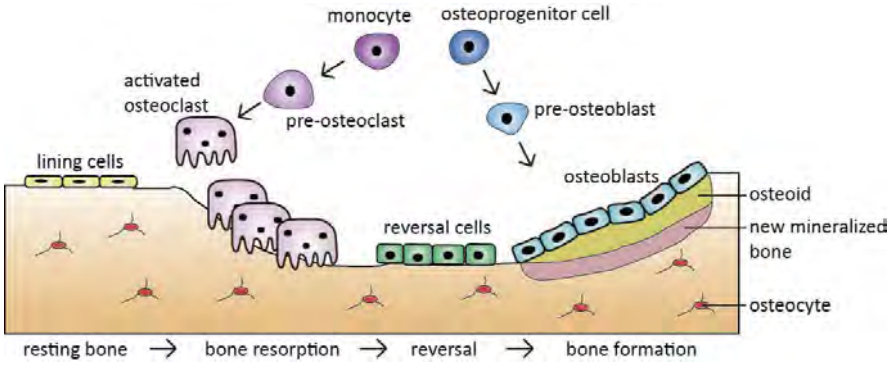

Figure 3 Schematic representation of bone remodeling and the bone cells involved.

ORN can occur spontaneously after irradiation, but the risk is highly increased when an insult is inflicted on previously irradiated bone, such as subsequent surgery or biopsy, tooth extractions or denture irritations[25]. Especially when patients suffer from xerostomia, the oral environment is more prone to develop dental caries that can lead to infection and consequently ORN. Even years after radiation, the risk to develop ORN remains high in previously irradiated bone.

PREVENTION AND TREATMENT OF RADIATION DAMAGE

Efforts have been made to prevent or treat radiation damage to the head and neck area, since the complications greatly affect quality of life. Obviously, prevention is the ultimate goal, since radiation damage is for the most part irreversible. Salivary gland damage is the most prominent complication of RT and the prevention of salivary gland damage and ORN are in close association since xerostomia is an underlying cause of ORN and dental decay.

Prevention

To reduce the risk for ORN and dental decay, teeth at risk will be removed before radiation therapy[26]. Minimizing the radiation dose delivered to salivary glands and bone is important in sparing their function. In the last decades, advances in the delivery of radiotherapy have been made to target the tumor tissue more efficiently. Fractionated radiation significantly reduces damage to adjacent tissues. Three-dimensional conformal RT (3D-CRT) uses highly collimated beams to target the tumor and minimize the treatment field and thus normal tissue damage. A greater repair-potential is achieved when using intensity modulated radiation therapy (IMRT), where radiation doses vary in intensity, resulting in parts of the tissue receiving lower exposure[27-29]. Especially the parotid glands can be spared to some extent using these techniques. Sparing submandibular glands remains more

challenging, since they are often located closer to the tumor[30].

Another way of preventing radiation induced normal tissue damage is by using agents that protect cells from radiation damage. The only cytoprotectant approved by the US Food and Drug Administration (FDA) is amifostine, which acts as a selective free radical scavenger for normal tissue. However, clinical trials on its effectiveness are conflicting and concern remains about its toxicity as serious adverse events such as hypotension and gastrointestinal disturbances have been reported[13, 31, 32].

Surgical transfer of salivary glands to the submental place outside the radiation field[33, 34] is an option that is not used frequently. In some patients, it is impossible to shield the submental place because of the proximity of the tumor. Furthermore, salivary gland transfer is only useful in patients that need postoperative RT, which is not always predictable[13].

Treatment

In the treatment of xerostomia and ORN, maintaining a strict oral hygiene is important to avoid oral infections and dental problems. To mimic saliva function, salivary replacements are available. They offer temporary relief of symptoms, but do not have the antibacterial and immunological properties of saliva and some patients prefer regular water intake[14].

Saliva secretion can be increased by sialogogues, which enhance any residual secretory function. Pilocarpine is the only sialogogue approved by the FDA. It is a cholinergic agonist that acts parasympathicomimetically, thereby stimulating the unaffected acinar cells to secrete saliva[13]. However, due to its general mechanism of action, caution should be taken with respect to its administration, especially in patients with cardiovascular disease, glaucoma or uncontrolled asthma[35].

For ORN, treatment options depend on the stage of the disease; early stage can be treated with local wound care and antibiotic therapy, whereas late stage patients need radical resection and reconstruction[26, 36].

Despite these prevention and treatment modalities, patients experience no full recovery from xerostomia and once bone or dental fragments are affected, full healing is not feasible anymore. The need for other therapies is therefore evident. Preclinical studies have investigated the possibility of gene transfer and stem cell therapy to restore salivary gland function[37, 38], but clinical studies have not been performed yet, and these therapies are therefore still far from clinical implementation.

1

HYPERBARIC OXYGEN THERAPY

Hyperbaric oxygen therapy (HBOT) is used as an adjunctive treatment for the management of ORN, or when tooth extraction has to take place in a previously irradiated region. Patients breathe 100% oxygen under elevated pressure (typically 2.4 atmospheres absolute) in specially built pressure chambers. One session lasts approximately two hours, and 20-40 daily sessions are carried out, depending on the indication.

History

The use of hyperbaric therapy dates back to 1662, when the British physician Henshaw built the first hyperbaric chamber. He used reduced pressure for the treatment of chronic illnesses and increased pressure for more acute illnesses. By the late nineteenth century, chambers were widely used for a variety of conditions. These early chambers used compressed air instead of oxygen, because of the reports of oxygen toxicity. In spite of this, Drager explored the use of pressurized oxygen in the treatment of decompression sickness, which was tested by the US Military and put into practice in the 1930s. Beneficial effects on a number of other diseases, investigated in the 1950s and 1960s, led to the widespread use of hyperbaric oxygen therapy. In 1976, the 'Committee on Hyperbaric Oxygen Therapy' was founded by the Undersea and Hyperbaric Medical Society (UHMS) to oversee the ethical practice of hyperbaric medicine[39]. This committee is currently the international authority on HBOT. The list of accepted indications includes delayed radiation injury (see table below).

UHMS* approved indications for HBOT	
Air or gas embolism	Intracranial Abscess
Carbon monoxide poisoning	Necrotizing soft tissue infections
Clostridial myositis and myonecrosis	Osteomyelitis (refractory)
Crush injury, compartment syndrome and other acute traumatic indications	Delayed radiation injury (soft tissue and bony necrosis
Decompression sickness	Compromised grafts and flaps
Arterial Insufficiencies	Acute thermal burn injury
Severe Anemia	Idiopathic sudden sensorineural hearing loss

*Undersea and Hyperbaric Medical Society

Mechanism of action

All living cells need oxygen to function and the oxygen demand of the tissue will rise when wound healing and regeneration needs to take place in response to

injury. Normally, oxygen is transported by the blood in two forms: by the reversible binding to hemoglobin proteins of red blood cells (98%), and dissolved in plasma (2%). Under normal circumstances, hemoglobin is saturated for 97%. The elevated pressure combined with pure oxygen breathing used in HBOT, results in an increase of the amount of oxygen dissolved in plasma[39]. The oxygen can diffuse further into tissues, and can reach obstructed places where red blood cells cannot pass (Figure 4). In irradiated tissue, this leads to a better oxygenation of hypoxic areas[40]. Oxygen is involved in numerous processes regarding wound healing and regeneration, such as microbial killing, cytokine release, apoptosis and angiogenesis[41-43]. Therefore it is expected that HBOT may have an effect on these processes. However, the precise effects and mechanism of action of HBOT remain poorly understood. Angiogenesis is the most studied process, and it is generally accepted to be positively influenced by HBOT[39, 44-48].

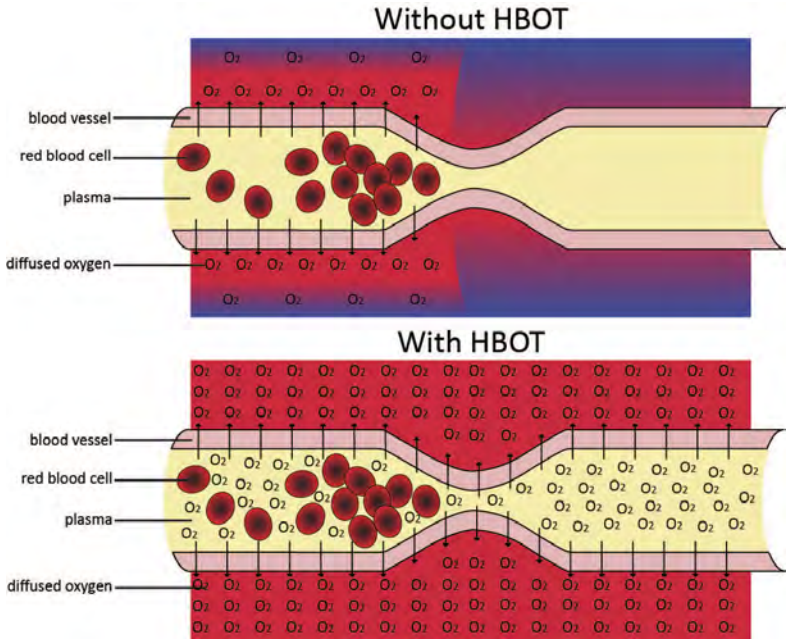

Figure 4 Mechanism of hyperbaric oxygen therapy (HBOT). A radiation-induced occlusion (damaged blood vessel) prevents red blood cells from passing (upper picture), resulting in hypoxic tissue (blue). Due to the administration of hyperbaric oxygen (lower picture), oxygen is dissolved in much higher amounts in the plasma, allowing diffusion into hypoxic areas resulting in much higher tissue oxygen tensions.

The effect of HBOT on radiation-induced hyposalivation has been studied clinically[49-54]. Moderately positive effects have been reported, but evidence remains scarce. Studies on the clinical use of HBOT to prevent or treat ORN are more prevalent[20, 36, 52-70], but there is no general consensus about the beneficial effects of the therapy.

Effects on tumor tissue

Patients that are treated with HBOT to repair or prevent radiation-induced tissue damage have a history of cancer. This has raised concerns about the promoting effects that HBOT might have on (residual) tumor tissue. If HBOT promotes angiogenesis in tumors, it could stimulate growth and recurrence of cancer[71-73]. On the other hand, hypoxic tumors are known to be more aggressive in terms of invasive growth, metastasis and therapy resistance than well-oxygenized tumors[74, 75]. In this case, if HBOT is able to oxygenize the tumor, it might positively influence the prognosis.

REGENERATING AGENTS

ReGeneraTing Agents (RGTAs) are clinically used in the management of non-healing wounds, although conclusive evidence of its beneficial effect needs to be obtained. RGTAs are designed to mimic heparan sulfates, which are normally localized in the extracellular matrix (ECM), bound at heparan sulfate binding sites that are present on macromolecules of the ECM, such as collagen, fibronectin and laminin. Heparan sulfates in their turn, can bind growth factors and cytokines, preventing them from being degraded, thereby maintaining normal tissue homeostasis. In the case of injury, however, the heparan sulfates are degraded by heparanases. This allows other proteases to degrade parts of the ECM and leads to a loss of growth factors and cytokines, which are essential for repair processes. RGTAs are engineered in such a way that they can bind at heparan sulfate binding sites and are able to bind growth factors. The difference with natural heparan sulfate is that RGTAs cannot be degraded by heparanases. In this way, growth factors and cytokines will be available in the injured site and can fulfill their action needed to repair the tissue[76].

Several animal studies have shown positive effects of RGTAs on tissue repair in bone[77], muscle[78, 79], skin[80, 81] and mucosa[82, 83]. Clinical studies are mostly small pilot studies and case reports, regarding various diseases [84-88] that advocate randomized controlled trials to be performed. Effects of RGTAs on radiation-induced damage have not been studied extensively, except for a study in which radiation-induced mucositis was prevented by RGTA in rats[82].

AIM & OUTLINE

The current thesis aims to give more insight in the prevention of radiation-induced damage to the tissues of the head and neck, including bone and salivary glands, by hyperbaric oxygen therapy, and to a lesser extent, by RGTAs. For this purpose, a mouse model was used, in order to be able to assess morphological, cellular and molecular changes in the tissues, since these parameters have not been

investigated extensively and will help to better understand working mechanisms. Effects of HBOT on irradiated tumor tissue are also addressed.

Chapter 2 gives an overview of the existing literature regarding hyperbaric oxygen therapy in the irradiated head and neck region.

Chapter 3 describes the hyperbaric oxygen chamber that was specially built to enable research with small laboratory animals, in our case mice. Hyperbaric chambers used for the treatment of humans are not suitable for experimental animal research and chambers for animal use are not abundantly available.

In **Chapter 4**, irradiated mandibular bone is characterized by means of microCT and histology, and the effect of HBOT on these parameters is studied.

In **Chapter 5**, the effects of HBOT on irradiated soft head and neck tissues were studied, focusing on the salivary glands. Salivary flow rate was measured, as well as proliferation, apoptosis and blood vessel density, up to 24 weeks after irradiation.

Chapter 6 focusses on RGTA, and its potential to influence irradiated salivary gland function and morphology, by investigating saliva production and composition and assessing the amount of functional acinar cells by histology.

Chapter 7 provides a more profound insight on the molecular pathways that are affected by HBOT in irradiated submandibular glands. Microarray analysis was used to detect differences in gene expression that lead to activation or inhibition of different molecular processes within the tissue.

The effects of HBOT on irradiated and non-irradiated tumor tissue are addressed in **chapter 8**. For this purpose, an orthotopic floor-of-mouth mouse model of head and neck squamous cell carcinoma was used. Tumor growth, vascular permeability, hypoxia and metastasis could be investigated using *in vivo* optical imaging techniques, in combination with histological methods and PCR.

Chapter 9 provides a general discussion on the results obtained in this thesis and offers suggestions for further research.

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Chapter 2

Hyperbaric oxygen therapy in the management of radiation-induced injury in the head and neck region: a review of the literature

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ABSTRACT

Purpose: Radiotherapy is generally used in the treatment of malignant tumors in the head and neck region. It causes a hypoxic, hypocellular, and hypovascular environment that leads to injury to surrounding normal tissue, both acute and chronic, ranging from xerostomia to osteoradionecrosis. These side effects are debilitating and greatly influence quality of life in these patients. Hyperbaric oxygen (HBO) therapy is clinically used to prevent or treat these side effects by enhancing oxygen pressure and thereby regeneration. Although this therapy is widely applied, its mechanism of action is still poorly understood, and controversy exists in the literature about its clinical use. This review therefore aims to analyze the existing experimental and clinical research on this topic.

Methods: A systematic search was performed in PubMed for experimental and clinical studies conducted regarding the use of HBO therapy in previously irradiated tissue, in the period from January 1990 to June 2009.

Results: Experimental research is scarce, and clinical studies are especially lacking in terms of randomized controlled studies. Although discussions on the subject are ongoing, most studies suggest a beneficial role for HBO in previously irradiated tissue.

Conclusion: Further research, both experimental and clinical, is necessary to unravel the working mechanism of HBO therapy and validate its clinical use.

INTRODUCTION

Malignant tumors in the head and neck region are generally treated by surgery in combination with chemotherapy and/or radiotherapy. Besides its direct influence on the malignant tumor, radiotherapy results in injury to surrounding normal tissue that appears either acutely or as a late effect. Late effects can be observed months to years after radiotherapy. Radiation causes mitotic cell death by the formation of free radicals that irreversibly disturb deoxyribonucleic acid replication. Furthermore, radiation induces stasis and occlusion of small blood vessels, resulting in hypoxia and hypovascularity. This hypoxic environment profoundly reduces the potential of cells to regenerate and survive. The occlusion of vessels also inhibits the infiltration of osteoprogenitor cells and endothelial progenitor cells, further compromising the regenerative capacity. The overall result is a hypoxic, hypovascular, and hypocellular environment comparable to that of a chronic, nonhealing wound[1].

Osteoradionecrosis

An early side effect of radiotherapy in the head and neck region is xerostomia. The mucus that is secreted by salivary glands thickens because of a change in composition. Patients with xerostomia have a dry mouth, which results in difficulties with eating and speech. Repair of salivary gland tissue is problematic, and provided that it occurs, will take months to years[2]. Xerostomia causes an environment that is more prone to develop dental caries, which in turn can lead to periapical infection and, ultimately, necrosis of the underlying bone, so-called osteoradionecrosis (ORN)[3]. ORN can occur spontaneously after radiation, when the dose exceeds 50 to 60 Gy[4], but the risk is increased when the previously irradiated region undergoes trauma (eg, tooth removal). ORN has a profound influence on the quality of life (QoL) of patients and leads to major difficulties with regard to reconstructive surgery. Reuther et al[5] reported the incidence of ORN to be 8.2% in a group of 830 head and neck tumor patients who received radiotherapy. ORN affects the mandible more often than the maxilla and, in fact, more than any other bone in the body, probably because of the poor blood supply in the mandible[6]. The risk of ORN developing after radiotherapy is highest during the first 3 to 24 months, but it persists throughout the patient's life and even increases over time for trauma-induced ORN[7].

Hyperbaric Oxygen Therapy

The delivery of oxygen is an essential process when considering (bone) tissue repair. Inadequate vascularity of bone reduces osteogenesis and thus bone mass. Most osteogenic factors stimulate angiogenesis, and when angiogenesis is inhibited during the repair of bone fractures, fibrous tissue is formed instead of bone[8]. Oxygen stimulates collagen synthesis, matrix deposition, angiogenesis,

epithelialization, and the eradication of bacteria[9]. The use of hyperbaric oxygen (HBO) therapy in improving wound healing is based on this principle. In HBO therapy patients will breathe 100% oxygen at increased pressure (2-3 atmospheres absolute). In the short term, this causes an increase in the tissue's internal oxygen pressure, leading to vasoconstriction, enhanced oxygen delivery, edema reduction, phagocytose activation, and an anti-inflammatory effect. The long-term effects are neovascularization, osteogenesis, and a stimulation of collagen production by fibroblasts, all of which promote wound healing[9].

It is estimated that in Europe approximately 500 patients are treated with HBO for radiotherapy-induced injury every year[10]. Most of these patients have had malignancies in the head and neck region and are being treated for some form of ORN. HBO can also be used prophylactically, for example, when a tooth extraction is performed in a previously irradiated region. Cellular, biochemical, and physiologic mechanisms of HBO are still not completely understood. Clinical studies regarding the effectiveness of HBO, especially with regard to prophylactic use, show contradictory outcomes, and the lack of randomized, controlled, double-blind trials further hampers assessment of the efficacy of this treatment. Therefore no consensus exists regarding both the mechanism of action and the effectiveness of HBO in the prevention and treatment of ORN. Subsequently, several theories on the pathophysiology of ORN have arisen.

Al-Nawas et al[11] propose that the essence of the development of ORN is the direct effect of radiation on osteoclasts, which occurs before vascular alterations. According to this theory, the suppression of bone turnover via increased osteoclast function is the most important factor in ORN, and consequently, the use of HBO therapy will not be valuable. Another theory focuses on radiation-induced fibrosis[12]. This theory states that the activation and dysregulation of fibroblastic activity, leading to atrophic tissue, are the key events in the progression of ORN within previously irradiated bone. The use of pentoxifylline together with tocopherol (vitamin E) is suggested to prevent ORN, because these 2 drugs act synergistically as potent antifibrotic agents, thereby possibly reducing the negative effect of radiation on tissues.

This review takes an in-depth look into the existing experimental and clinical literature on the effect of HBO in irradiated bone, with special focus on the head and neck region. Furthermore, we aim to present a guideline for the direction of future research on HBO that is needed to validate the use of this therapy.

MATERIALS AND METHODS

A systematic search of the literature was performed by use of the PubMed database with the following key words, as well as combinations of these terms: hyperbaric oxygen, irradiation, radiation, radiotherapy, maxillofacial, craniofacial, head and neck, bone, mandible, soft tissue, salivary glands, and mucosa. Only articles written in English within the time period from January 1990 to June 2009 were included. Experimental and clinical studies were included, whereas case reports were excluded. References of the articles found were checked regarding importance and obtained when useful.

RESULTS

Working mechanism of HBO

Experimental studies regarding the working mechanism of HBO in general were evaluated to give a brief overview of the existing knowledge. In vitro[9, 13-15] and in vivo[16-20] studies were included, and the results of these studies will be reviewed in the "Discussion" section.

Experimental research

The next step was to identify experimental studies that consider the effect of HBO on irradiated bone tissue. Five studies were found that focused on the cancellous bone of the hind legs of rabbits or mice, either with or without placement of implants[21-25]. Eight studies were carried out in the head and neck region, 7 of which combined irradiation and HBO therapy with distraction osteogenesis (DO) in rabbit mandibles[26-32]. Williamson[33] studied the effect of HBO on irradiated tissue regeneration in the mandible of the rat, when given 1 week after the completion of radiotherapy.

Clinical research

Twenty clinical studies were found that were conducted to study the effect of HBO therapy on previously irradiated head and neck tissue[34-53]. The studies varied widely in protocols and conclusions, of which the most important parameters are listed in Table 1 (see next page). The term "surgery" in this table comprises implant placements as well as tooth extractions, with perioperative or postoperative HBO therapy. Furthermore, the HBO use was either prophylactic, to prevent radiation-induced injuries, or therapeutic, when these injuries already existed.

Table 1 Parameters and conclusions of clinical studies regarding effect of HBO on previously irradiated head and neck tissue.

First Author and Year of Publication	Sample Size	Study Type		Surgery*		HBO Use		Control Group		Statistics Used		HBO Effective According to Authors	
		Prospective	Retrospective	Yes	No	Prophylactic	Therapeutic	Yes	No	Yes	No	Yes	No
Ang et al[34] (2003)	21		X	X			X	X			X	X	
Annane et al[35] (2004)	68	X			X		X	X		X			X
Arcuri et al[36] (1997)	4†	X		X		X			X		X	X	
Bui et al[37] (2004)	28‡		X		X		X		X		X	X	
Chavez and Adkinson[38] (2001)	40	X		X		X			X		X	X	
David et al[53] (2001)	75		X	X	X	X	X		X		X	X	
Gal et al[40] (2003)	30		X	X			X	X		X			X
Gerlach et al[41] (2008)	21	X			X	X	X		X	X		X	
Granstrom et al[42] (1999)	78		X	X			X	X		X		X	
Harding et al[43] (2008)	66		X	X	X	X	X		X	X		X	
van Merkesteyn et al[50] (1995)	29		X	X			X		X		X	X	
McKenzie et al[44] (1993)	26		X	X	X		X		X		X	X	
Mounsey et al[45] (1993)	41		X		X		X		X		X	X	
Narozny et al[46] (2005)	8		X		X		X		X		X	X	
Schoen et al[47] (2007)	26	X		X		X		X		X			X
Shaw et al[48] (2005)	38§		X	X		X		X			X		X
D'Souza et al[39] (2007)	23		X		X		X	X			X		X
Teguh et al[49] (2009)	19	X			X	X		X		X		X	
Vudiniabola et al[52] (2000)	37		X		X		X		X	X		X	
Vudiniabola et al[51] (1999)	14	X		X		X			X		X	X	
Total	695	7	13	12	11	9	14	8	12	8	12	15	5

*The term "surgery" comprises implant placements as well as tooth extractions, with perioperative or postoperative HBO therapy.
†Four patients received a total of 18 implants, of which the successful osseointegration was analyzed.
‡Only patients with late complications in the head and neck region were included.
§Only patients who received radiotherapy were included.

DISCUSSION

HBO has been used since the late 1950s to treat a variety of conditions, such as syphilis, multiple sclerosis, myocardial infarction, gangrene, and decompression sickness[54]. ORN—a late irradiation complication— could, in theory, benefit from HBO use, either prophylactically or therapeutically. However, conflicting opinions about the efficacy exist, hence this review on experimental and clinical studies reported in the literature.

Marx, the founder of the so-called 3H model (hypoxia, hypocellularity, hypovascularity) of the pathogenesis of ORN, and colleagues[55] conducted a classical study regarding the effect of HBO on the prevention of ORN. They compared the effect of HBO in irradiated mandibles that required dental extraction with that in a control group and found a decrease in the incidence of ORN from 29.9% in the control group to 5.4% in the HBO group. These findings paved the way for more studies to come and, concurrently, the clinical use of HBO in irradiated head and neck tissue.

Working mechanism of HBO

The exact working mechanism of HBO is not completely understood. With regard to bone tissue, radiotherapy decreases bone-forming capacity by decreasing the numbers of osteocytes and osteoblasts, increases bone resorption by increasing the number of osteoclasts, and decreases the capillary density[56]. Multiple in vitro studies therefore focused on the effects of HBO on osteoblasts, the bone-forming cells. Wong et al[14] found an inhibition in the growth of osteoblasts. In the study of Wu et al[15], proliferation of osteoblasts was either stimulated or reduced, depending on the type of culture medium. The mineralization was stimulated in both types of mediums, and it was thus suggested that HBO results in differentiation of osteoblasts to an osteogenic phenotype, rather than increasing cellular proliferation. These conclusions were supported by Tuncay et al[13]. They compared
hypoxic (10% O2) and hyperoxic (90% O2) conditions in osteoblast-enriched cultures from fetal rat calvarias and showed that hyperoxia suppressed cellular proliferation whereas alkaline phosphatase activity and collagen synthesis (both markers for bone forming) were increased. Effects of hypoxia were opposing, and when hypoxic cells were switched to a hyperoxic environment, their metabolic activities were abruptly reversed. This suggests a triggering role for oxygen tension in bone remodeling. Tompach et al[9] concentrated on the effect of HBO on endothelial cells and fibroblasts and found an increase in proliferation for both cell types.

In vivo experiments with HBO supported the findings of the in vitro studies. Levin et al[19] showed an enhancement of fibroblastic, osteoblastic, osteoclastic, and angioblastic activities in the femoral heads of rats, and Jan et al[18] found a

significantly higher boneforming capacity in critical-sized defects in rabbits' parietal bones. The regenerate of the HBO-treated group in this latter study also showed a higher capillary density than the control group, at both 6 weeks' and 12 weeks' follow-up. Some studies shed light on how the increase in angiogenesis due to HBO can be explained. Two studies conclude that HBO mobilizes endothelial progenitor cells through induction of bone marrow nitric oxide[17, 20]. Fok et al[16] found HBO to increase vascular endothelial growth factor, one of the primary growth factors responsible for neovascularization during wound healing.

The finding that HBO promotes/stimulates angiogenesis led to some concern regarding its use in cancer patients. Tumors are dependent on blood supply to grow; therefore, increased angiogenesis may promote (recurrent) tumor growth. However, in a thorough systematic review, Feldmeier et al[57] concluded that there is no reason to presume that a history of malignancy must be considered a contraindication for HBO therapy.

Experimental research

HBO and Cancellous Bone

Cortical bone, of which the craniofacial bones are predominantly composed, differs from cancellous bone in composition as well as repair mechanism. Cortical healing is slower than cancellous healing[58, 59], so it is likely that radiation has a more profound effect in the former. On the other hand, cancellous bone is highly cellular and usually has a higher rate of metabolic activity than cortical bone[60], which can increase the negative effect of radiotherapy. Therefore it is important to distinguish these 2 bone types in research and be careful when applying results of studies undertaken in cancellous bone to bone of the head and neck region and vice versa.

Two studies that emerged from our literature search investigated the effect of HBO in irradiated hind legs of rabbits and mice, respectively, and reported various results[22, 25]. No significant effect of irradiation or HBO on bone mineral density was found in the study of Johnsson et al[22], probably because of high variations in bone-forming capacity between animals and a limited number of animals. They could only report a tendency toward improved bone formation after HBO therapy in nonirradiated tissue. Wang et al[25] found that HBO significantly reduces the retardation of bone growth induced by irradiation but only at radiation doses of 10 and 20 Gy. At a higher dose (30 Gy), the damage from irradiation could not be alleviated by the use of HBO. It must be noted that in this study and, actually, in all aforementioned in vivo experimental studies, the radiation dose and fractionation may not replicate the radiation pathology in humans, because tissue response is species specific[61].

In 3 studies implants were placed in irradiated hind legs of rats or rabbits, and

the effect of HBO on the tissue reaction around these implants was investigated[21, 23, 24]. Results varied from slightly improved trabecular bone formation and improved implant-bone contact[21] to improved bone formation and maturation[23] and an increase in the biomechanical force necessary to unscrew titanium implants[24].

HBO in Head and Neck Region

Literature regarding HBO therapy in previously irradiated head and neck tissue in animals proved to be scarce. Only 1 study was exclusively designed to determine whether HBO is effective in reducing the long-term side effects of therapeutic irradiation treatment on the rat mandible[33]. In this study HBO therapy was given to rats 1 week after completion of radiotherapy. The follow-up period was up to 36 weeks to obtain a clear view on the late effects of irradiation (and HBO). Furthermore, not only bone was investigated, but also salivary glands were taken into account. A dysfunction of these glands can cause xerostomia, a very debilitating side effect of irradiation. The results of this study indicate an HBO-influenced reduction in the acute inflammatory damage caused by radiotherapy. This leads to an increase in the preservation of specialized tissues, which can explain a reduction in the long-term complications of radiotherapy.

In the studies of Muhonen et al[27-32], all animals were subjected to DO. This technique is clinically used in the treatment of various deficiencies of the maxillofacial skeleton. An osteotomy is performed in hypoplastic bone, after which the bone segments are slowly driven apart by a distraction device. Thus the growth of new bone tissue between the bone segments is stimulated, resulting in lengthening of the distracted bone. The force that is generated during DO causes severe damage to irradiated bone and delays its regeneration. The authors, therefore, investigated whether the use of HBO can help to successfully perform DO in irradiated bone of the head and neck region. Three of the studies investigated the osteoblastic activity in the bone of the mandible[27-29], whereas another 3 studies focused on the temporomandibular joint[30-32], of which one was specially focused on the condylar cartilage[32]. Different methods were used to measure osteoblastic activity, including fluoride [18F] positron emission tomography, morphometry, and immunohistochemistry. Two of the studies carried out in the temporomandibular joint concluded that HBO did not have a positive effect or, at best, a scant limitation to the radiation-induced damage[30, 32]. However, the study that used positron emission tomography to measure osteoblastic activity found an increase in this activity in high-dosage radiotherapy, albeit to a lesser extent than in lower-dosage radiotherapy, and thus concluded HBO to be beneficial[31]. The 3 other studies focusing on the mandible resulted in more positive conclusions regarding the effect of HBO[27-29]. In general, all reported a higher osteoblastic activity because of HBO, although this activity never reached the level of nonirradiated bone.

One study did not find a direct effect of HBO on the osteogenic rate but hypothesized that HBO might improve bone healing because of a lengthening of the period of high osteoblastic activity, rather than an increase of the activity itself[29]. Remarkably, in the control mandibles (without DO), the osteoblastic activity did not differ between all groups. Neither irradiation nor HBO had an effect on the basic osteogenic rate, which could be expected. It has to be noted that all of the studies were done by the same research group. Clark et al[26] performed a similar study but were unable to show a significant difference in percentage of bone fill and bone mineral density of irradiated bone with or without HBO therapy. A general remark on the previously mentioned studies is that the long-term effects were not considered, because the animals were sacrificed 4 weeks after the DO was completed.

In the experimental studies mentioned, HBO had either no effect or a positive effect on the irradiated tissue, whereas no negative effects were reported. There is no general consensus on the mechanism of action and the efficacy of HBO.

Clinical research

Reports on clinical research regarding the effect of HBO on previously irradiated tissue are more abundant than experimental studies. Twenty clinical studies were found that met our criteria. Different strategies were used to investigate the effect of HBO (Table 1). Thirteen studies were retrospective, and the findings should therefore be viewed with some caution. As Hess[62] stated, results of retrospective studies are, at best, hypothesis generating. Indeed, conclusions are carefully described, ranging from "HBO seems to be an efficacious treatment modality for many radiation- induced late side effects[37]" to "this paper supports existing literature on the potential benefit of HBO as a prophylactic agent and adjunctive treatment of ORN[39]".

Another serious limitation is that 60% (12) of the studies lack a control group. This is because of the retrospective nature of these studies and ethical considerations. Because HBO therapy has become a common recommendation for most patients with radiation-induced late side effects, it is considered unethical to withhold them from this therapy[53, 63]. In contrast, Annane et al[35] terminated their prospective clinical trial because of potentially worse outcomes in the HBO-treated group and concluded that HBO should not be recommended to treat patients with overt mandibular ORN. However, a discussion arose regarding the thoroughness of this study. Laden[64] states that, because of the termination, at least 75% of the treatment group did not receive the protocol minimum of 30 treatments. The study is "under-powered," and; therefore, Laden is surprised by the unequivocal statement made by Annane et al. Rogers[65] shares the same concern, by wondering whether a single trial with 30 patients in each group can decide policy. In his opinion, this study highlights the need for robust randomized trials, rather than concluding that the use of HBO

should cease in this field. van Merkesteyn and Bakker[66] criticize the study protocol by stating that twice-daily HBO treatment is in contrast to the majority of reports in the literature, in which once-daily treatment is used.

Some studies used QoL questionnaires to evaluate the effect of HBO[37, 44, 48, 50]. Despite the fact that these questionnaires are validated, the subjective character of these data plays a major role. Bui et al[37] used QoL scores in a retrospective study, relying on the patient's ability to recall symptoms they experienced. However, results were compared with notes of the HBO physician who prospectively documented the progress of major symptoms, and they correlated with the patients' response in 98% of cases. This is probably because late radiation side effects are severe and therefore easily remembered. In a prospective study, difficulties with QoL scores can also arise, as is seen in the study of Teguh et al[50]. They found a difference in baseline value for the European Organization for Research and Treatment of Cancer (EORTC) Head and Neck Cancer Module (H&N35) QoL questionnaire dry mouth questions. The patients in the non-HBO group had higher scores (subjective dryer mouth) than the HBO group before treatment was started. It is discussed that a possible reason could be that some of the patients who knew they were not scheduled for HBO argued that they must have a dry mouth to some extent because the purpose of the study was to investigate potential successful treatment of xerostomia with HBO therapy. Teguh et al concluded that a placebo effect could not be totally disproved. Another bias in research that uses QoL scores, according to Schoen et al[48], is that the HBO treatment is an extra burden and, especially if small effects are achieved, this could negatively influence QoL results in these patients. However, this indicates that the effect of HBO is not worth the effort according to the patients, rather than being a bias.

It must be noted that some of the studies have a rather small sample size, diminishing the power of these studies. Furthermore, in 60% (12) of the studies, no statistical analysis was conducted. Conclusions in these studies are based on the comparison of percentages.

In general, 75% (15) of the studies mention a positive effect of HBO therapy on the prevention or treatment of ORN, as well as on the survival rate of implant placement in previously irradiated head and neck tissue. As outlined previously, findings must be read with caution, and in some of the studies, conclusions are merely suggestions rather than statements. It is remarkable that, although almost all studies suggested the use of an extensive randomized controlled trial to investigate the efficacy of HBO, until now, no such trial has been executed. This is probably because of the difficulties that arise when planning such an elaborate investigation, ethical issues included. The only authors who present skepticism about this idea are Schoen et al[48]. They state that their small randomized trial shows that a very large population is needed to detect a clinically significant benefit of HBO treatment and

even doubt whether such a large randomized controlled trial will actually prove beneficial.

Despite the lack of randomized controlled trials, the literature suggests a positive role for HBO in the treatment and prevention of ORN. However, opposing views on this subject exist. Several review articles advocate the use of HBO[67-69], whereas others build a case against it[60, 70, 71]. Arguments against the use of HBO mainly focus on the scant evidence for the necessity of HBO treatment and on the high costs of the therapy.

This review aims to give an overview of experimental and clinical studies regarding the effect of HBO therapy on previously irradiated head and neck tissue in the last 2 decades. Experimental research is scarce, and the power of clinical research is limited because of the lack of randomized controlled trials. HBO is used clinically, which is remarkable considering the existing opposing views on its efficacy and value. It is therefore concluded that more research, both clinical and experimental, is necessary before solid conclusions can be drawn. In experimental studies the mechanism of action of HBO should be further unraveled, the results of which can possibly be implemented in clinical research to generate an accurate protocol for the prevention and treatment of radiation- induced late side effects in the head and neck region.

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Chapter *3*

A hyperbaric oxygen chamber for animal experimental purposes

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ABSTRACT

Facilities for hyperbaric oxygen therapy that are suitable for animal experimental research are scarce. In this paper, the authors introduce a hyperbaric oxygen chamber that was developed specifically for animal experimental purposes. The hyperbaric oxygen chamber was designed to meet a number of criteria regarding safety and ease of use. The hyperbaric oxygen chamber conforms to 97/23/EC (Pressure Equipment Directive), Conformity Assessment Module G Product Group 1. It provides easy access, and can be run in manual mode, semi-automatic mode and full-automatic mode. Sensors for pressure level, oxygen level, temperature, humidity and carbon dioxide level allow full control. This state-of-theart hyperbaric oxygen chamber for animal experimental purposes permits the investigation of the biological mechanisms through which hyperbaric oxygen therapy acts at a fundamental level.

INTRODUCTION

Patients who are treated for head and neck cancer often undergo a combination of tumour resection and radiotherapy. Tumour resection often involves surgical removal of parts of the surrounding normal tissues, such as jaw bone, tongue and floor of the mouth as well. This may result in large tissue defects, which need to be corrected by means of reconstructive surgery. Concurrent radiotherapy may result in decreased regenerative capacity of these normal tissues. As a result, wound healing after reconstructive surgery is compromised, which can have negative consequences for the patient's quality of life.

Hyperbaric oxygen therapy (HBOT) is considered to be able to partly or completely reduce radiation-induced tissue damage by stimulating cell proliferation, neovascularisation, and oxygenation in the irradiated tissues. Thus, HBOT improves the success rate of reconstructive surgery in previously irradiated tissues. Although HBOT has been clinically applied for several years, the exact biological mechanisms through which HBOT acts are not completely understood. The scientific literature shows that there is need for further pre-clinical research on this topic[1].

Animal studies that focus on investigating the effects of HBOT on irradiated tissues of the head and neck area are desired but HBOT facilities that are suitable for animal experimental research are scarce. Clinical HBOT facilities are often not suitable for various reasons. In this paper, the authors introduce a hyperbaric oxygen chamber that was developed specifically for animal experimental purposes.

CHARACTERISTICS OF THE HYPERBARIC OXYGEN CHAMBER

The hyperbaric oxygen chamber (HBO Test Vessel P1460) (Fig. 1) has been developed in collaboration with a company with extensive expertise in the field of hyperbaric technology (Hytech BV, Raamsdonksveer, the Netherlands, http://www.hyperbaric-technology.com). The hyperbaric oxygen chamber was designed to meet a number of criteria regarding safety and ease of use. The HBO chamber conforms to 97/23/ EC (Pressure Equipment Directive), Conformity Assessment Module G Product Group 1. It has been certified by TÜV (TÜV Industrie Service GmbH, TÜV SÜD Gruppe, Dampf- und Drucktechnik, München, Germany). The laboratory in the Erasmus Laboratory Animal Science Centre, where the HBO chamber is located, has been modified to meet the requirements for safe installation of hyperbaric oxygen chambers. The HBO chamber is connected to the central oxygen supply system of the laboratory, which provides pressurized pure oxygen so separate oxygen bottles are not needed to supply the HBO chamber with oxygen. This eliminates the risk of running out of oxygen during hyperbaric oxygen treatment because of the limited volume of an oxygen bottle. There is no need to replace depleted oxygen bottles

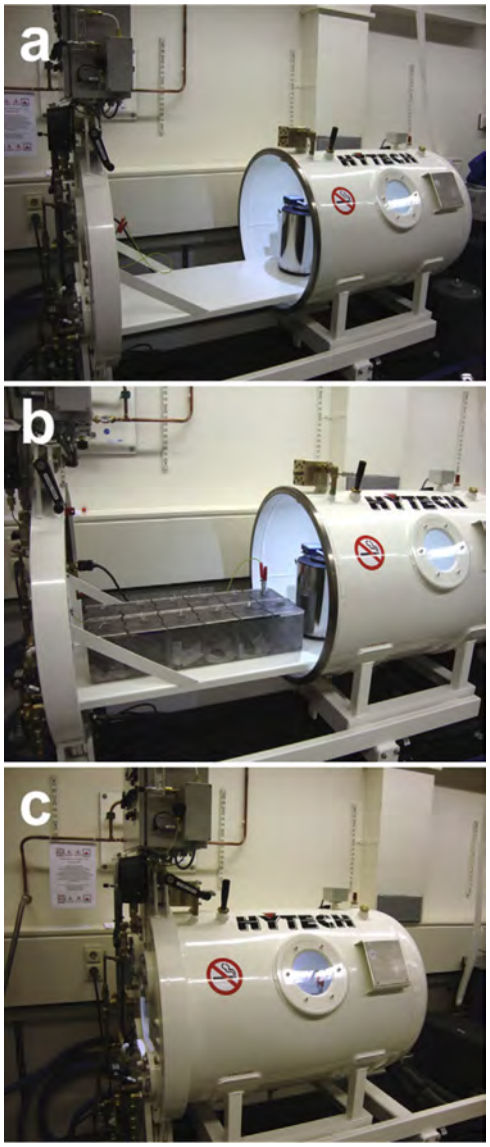

Figure 1 The hyperbaric oxygen chamber is large enough to accommodate the most commonly used experimental animals. (a) In the open position, the platform on which the animals are placed is easily accessible. (b) Compartmentalized cages are used to treat up to 24 mice simultaneously. (c) In the closed position, a safety pin and valve prevent the chamber from opening whilst pressurized. High-pressure proof acrylic windows and two bright LED lights allow a clear view into the chamber.

with full ones, which saves time and minimizes the chances of operator errors whilst changing the oxygen bottles.

The cylindrical HBO chamber is 70 cm in diameter and 115 cm long. The HBO chamber has a volume of 420 l and weighs 600 kg. Inside the chamber there is a level, rectangular platform, on which animal cages can be placed, measuring 95 cm in length and 47.5 cm in width. Despite its weight, the roll-out unit and hand grip allow the HBO chamber to open and close smoothly. A safety pin is integrated to prevent the HBO chamber from opening whilst under pressure. The maximum

working pressure for this vessel is 5 bar (equivalent to approximately 5 atmosphere absolute, whereas the normal air pressure at sea level is 1 atmosphere absolute). A safety valve is present which gradually reduces the pressure in case it exceeds 5 bar.

By installing the HBO chamber on a storage trolley suitable for its dimensions and weight, the HBO chamber is mobile and can be easily transported if necessary. All the materials used are suitable for exposure to pure oxygen and high pressure. The materials used should be able to withstand corrosion and should not be flammable in contact with pure oxygen. The maximum working pressure of 5 bar is equivalent to the weight of a column of water at approximately 50 m depth, therefore the materials used should be able to withstand such high pressure.

When the connector is plugged into a single-phase 230 V AC electric socket, all electrical devices on the HBO chamber are supplied with electricity. These electronic devices can be controlled from the control box and include a water machine (for circulating cool or hot water into the heating/cooling system), two LED lights (5 W each) inside the chamber, a gas/pressure analyser, a temperature analyser, a ventilator, a heating/cooling system, an alarm buzzer, and a data recorder (Fig. 2).

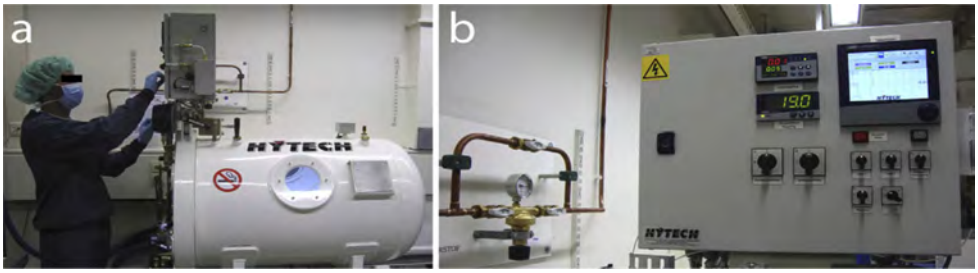

Figure 2 (a) A control box allows the operator to control the parameters inside the chamber. (b) The control box integrates switches, screens, and a data recorder, to control, visualize and record the various parameters.

The HBO chamber was designed to fit the most commonly used small laboratory animals, ranging from mice to rabbits. It is large enough to accommodate multiple animals simultaneously (the total number depending on the size of the animals used). There is easy access to the HBO chamber for placing the animals in the vessel, and the inner surfaces are easy to clean after use. A clear view into the vessel is provided by high-pressure proof acrylic windows, whilst the two LED lights provide sufficient light to be able to monitor the behaviour of the animals during treatment.

Human subjects who undergo hyperbaric oxygen therapy are usually placed in a chamber that is pressurized with normal air, whilst the pure oxygen is administered through oxygen masks. Animal subjects often do not tolerate such oxygen masks, so the HBO chamber is designed as a chamber in which the complete volume of air inside the vessel is replaced by pure oxygen. In this case, no oxygen masks are necessary.

Sensors are able to measure the oxygen concentration, the carbon dioxide concentration, the pressure, the temperature and the humidity inside the HBO chamber. A data recorder with thin-film transistor (TFT) screen is integrated to store the data on internal hard disk, compact flash memory card, or external USB device.

The system can run in three different modes: manual, semi-automatic, and fully automatic. When operating in manual mode, the operator controls the pressure level and oxygen level by adjusting the oxygen valves and purge flow meter by hand. The fully automatic mode relieves the operator from continually having to adjust the valves, by executing a program with pre-set values for pressure build-up time and desired oxygen level. The HBO chamber allows the operator to store up to ten pre-set programs. The semi-automatic mode can be used when the user needs to adjust the pressure and oxygen level manually at first, and let the automatic system take over to maintain the manually achieved oxygen and pressure levels.

The integrated climate control system helps to maintain a constant temperature in order to minimize temperature-related stress in the animals, as the temperature will fluctuate as a result of changes in pressure. A purge flow meter enables the operator to control the refresh rate of pure oxygen. This is important, as living creatures exhale carbon dioxide during their treatment inside the HBO chamber. By adjusting the purge flow meter, it is possible to maintain an oxygen concentration level of 100%.

DISCUSSION

Animal experimental studies allow the study of various parameters in a statistically relevant number of subjects, but facilities to study the effects of HBOT in animal models are often not available. Clinically used HBO chambers are often not suitable for use with animals for logistical reasons (e.g. no animal experimental work can be done during the time that the HBO chamber is in use by human patients), for ethical reasons (e.g. patients may reject the idea that the same facilities for human subjects are also used for animal experiments), or for practical reasons (e.g. the oxygen masks that are used for oxygen administration are suitable for human subjects but are not tolerated by animal subjects).

HBO chambers specifically suitable for animal experimental purposes are scarce. To the authors' knowledge, only Rech et al[2] have recently published a similar technical note on a hyperbaric oxygen chamber for animal use. Such an HBO chamber needs to comply with strict safety regulations, because high pressure and pure oxygen are involved. Such devices should only be manufactured by companies with extensive expertise in hyperbaric technology. The financial costs of developing such a device are high. The authors' research group has developed a state-of-the-art, safe and easy-to-use HBO chamber specifically for animal experimental purposes. This

allows for the investigation of the effects of hyperbaric oxygen therapy on tissue repair and regeneration on a fundamental level in the most commonly used small animal models.

Many animal experimental studies involve small animal models, such as mice and rats. To be able to keep mice safely in the HBO chamber, compartmentalized cages were fabricated to accommodate up to 12 mice per cage simultaneously. These cages are also suitable to accommodate rats; the compartments can be enlarged by removing the partitions between the compartments. A cage can provide space for up to 6 rats simultaneously. In the HBO chamber, there is room for two such cages, suitable for up to 24 mice or 12 rats simultaneously. The cages are made of corrosion-resisting steel, because of the corrosive nature of pure oxygen. The steel plates are punctured, giving a meshed appearance. This allows the oxygen to enter every single compartment, and also allows viewing inside the compartments to monitor the behaviour of the animals. Such custom-made cages can be fabricated for any other suitable small laboratory animal.

Regarding the effects of HBOT on the regeneration of irradiated head and neck tissues, the number of experimental papers is limited[1]. HBOT has been used clinically to treat radiation-induced damage for many years, but there remain opposing views on its efficacy[3-9]. This reflects the need for more in-depth animal experimental research on HBOT.

The authors' current pre-clinical research focuses on the effects of HBOT on radiation-induced oral tissue damage, but HBOT can be used as adjunctive or main treatment for various other medical indications. The authors' HBO chamber can be of value to many members of the scientific community who are interested in carrying out animal experimental research on hyperbaric oxygen therapy.

In conclusion, the authors have developed a state-of-the-art hyperbaric oxygen chamber specifically for animal experimental purposes. It meets the criteria regarding safety regulations and ease of use. This will create new possibilities for further in-depth research and strong collaborations in the field of hyperbaric oxygen therapy.

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Chapter 4

Hyperbaric oxygen therapy as a prevention modality for radiation damage in the mandibles of mice

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ABSTRACT

Background: Radiation therapy (RT) as part of the treatment of head and neck cancers often leads to irradiation of surrounding normal tissue, such as mandibular bone. A reduced reparative capacity of the bone can lead to osteoradionecrosis (ORN). Hyperbaric oxygen therapy (HBOT) is used to treat ORN, based on its potential to raise the oxygen tension in tissues. However, prevention of radiation-induced damage is of optimal interest. Our purpose was to investigate whether HBOT could prevent radiation-induced damage to murine mandibles.

Methods: Twenty-eight mice were irradiated in the head and neck region with a single dose (15 Gy) and half of them were subsequently subjected to HBOT. Another 14 mice did not receive any treatment and served as controls. Ten and 24 weeks after RT, mandibles were harvested and analysed histologically and by microcomputed tomography (micro-CT).

Results: Micro-CT analysis showed a reduction in relative bone volume by RT, which was partly recovered by HBOT. Trabecular thickness and separation were also positively influenced by HBOT. Morphologically, HBOT suppressed osteoclast number, indicating decreased resorption, and decreased the amount of lacunae devoid of osteocytes, indicating increased bone viability.

Conclusions: HBOT was able to partly reduce radiation-induced effects on microarchitectural parameters, resorption and bone viability in mouse mandibles. HBOT could therefore potentially play a role in the prevention of radiation-induced damage to human mandibular bone.

INTRODUCTION

Radiation therapy (RT) is a standard component in the protocol treatment of head and neck cancer. Inevitably, normal surrounding tissues, including maxillofacial bones such as the mandible, will also be exposed to RT. Radiation damages small arteries, reducing the circulation to the, already relatively poor vascularized mandible. This causes an impaired remodeling capacity of the bone, that can lead to a reduction in bone mass and bone density, and thus to more vulnerable bone[1]. The impaired reparative capacity of the bone especially poses a risk when trauma, such as subsequent surgery, biopsy or tooth extraction and consecutive implant placement, is inflicted on the previously irradiated bone. Due to the vascular damage, a hypoxic and hypovascular environment exists in which bone is more prone to inflammation, which can eventually lead to destruction of bone, so called osteoradionecrosis (ORN)[2].

ORN of the mandible is a serious long term side effect in patients that receive radiation as part of the treatment for head and neck cancer. It is a very painful condition that can present itself even years after RT and is difficult to treat. Treatment regimens depend on the grade of ORN; lower grade can be treated effectively by long term oral antibiotic therapy, while for more severe cases, removal of the affected bone might be necessary.

Hyperbaric oxygen therapy (HBOT) is used as an adjuvant therapy to treat ORN, or is used in a preventative manner when minor or major surgery is performed in irradiated bone. The rationale is that HBOT, in which patients breathe 100% oxygen under elevated pressure, raises the oxygen tension and can thereby positively affect the healing process. Although clinical studies report positive effects, a general consensus about the effectiveness of the therapy remains to be achieved[3, 4]. Experimental studies on the effects of HBOT on the treatment or prevention of ORN are especially scarce, while these kind of studies would provide a better understanding of the effects and working mechanism of HBOT. Furthermore, the potential of HBOT to protect bone from radiation damage before complications have arised, has not been investigated thoroughly.

In this study, we investigated the effects of HBOT, when given directly after RT, on irradiated mandibular bone of mice, by means of micro-CT and histology. We were particularly interested in the effect on bone microarchitecture and viability in non-traumatised bone, to assess if HBOT is able to prevent radiation-induced bone damage.

MATERIALS AND METHODS

Animals

Female C3H mice (Harlan Netherlands BV, Horst, the Netherlands), 7-9 weeks old at the start of experimentation were kept under standard housing conditions with free access to food pellets and acidified water. The mice were divided into three groups: control, radiation therapy (RT) and radiation therapy followed by hyperbaric oxygen therapy (RT+HBOT). At 10 and 24 weeks after RT, 7 mice of each group were sacrificed and mandibles were harvested for ex-vivo micro-CT scanning, after which they were used for histology. Animals were weighed frequently and given soft crushed food pellets to allow sufficient food intake after RT. The experimental protocol was approved by the Animal Care Committee of the Erasmus MC, Rotterdam, the Netherlands, under the National Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EC.

Radiation- and hyperbaric oxygen therapy

Radiation therapy and hyperbaric oxygen therapy were given as previously described[5]. In short, RT consisted of a single 15 Gy dose administered to the head and neck region of anesthetized mice. Mice in the RT+HBOT group received the first HBOT session the day after RT. In a HBOT session, mice breathed 100% oxygen at 2.4 atmospheres absolute during one hour in a hyperbaric chamber suitable for small laboratory animals [6]. Twenty consecutive sessions were carried out daily, except at saturdays and sundays.

Micro-CT scanning

Immediately after sacrifice by CO_2-asphyxiation, at 10 and 24 weeks after RT, mandibles were harvested and fixated in 10% buffered formalin. Microcomputed tomography (micro-CT) was used to analyze bone parameters. Micro-CT scans of the tissue blocks were made with a SkyScan 1076 in vivo microCT scanner (SkyScan, Aartselaar, Belgium) and manufacturer's scanning software. Examination consisted of a scout view, selection of region of interest, off-line reconstruction, and evaluation. Serial transverse scan images were made at a resolution of 18 µm. Nrecon 1.3 (SkyScan, Aartselaar, Belgium) and CT Analyser 1.3.2.2 (SkyScan, Aartselaar, Belgium) software were used to reconstruct the data for analysis. A 3-dimensional volume of interest was created by applying interpolation between 2-dimensional free-hand selections of the mandibular bones. Within the volume of interest, the relative bone volume (BV/TV) was determined to quantify new bone formation, as well as trabecular number (TbN), trabecular thickness (TbTh) and

trabecular separation (TbSp).

Histology

After micro-CT scanning, mandibles were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 12 days, after which they were dehydrated and embedded in paraffin blocks. 5 µm slides were sagitally cut and standard hematoxylin eosin (HE) staining was performed. Per slide, the amount of empty lacunae and osteocytes were counted in 2-3 fields (20X magnification). Empty lacunae were expressed as a percentage of the total count of osteocytes. Adipocyte density in bone marrow (number and area of adipocytes per mm^2 bone marrow) was quantified using Image J version 1.45b (National Institutes of Health, Bethesda, USA). Tartrate-resistant acid phosphatase (TRAP) staining was used to stain osteoclasts. Slides were first incubated for 20 minutes in 0.2M acetate buffer with 50mM L(+) tartaric acid (ICN Biomedicals Inc, Aurora, USA). Then, 0.5 mg/ml naphthol AS-MX phosphate (Sigma-Aldrich Corp., St. Louis, USA) and 1.1 mg/ml fast red TR salt (Sigma-Aldrich Corp., St. Louis, USA) were added, after which slides were incubated for 60 minutes at 37°C, rinsed in distilled water, counterstained with hematoxylin and embedded with vectamount. The number of positively stained osteoclasts per mm bone marrow perimeter was counted.

Statistical analysis

All data are expressed as mean values with standard error of the mean (SEM), and were analysed using SPSS PASW 21.0 for Windows (SPSS Inc., Chicago, USA). The Shapiro-Wilk test was used to test for normality, followed by the Mann-Whitney *U* test for the comparison of non-normally distributed data, while Student's T-test was used for normally distributed data. $P<0.05$ indicated significant differences.

RESULTS

Micro-CT

Figure 1 shows bone parameters quantified by micro-CT scanning in the mandibles of mice, 10 and 24 weeks after radiation therapy. Figure 1A shows the volume of interest, between the red lines, that was selected. At ten weeks post-RT, no differences between groups were found in bone volume (Figure 1B), trabecular thickness (Figure 1C), trabecular separation (Figure 1D) and trabecular number (Figure 1E). However, porosity (Figure 1F) was significantly increased in the RT-group (36.6 ± 1.1%) compared to controls (31.3 ± 2.0%; $p<0.05$). HBOT given after RT reduced the percentage of porosity towards control levels (30.7 ± 2.0%; $p<0.05$).

Twenty-four weeks after RT, no differences in the percentage of porosity were present anymore. However, the effect of RT became evident in the other parameters

measured. Relative bone volume (BV/TV) significantly decreased in the RT-group (57.2 ± 0.6%) compared to control (63.0 ± 1.1%; p<0.01). When HBOT was given after RT, relative bone volume increased towards control levels (62.2 ± 3.0; p<0.05 for RT vs. RT+HBOT). The same pattern was seen for the trabecular thickness (control 0.171 ± 0.005 mm; RT 0.145 ± 0.003 mm; RT+HBOT 0.157 ± 0.007 mm; p<0.001 for control vs. RT; p<0.05 for RT vs. RT+HBOT). Trabecular separation was inversely affected, with a higher separation of trabeculae in RT (0.108 ± 0.002) compared to control (0.100 ± 0.002 mm; p<0.05) and oppositely a reduction in trabecular separation due to HBOT (0.096 ± 0.008 mm; p<0.05). Trabecular number was increased by RT (3.96 ± 0.06 vs. 3.70 ± 0.04 in controls; p<0.01), whereas the administration of HBOT after RT did not influence trabecular number (3.95 ± 0.04) compared to RT.

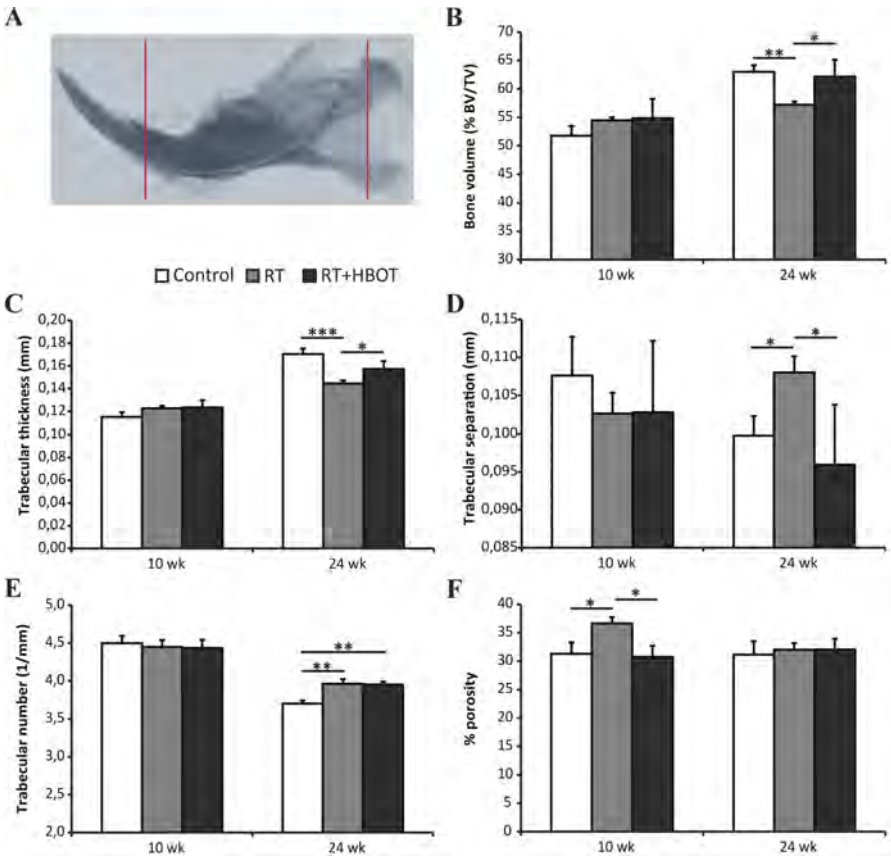

Figure 1 Micro-CT analysis. Micro-CT analysis was performed in mandibles of mice; the volume of interest is indicated between red lines (**A**). Relative bone volume (**B**), trabecular thickness (**C**), trabecular separation (**D**), trabecular number (**E**) and percentage porosity (**F**) were calculated, at 10 and 24 weeks after radiation therapy. RT = radiation therapy, HBOT = hyperbaric oxygen therapy. Lines indicate significant differences between groups.

Histology

The amount of empty lacunae, devoid of osteocytes, reflects bone viability and has been reported to be increased after RT[7,8]. In our study, the percentage of empty lacunae increased at 24 weeks after RT (3.69 ± 0.41% vs. 1.26 ± 0.43% in controls; p<0.01), while no effect was seen at 10 weeks post-RT (Figure 2A). HBOT had a positive effect since a lower percentage of empty lacunae was found at 24 weeks post-RT (2.34 ± 0.31%; p<0.05 for RT vs. RT+HBOT).

Adipocyte density (Figure 2B) of bone marrow was increased by RT, at both 10 weeks (62.1 ± 14.6 vs. 8.2 ± 4.4 in controls; p<0.05) and 24 weeks post-RT (49.88 ± 13.5 vs. 13.8 ± 7.2 in controls; p<0.05). HBOT did not have an effect on adipocyte density.

The number of bone-resorbing osteoclasts (Figure 2C) was increased by RT at both time-points, however not significantly at 24 weeks post-RT (1.68 ± 0.34 vs. 0.07 ± 0.04 in controls at 10 weeks, p<0.05; 1.48 ± 0.55 vs. 0.66 ± 0.17 in controls at 24 weeks). HBOT decreased osteoclast number at 24 weeks post-RT compared to RT treatment alone (0.38 ± 0.17 in RT+HBOT vs. 1.48 ± 0.55 in RT; p<0.05).

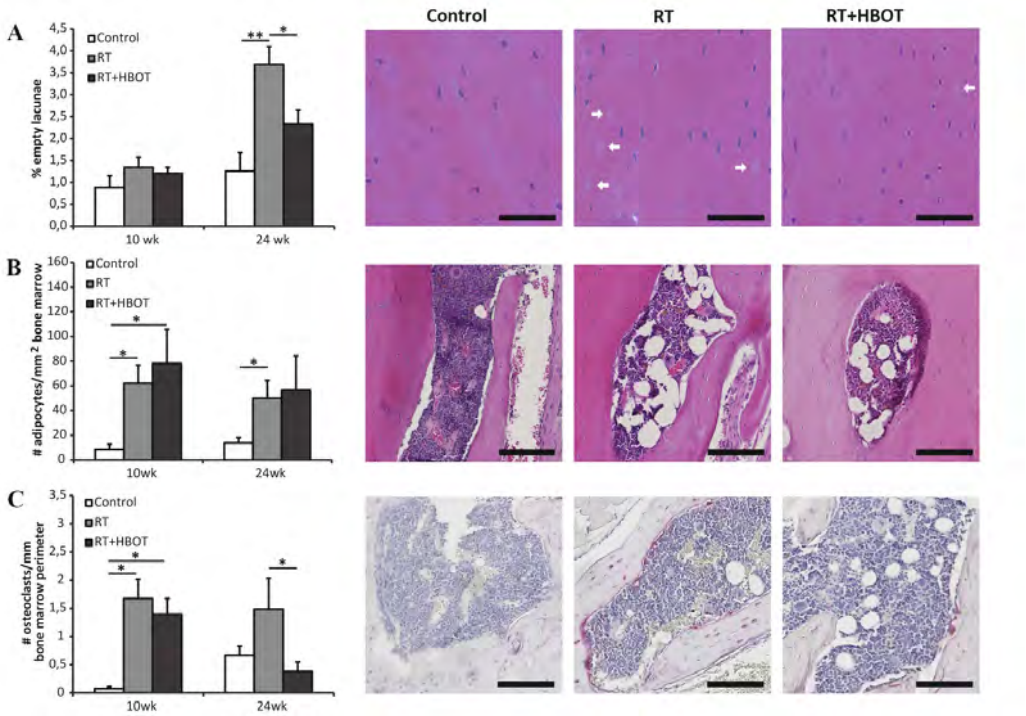

Figure 2 Morphological analysis. Quantification of morphological parameters, with pictures from different groups at 24 weeks postRT. The percentage of empty lacunae is shown in **A**. White arrows depict empty lacunae, which are more abundant in the RT-group. Scale bars represent 50 µm. Adipocyte density is shown in **B**, with an increased density in both RT-treated groups. Scale bars represent 100 µm. **C** represents the number of osteoclast per mm bone marrow perimeter. Osteoclasts are stained red by TRAP staining and are more abundant in the RT-group. Scale bars represent 100 µm. RT = radiation therapy, HBOT = hyperbaric oxygen therapy. Lines indicate significant differences between groups.

DISCUSSION

Radiation therapy, used as a standard component in the protocol treatment of head and neck cancer, inevitably exposes surrounding normal tissues to radiation. Maxillofacial bones that are in the field of irradiation become more vulnerable to fractures and have a decreased regenerative capacity. Reconstructive surgery on irradiated bones therefore often gives suboptimal results[9,10]. Treatments that can improve the condition of tissues that suffer from radiation-induced damage are desired.

Hyperbaric oxygen therapy is used in the treatment of osteoradionecrosis, as well as necrosis of the bone due to bisphosphonates[11]. It is also used in a preventative manner when dental alveolar surgery and implantology need to be performed in previously irradiated mandibular bone. However, its effectiveness remains debatable, with an increasing amount of studies reporting a lack of effect and advocating the need for randomized controlled trials[3, 12-14]. Also, the mechanism of action is not fully understood[4, 15].

In the present study, we investigated if HBOT was able to protect the mandibular bone from long-term radiation effects. For this purpose, microarchitecture and morphology were assessed at 10 and 24 weeks after RT and compared with controls.

Micro-CT analysis is a powerful and non-destructive tool to quantify bone properties on a microstructural level. Its advantage over histologic analysis is that this technique enables the measurement of various parameters in larger volumes of bone, whereas histological assessment of bone microarchitecture relies on the extrapolation of measurements performed in a few slides [16]. Relative bone volume (BV/TV), trabecular number (TbN), trabecular thickness (TbTh) and trabecular separation (TbSp) are the most common parameters used to describe bone microarchitecture. BV/TV, TbN and TbTh have been shown to decrease in response to radiation in animal models, whereas TbSp increases after RT[7, 16-18]. These changes in bone microarchitecture result in reduced strength of the bone, which thus becomes more vulnerable to fractures.

Our study is in accordance with previous studies and showed a decrease of BV/TV and TbTh 24 weeks after radiation therapy, whereas TbSp was increased. Surprisingly, TbN was increased due to radiation. Apart from the percentage of porosity, no effect was seen at 10 weeks after RT, while other studies showed changes in microstructure at the same or earlier time-points[7, 16-18].This could be due to the use of different animal models with varying RT dose and administration in these studies, making it difficult to compare time-points. Most studies are performed in hind limbs, while the effects of RT on mandibular bone are less well studied. Damek-Poprawa and colleagues[7] have established that the onset of osteoradionecrosis is more rapid in

the mandibles than in the tibias of rats. A 50 Gy single dose, administered locally to the mandible was used in this study, whereas we used a 15 Gy single dose which comprised the head and neck area. These differences could account for the fact that in our study, effects on bone microarchitecture were only visible 24 weeks after RT, as compared to effects seen on 10 weeks after RT in the study of Damek-Poprawa.

In addition to the observed microarchitectural changes, morphological changes due to RT were also observed. Adipocyte density in the bone marrow increased at both time points studied. The increase of adipocytes in bone marrow as a consequence of radiation has been reported[19]. Mesenchymal stem cells (MSCs) in the bone marrow can differentiate into osteoblasts and adipocytes[20]. Radiation probably targets the MSCs and disrupts the balance between MSCs osteoblast- and adipocyte differentiation. Adipocytes secrete anti-osteogenic factors and therefore decrease bone formation and bone mass[21], probably due to an induction of apoptosis of bone-forming osteoblasts and increased proliferation and differentiation of bone-resorbing osteoclasts[22]. Osteoclast number was indeed also increased after RT in our study, indicating increased bone resorption.

The number of empty lacunae, which represents loss of osteocytes, increased under the influence of RT at 24 weeks post-RT. Osteocytes derive from osteoblasts that have become 'trapped' in the matrix they have produced themselves. They keep contact with each other and with the osteoblasts and osteoclasts that reside at the bone surface by cytoplasmic extensions called dendrites, that extend into channels in the matrix[23]. In this way, osteocytes send signals for bone resorption and formation to osteoclasts and osteoblasts, respectively, and therefore play an essential role in bone maintenance and remodeling[24]. Dying or dead osteocytes have been suggested to stimulate resorption[25, 26]. Various studies on the effects of radiation on bone use the increased amount of empty lacunae as a measure for radiation damage[7, 8, 27-29] and our results confirm the radiation-induced loss of osteocytes.

Effects of hyperbaric oxygen on (irradiated) bone have not been studied extensively. Experimental animal studies are mainly conducted in either distracted, previously irradiated, bone or include the placement of implants. Moderately positive effects of HBOT on osteoblastic activity have been reported in irradiated distracted bone (reviewed in [4]). Implantology is usually performed in the hind legs of rats, mice or rabbits, which differ from the predominantly cortical bones of the head and neck region, since they mainly consist of cancellous bone. Small improvements in bone formation, implant-bone contact and force needed to unscrew implants due to HBOT were reported[4]. Williamson and colleagues subjected rats to RT followed by HBOT and investigated bone 36 weeks after RT[30]. They counted the number of

lacunae filled with osteocytes and reported a positive effect (i.e. higher number of filled lacunae) in the group treated with RT and HBOT compared with the group that was irradiated. Our findings, at 24 weeks after RT, correspond with these results, as HBOT was also able to positively influence osteocyte count and thus the viability of mandibular bone. In addition, we found positive effects of HBOT on microstructural parameters that were negatively influenced by RT, and a suppression of osteoclasts.

The effects of HBOT on osteogenic cells, i.e. bone-forming osteoblasts and bone-resorbing osteoclasts, have been studied *in vitro*. Positive effects of HBOT on proliferation and/or differentiation of osteoblasts have been described[31, 32], while Wong and colleagues found no effect of HBOT on normal or irradiated osteoblasts[33]. Osteoclast formation has been shown to be suppressed due to HBOT[34]. In our *in vivo* study, osteoclast number was decreased by HBOT, at 24 weeks after RT, indicating a reduction of bone resorption. Together with the decreased number of empty lacunae, this may have led to the increased relative bone volume seen by micro-CT analysis.

The clinical relevance of our results remains to be elucidated. It is to be defined if the single radiation dose of 15 Gy (biologically equivalent to a cumulative dose of 32 Gy) that was used in our experimental model leads to the same degree of bone damage as the fractionated radiation dosing schemes that are clinically used. Furthermore, it is clinically important to investigate whether the observed HBOT induced changes cause stronger bone and an improved regenerative capacity when trauma is inflicted. Further experimental investigation followed by randomized clinical trials are therefore desired.

CONCLUSION

Radiation irreversibly damages bone and other specialized tissues. Therefore, the prevention of radiation-induced damage to bone would be preferable to the treatment of complications. Our results showed that HBOT was able to positively influence microarchitectural parameters of irradiated mandibular bone of mice, suppress osteoclast number and increase bone viability. This could indicate that HBOT has potential to be used as a prevention modality for radiation-induced bone damage. Further research will be necessary to evaluate whether the observed microstructural and morphological changes of irradiated bone can indeed cause stronger bone, less vulnerable to microfractures or other trauma which can lead to ORN.

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Chapter 5

Effects of hyperbaric oxygen therapy on the viability of irradiated soft head and neck tissues in mice

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ABSTRACT

Objectives: Hyperbaric oxygen therapy (HBOT) is used clinically in irradiation-induced injury to healthy tissues, but the effectiveness and working mechanism remain unclear. This study examined the effects of HBOT on irradiated salivary glands and tongue in a mouse model.

Materials and methods: Mice were irradiated with a single dose (15 Gy) in the head and neck region and subjected to HBOT, either before or after irradiation. During the course of the treatments, salivary flow rates were measured and at different time points after radiation (2, 6, 10 and 24 weeks), salivary glands and tongue were harvested and (immuno) histochemically analysed.

Results: Proliferation and blood vessel density in salivary glands were enhanced by HBOT in the medium term (10 weeks after irradiation), while salivary flow rates were not influenced. In the long term, irradiation-induced proliferation in the muscle tissue of the tongue was decreased by HBOT.

Conclusion: Hyperbaric oxygen therapy (HBOT) appears to stimulate regeneration or protection of salivary gland tissue following radiation therapy. Possible implications of the effect of HBOT on muscle tissue of the tongue for the prevention of dysphagia and trismus are discussed. This study provides insights on the cellular changes after HBOT and encourages further research on this topic to achieve a better implementation of the therapy in humans.

INTRODUCTION

Head and neck cancer is most commonly treated by radiotherapy (RT), alone or in combination with chemotherapy or surgery. RT causes not only injury to the malignant tumour, but also to surrounding healthy tissues. The salivary glands and tongue are often in the radiation field and suffer from the adverse effects of RT. Acute effects include mucositis and hyposalivation, of which the latter often persists into a chronic stage called xerostomia[1]. Patients with xerostomia have difficulties with speech, mastication, swallowing and suffer from impairment of their taste and sleep patterns[2-4]. Therefore, the quality of life of these patients is significantly reduced[5]. To date, no prevention modality or treatment exists that offers protection or full recovery from xerostomia. Prevention strategies currently used for xerostomia include the surgical transfer of major salivary glands outside the radiation field, intensity modulated radiotherapy to spare salivary glands and the use of cytoprotectants such as amifostine[1, 6]. However, these interventions are not applicable to all patients and, in the case of amifostine, there is still controversy regarding possible tumour protection and toxic side effects[7].

Current treatments of xerostomia rely on the stimulation of the residual secretory capacity of salivary glands via sialogogues, or, if this is insufficient, the use of saliva substitutes[1]. However, these have to be taken for the rest of the patients' lives, with their associated side effects, as the therapeutic effect directly ceases when the administration is stopped. Therefore, the need for a therapy that stimulates salivation is evident.

Other debilitating consequences of RT in the head and neck region include dysphagia (swallowing problems) and trismus (an inability to normally open the mouth due to muscle rigidity)[8, 9]. Damage of the tongue can be a factor in the development of RT-induced dysphagia[10]. Dysphagia can result in aspiration pneumonia and permanent or long-term feeding tube dependence, whereas patients with trismus often suffer from malnutrition, difficulty in speaking and compromised oral hygiene.

Hyperbaric oxygen therapy (HBOT) has been used to treat radiation injuries since the early 1970s[11]. Patients breathe 100% oxygen at elevated pressure, typically 2–3 atmospheres absolute (ATA). The increased pressure, combined with pure oxygen breathing, will increase the portion of oxygen that is carried in solution. This leads to a 10-fold increase in the oxygen tension in tissues[12]. As the oxygen is in solution, it can also reach obstructed areas where red blood cells cannot pass and it can diffuse further into poorly vascularised regions. Hyperoxia has been shown experimentally to increase angiogenesis by elevating vascular endothelial growth factor (VEGF) levels[13] and to facilitate the deposition of collagen fibres around newly formed blood vessels[14, 15]. In this way, HBOT may be able to overcome the progressive loss of

the microvasculature resulting in chronic tissue hypoxia that is present in radiation-induced damage.

However, experimental as well as clinical evidence on the effects and possible risks of HBOT is still scarce, and in addition, its working mechanism not completely understood[16, 17]. In this study, the effects of HBOT, given before or after irradiation, on salivary glands and tongue were studied. In order to do this, we used a mouse model in which the animals were followed for up to 24 weeks after RT to the head and neck region to assess the effects on chronic radiation damage.

MATERIALS AND METHODS

Animals

Female C3H mice, 7–9 weeks old at the time of experimentation, were purchased from Harlan (Harlan Netherlands BV, Horst, the Netherlands) and were kept under standard housing conditions with free access to food pellets and acidified water. They were allowed to acclimatise for at least one week before experimentation started. The study comprised two consecutive experiments. For the 10-week experiment, 84 mice were divided into four groups: HBOT only (HBOT, n = 21), RT only (RT, n = 21), RT followed by HBOT (RT+HBOT, n = 21) and HBOT followed by RT (HBOT+RT, n = 21). At 2, 6 and 10 weeks, seven mice from each experimental group were sacrificed by CO2 asphyxiation. Five additional animals served as non-treatment controls. For the 24-week experiment, 21 mice were evenly assigned to three groups (HBOT, RT and RT+HBOT) and sacrificed at 24 weeks. Furthermore, seven mice did not receive any treatment and were sacrificed after 24 weeks. Schematic representation of the study design is shown in Figure 1. To assure sufficient food intake, animals were weighed frequently and all irradiated animals were given crushed food pellets mixed with water. The experimental protocol was approved by the Animal Care Committee of the Erasmus MC, Rotterdam, the Netherlands, under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of 'Guidelines on the protection of experimental animals' by the Council of Europe (1986), Directive 86/609/EC.

Radiotherapy

Mice were anesthetised intraperitoneally (i.p.) with a mixture of ketamine and xylazine (120 mg kg^{-1} and 6 mg kg^{-1} body weight, respectively). The head and neck region was locally irradiated with a single dose of 15 Gy by a 250 kV orthovoltage irradiator (Philips RT250, Philips Medical Systems, Brussels, Belgium) using a Cu filter and a dose rate of 1.9 Gy min^{-1}. The rest of the body was shielded by a 0.5 cm lead plate.

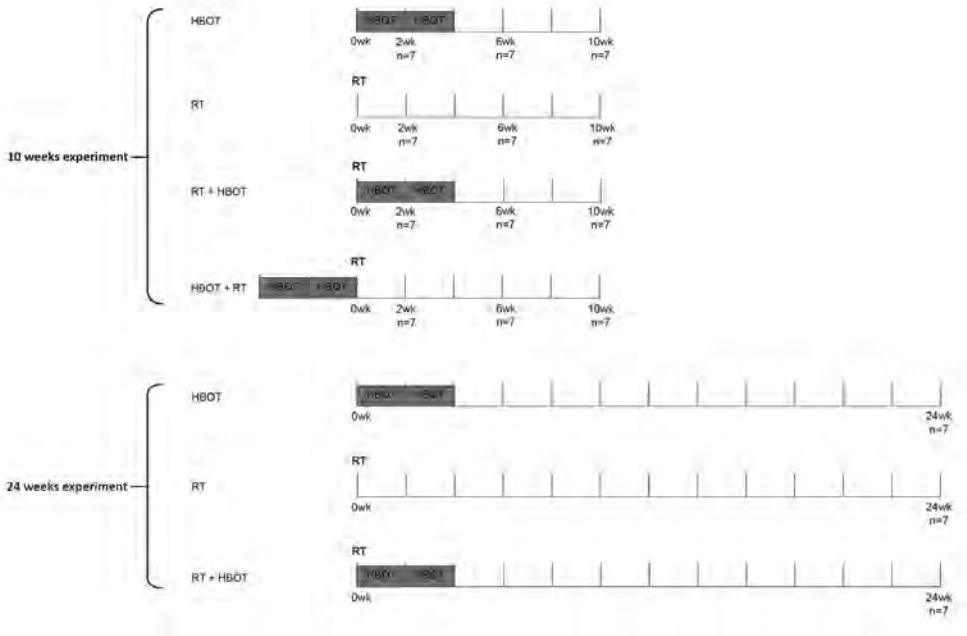


Figure 1 Study design. Mice received either RT, HBOT, or a combination and were sacrificed at 2, 6, 10 or 24 weeks (n = 7 for each group and timepoint) whereafter tissues were harvested for immunohistological analyses. Salivary flow rates were measured the day before sacrifice, and every other week in the 24-week experiment and controls. HBOT consisted of 20 sessions divided over a 4-week time period, and RT consisted of a single dose of 15 Gy to the head and neck area. HBOT, hyperbaric oxygen therapy; RT, radiotherapy.

Hyperbaric oxygen therapy

The hyperbaric oxygen chamber used in this study was custom-built for small laboratory animals (Hytech BV, Raamsdonksveer, the Netherlands)[18]. HBOT consisted of 20 daily sessions, given over a 4-week time period. Each session started with a compression phase of 30 min, during which the pressure in the chamber was elevated to 2.4 atmospheres absolute (ATA) and the oxygen level to 100%. These parameters were kept constant during the isopression phase, which lasted 1 h. Finally, decompression to 1 ATA took another 15 min. All sessions were carried out in the morning. For group RT+HBOT, the HBOT started the day after RT; for group HBOT+RT, RT was given the day after completion of HBOT.

Saliva collection

Whole saliva was collected the day before animals were sacrificed (10-week experiment) or every other week starting at 10 weeks (24-week experiments). In 24-week control mice, saliva was collected at the same time points used in the 10- and

24-week experiments. Mice were injected i.p. with pilocarpine (2 µg g^{-1} bodyweight, Sigma-Aldrich BV, Zwijndrecht, the Netherlands). Animals were manually fixed and saliva was collected for 10 min, starting 2 min after pilocarpine injection. Saliva was pipetted directly from the mouth and collected in preweighed Eppendorf tubes. Saliva volume was determined gravimetrically, assuming a density of 1 g ml^{-1} of saliva[19], and flow rates (µl min^{-1}) were calculated.

Histology and immunohistochemistry

Immediately after sacrifice, parotid glands, submandibular glands and tongue were excised and stored in 10% buffered formalin for 24–36 h. All tissues were then dehydrated, embedded in paraffin blocks, and 5-µm slides were cut. A standard haematoxylin–eosin staining was carried out to address overall morphology, whereas the periodic acid–Schiff (PAS) staining was used to identify functionally active acinar cells in the salivary glands. In short, slides were rehydrated, quenched in 0.5% periodic acid solution for 5 min, followed by 15 min in Schiff's reagent, before dehydration and embedding with Permount® (Fisher Scientific, Pittsburgh, PA, USA). For immunohistochemistry, sections were probed with primary antibodies against Ki67 (Novus Biologicals Ltd., Cambridge, UK), cleaved caspase-3 (Cell Signaling Technology Inc., Danvers, MA, USA) and CD-31 (Abcam, Cambridge, UK) to assess proliferation, apoptosis and blood vessel density, respectively. Biotinylated goat antirabbit IgG (Dako, Carpinteria, CA, USA) was used as secondary antibody. Detection of the antibody complex was performed with streptavidin–peroxidase (R&D Systems, Oxon, UK) and 3,3'-diaminobenzidine (Dako). Haematoxylin served as counterstain.

Quantification

Slides were scanned using a slide scanner (Hamamatsu Photonics K.K., Hamamatsu, Japan). For both submandibular and parotid glands, Ki67-positive cells or CD31-positive blood vessels were counted in a representative field (magnification 10x). For the tongue, Ki67-positive cells were counted in three fields per slide (magnification 10x), taken from dorsal, medial and ventral parts. Caspase-3-positive cells were counted in sections of the whole submandibular gland and expressed as the number of positive cells per mm^2 gland tissue.

Statistical analysis

All data are expressed as mean values with standard deviation (s.d.) and were analysed using SPSS PASW 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Univariate tests with *post hoc* Bonferroni correction were used to identify statistical differences ($P < 0.05$) between groups within the same time point. *T* tests were used to compare individual groups with non-treated controls.

RESULTS

Effects of HBOT in the short/medium term

To investigate the effects of HBOT on head and neck tissues in the short and medium term after RT, salivary glands and tongue of locally irradiated mice were analysed at 2, 6 and 10 weeks after RT.

Salivary glands

Salivary flow rate was reduced by half at 6 and 10 weeks ($P < 0.001$) after RT and was not positively influenced by HBOT (Figure 2a). Overall morphology of irradiated submandibular glands is depicted in Figure 2b for the different time points. At 10-week post-RT, some enlarged nuclei were present and the tissue showed some signs of disorganisation, but no gross changes in the tissues were visible. HBOT treatments did not influence morphology (data not shown).

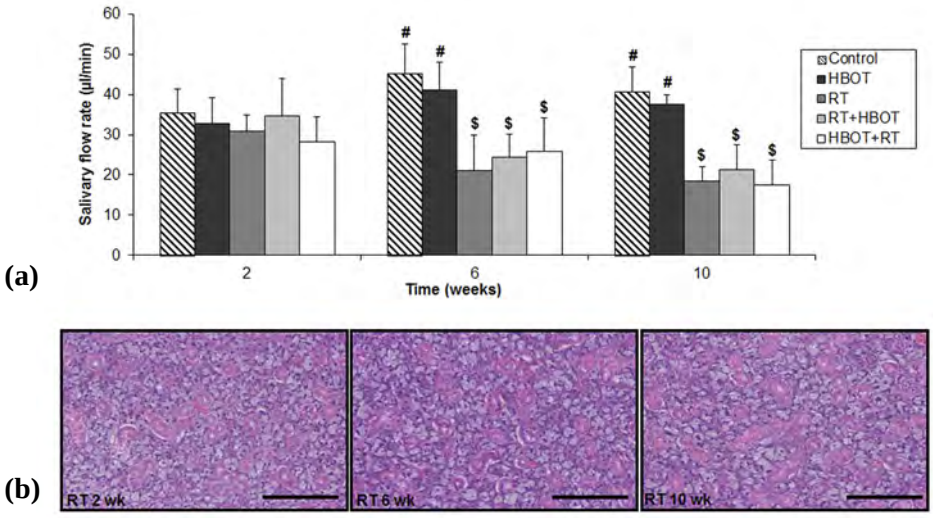


Figure 2 Short-/medium-term effects of RT and HBOT on salivary flow rates and overall morphology of submandibular glands. (a) Salivary flow rates following pilocarpine injection expressed as µl min-1 (mean + s.d.). Within each time point, groups with different symbols (#, $ or o) are statistically different from each other. (b) H&E stainings of submandibular gland tissue of RT group at different time points. At 10 weeks after RT (right panel), slight disorganisation of acinar cells with some enlarged nuclei is visible. Scale bar 200 µm. HBOT, hyperbaric oxygen therapy; RT, radiotherapy.

Proliferation, as measured by the number of Ki67-positive cells (Figure 3a), decreased after RT compared with non-treated controls in both submandibular- and parotid glands (submandibular gland: $P < 0.01$ at 6 weeks and $P < 0.05$ at 10 weeks; parotid gland $P < 0.01$ at 6 and 10 weeks). HBOT given after RT increased proliferation compared with RT alone for the submandibular gland at 10 weeks ($P <$

0.001) and for the parotid gland at 6 and 10 weeks ($P < 0.001$). HBOT given before RT increased proliferation compared with RT alone in both glands at 6 and 10 weeks after RT (submandibular gland: $P < 0.05$ at 6 weeks and $P < 0.001$ at 10 weeks; parotid gland: $P = 0.001$ at 6 and 10 weeks). The proliferation levels of HBOT-treated irradiated groups were comparable to nontreated control levels.

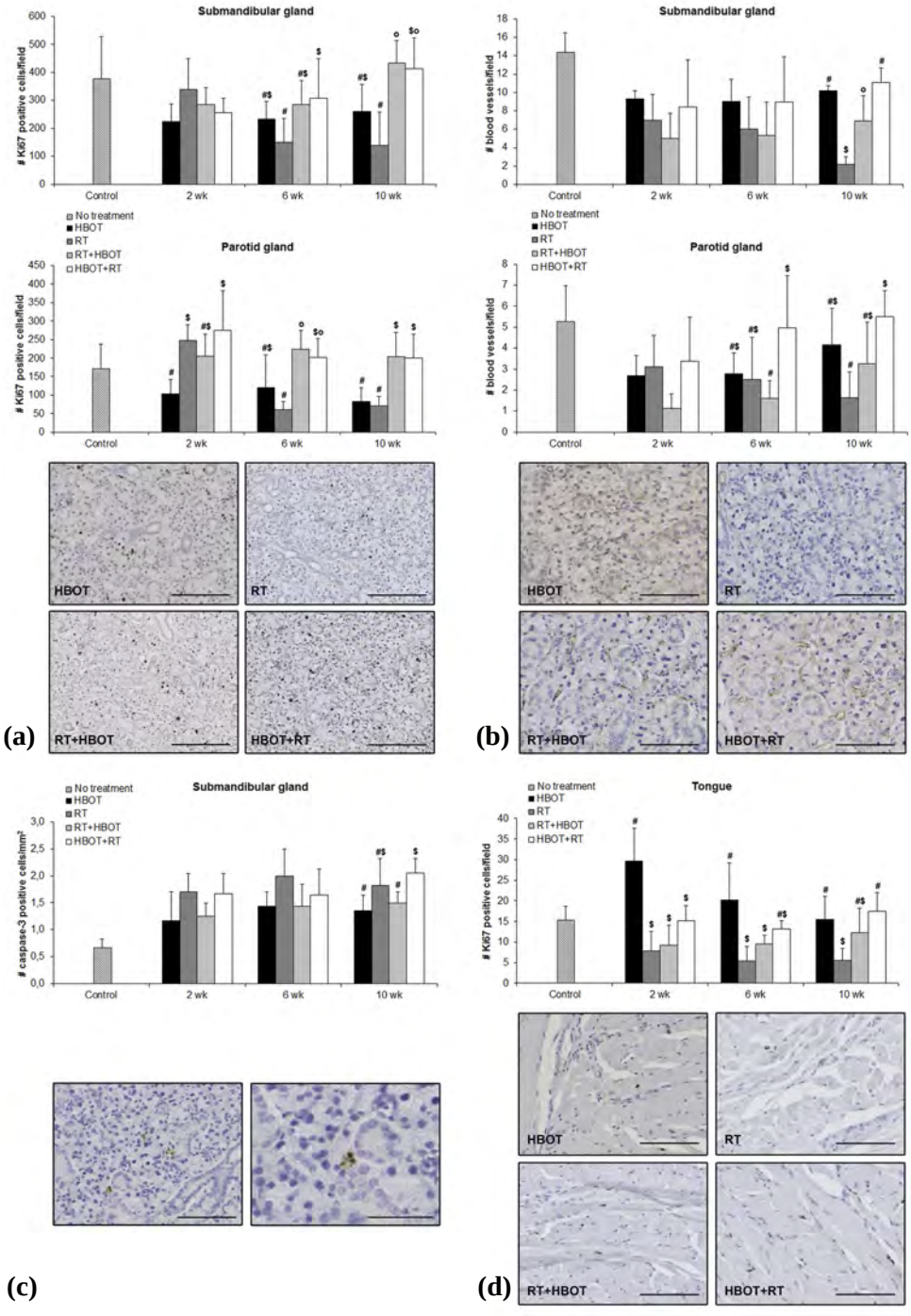
In the submandibular gland, blood vessel density (Figure 3b) decreased after RT ($P < 0.001$). When HBOT was given after RT, blood vessel density was higher compared with RT alone at 10 weeks (threefold, $P < 0.001$). The effect of HBOT on blood vessel density on this time point was even higher when HBOT was given before RT (fivefold, $P < 0.001$). The same pattern was seen in the parotid gland, with the largest effect of HBOT on blood vessel density when it was given before RT ($P < 0.01$).

Staining for cleaved caspase-3 showed no significant effects of HBOT on the degree of apoptosis in irradiated submandibular glands (Figure 3c). Only at 10-week post- RT, HBOT given before RT showed significantly more apoptosis compared with when it was given after RT ($P < 0.05$).

Tongue

No differences in proliferation of the basal layer of the tongue were seen (data not shown). In the muscle tissue, the level of proliferation (Figure 3d) decreased after RT ($P < 0.01$ at 2 and 6 weeks, $P < 0.001$ at 10 weeks). However, when HBOT was applied before or after RT, at 10 weeks after RT, proliferation values were higher ($P < 0.01$ for HBOT+RT) and were comparable to the non-treated groups.

Figure 3 (*next page)* Immunohistochemical analysis of the short-/medium-term effects of RT and HBOT. (a) Quantification of proliferating (Ki67-positive) cells in submandibular and parotid glands (mean + s.d.) and Ki67 immunostaining of submandibular gland tissue from different groups at 10 weeks (scale bar 200 µm). (b) Quantification of CD31-positive blood vessels in submandibular and parotid glands (mean + s.d.) and CD31 immunostaining of submandibular gland tissue from different groups at 10 weeks (scale bar 100 µm). (c) Quantification of apoptotic (caspase-3-positive) cells in submandibular gland (mean + s.d.) and immunostaining of caspase-3-positive cells (scale bar left panel 100 µm, right panel 50 µm). (d) Quantification of proliferating (Ki67-positive) cells in the muscle tissue of the tongue (mean + s.d.) and Ki67 immunostaining in this tissue from different groups at 10 weeks (scale bar 100 µm). Within each time point of each graph, groups with different symbols (#, $ or o) are statistically different from each other. HBOT, hyperbaric oxygen therapy; RT, radiotherapy.


Submandibular gland
Ki67 positive cells/field
Control
2 wk
6 wk
10 wk
No treatment
HBOT
RT
RT+HBOT
HBOT+RT
Parotid gland
blood vessels/field
HBOT
RT
RT+HBOT
HBOT+RT
(a)
(b)
caspase-3 positive cells/mm²
Tongue
(c)
(d)

Effects of HBOT in the long term

In order to investigate whether the observed HBOTinduced short-/medium-term changes in the irradiated tissues could ultimately lead to better functionality, a subset of conditions were also analysed at 24-week post-RT.

Salivary glands

Salivary flow rates decreased after RT, as was also seen in the 10-week experiment, and showed no sign of improvement during the time frame of our study, which confirms the chronicity of hyposalivation. HBOT given directly after RT did not increase salivary flow rates, and the administration of HBOT alone resulted in salivary flow rates comparable to controls (Figure 4a). HE and PAS stainings clearly showed the effect of radiation on the submandibular gland at 24-week post-RT (Figure 4b). The tissue was notably disorganised, with many enlarged nuclei, inflammatory cells and a striking decrease in acinar cells. The administration of HBOT did not influence overall morphology as seen in HE and PAS stainings (data not shown).

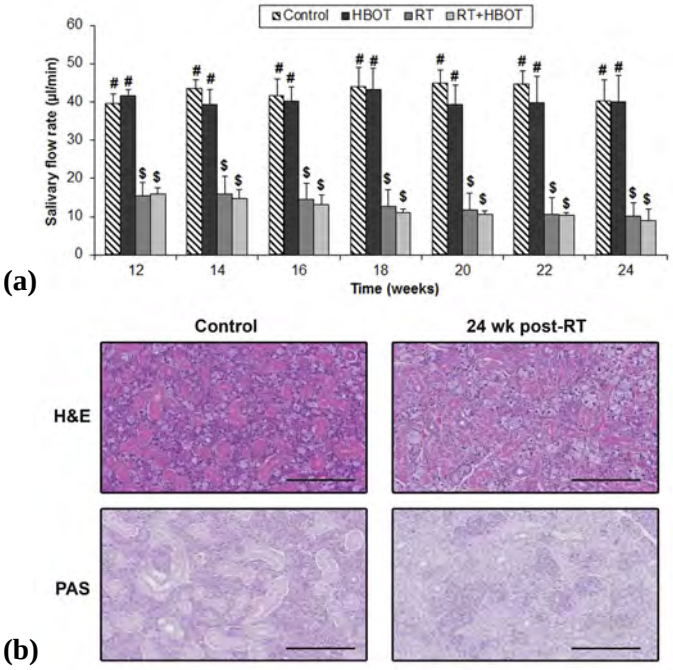


Figure 4 Long-term effects of RT and HBOT on salivary flow rates and overall morphology of the submandibular gland. (a) Salivary flow rates following pilocarpine injection expressed as µl min-1 (mean + s.d.). Within each time point, groups with different symbols (# or $) are statistically different from each other. (b) Haematoxylin eosin (top panels) and periodic acid–Schiff stainings (bottom panels) of control (left panels) and RT-treated (right panels) submandibular gland tissue. Scale bars 200 µm. HBOT; hyperbaric oxygen therapy, RT, radiotherapy.

Radiotherapy (RT) caused no difference in proliferation rate 24-week post-RT when compared with controls in both salivary glands (Figure 5a,b). HBOT did not affect proliferation rates of irradiated tissue, but remarkably, in the non-irradiated submandibular gland, proliferation was significantly reduced after HBOT compared with all other groups ($P < 0.001$, Figure 5a). Blood vessel density was increased when HBOT was given after RT in the parotid gland compared with non-irradiated tissues ($P \leq 0.001$, Figure 5b). Apoptosis was only measured in submandibular glands and showed to be decreased in all groups compared with control ($P = 0.01$ for HBOT, $P = 0.001$ for RT and $P = 0.000$ for RT+HBOT, Figure 5a).

Tongue

As in the short-/medium-term experiment, no differences were seen in proliferation rates of the basal layer of the tongue mucosa (data not shown). The muscle tissue showed a significant five times increase in the amount of proliferating cells when irradiated tissue was compared with control tissue ($P < 0.001$, Figure 5c). HBOT significantly decreased this number ($P < 0.01$), whereas on healthy tissue, HBOT did not have an effect.

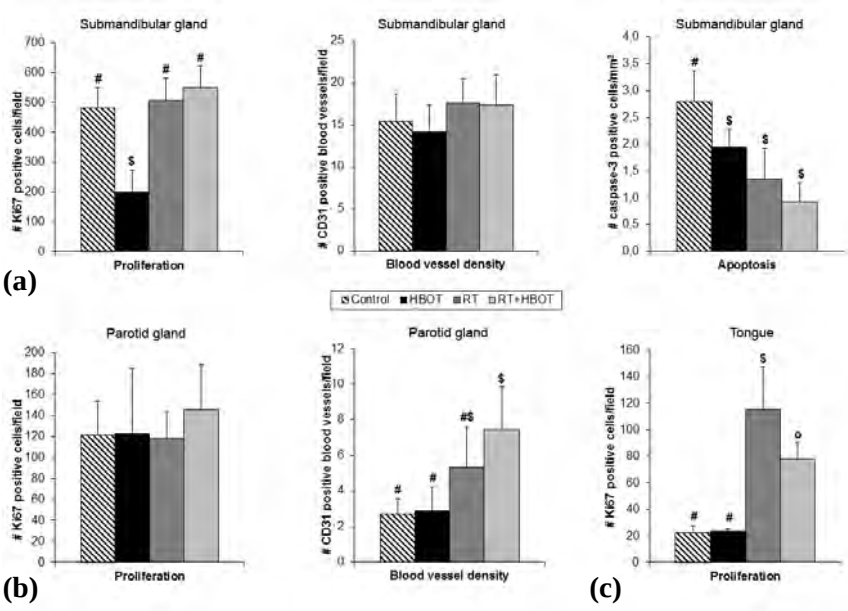


Figure 5 Immunohistochemical analysis of long-term effects of RT and HBOT. (a) Quantification of proliferation (left), blood vessel density (middle) and apoptosis (right) in submandibular gland tissue. (b) Quantification of proliferation (left) and blood vessel density (right) in parotid gland tissue. (c) Quantification of proliferation in tongue tissue. Within each graph, groups with different symbols (#, $ or o) are statistically different from each other. HBOT, hyperbaric oxygen therapy; RT, radiotherapy.

DISCUSSION

Hyperbaric oxygen therapy (HBOT) is used to treat radiation-induced damage, based on the principle that increased oxygen tension in the damaged tissues will facilitate repair and regeneration. Still, controversy exists about the efficacy and exact working mechanism of this therapy[17]. Our study therefore focused on the ability of HBOT to prevent radiation-induced damage to head and neck tissues, namely salivary glands and tongue.

Clinical studies have investigated the ability of HBOT to treat or prevent radiation-induced damage to salivary glands by measuring salivary flow rates and/or patient-scored xerostomia[20-22]. Despite the fact that some of these studies report a positive influence of HBOT on xerostomia complaints[17], we do not find an effect of HBOT on salivary flow rate in our mouse model. However, most clinical studies only measured patient-scored xerostomia and did not objectively determine salivary flow rates. When both parameters are investigated, there is often no correlation between the two[20]. Possibly, other factors such as saliva composition contribute more to the dry mouth feeling than salivary flow rate.

To our knowledge, there is only one animal study in which the effect of HBOT on irradiated salivary glands was investigated[23]. This study examined overall gland morphology, without measuring salivary flow rates. In our study, salivary flow rates declined after RT and were not stimulated by HBOT given either before or after RT. The decrease in salivary flow rate preceded histological changes as flow rate dropped already with more than 50% at 6-week post-RT, whereas morphological changes were most dominantly present at 24-week post-RT. This dissociation between structural and functional changes after irradiation has been shown before[24-27] and supports the theory that the acute drop in salivary flow results from damage to the membrane of acinar cells, whereas at later stages, acinar cell number decreases due to DNA damage.

More profound effects of HBOT were seen when the salivary gland tissue was investigated immunohistochemically. Proliferation markedly increased by HBOT given before or after RT, in both the submandibular gland and the parotid gland. Increased proliferation in response to HBOT has been observed in other tissues[15, 28-30]. In these studies often only short-term effects are addressed, whereas we show that in slowly dividing submandibular gland tissue, proliferation was elevated by HBOT at 10-week post-RT to levels comparable to non-treated controls. Proliferation was dominantly seen in the acinar cells, which are differentiated cells that have retained the ability to replicate. No increased proliferation was observed in the intercalated ducts where the stem cells reside[31]. This suggests an effect of HBOT on normal homeostasis mechanisms, which eventually could lead to improved regeneration of the salivary gland after RT.

Blood vessel density in irradiated salivary glands was higher after HBOT at 10-week post-RT, which suggests a protective effect of HBOT on radiation-induced blood vessel loss, or a stimulatory effect on angiogenesis in the medium term. The angiogenic potential of HBOT has been proposed before[13-15, 30, 32, 33].

Remarkably, in the muscle tissue of the tongue, proliferation was increased at 24 weeks in the RT group, whereas RT+HBOT proliferation levels were significantly lower. This long-term effect has not been reported before, and the consequences could have interesting implications. As has been shown in other tissues[34, 35], higher proliferation rates may be the result of an increased rigidity in the extracellular matrix, a well-known phenomenon associated with radiation injury. The lower proliferation levels observed after HBOT could indicate that tissue rigidity is reduced and that HBOT thus might have a positive effect on side effects of radiation injury such as trismus or dysphagia. Some clinical studies indeed mention positive effects of HBOT on swallowing[21, 36], but further research will be needed to elucidate this possible mechanism of action.

The use of HBOT either before or directly after RT gave comparable patterns of response regarding proliferation in the short-/medium-term experiment. The stronger effect of HBOT given before RT on blood vessel density could be due to the fact that angiogenesis is already triggered in the healthy tissue before radiation exerts its effects. Apoptosis was higher when HBOT was given before RT compared with when it was given after RT at 10 weeks. Different studies describe the effect of HBOT on apoptosis in various tissues; both induction and attenuation of apoptosis in the short term have been reported, while the long-term effect is often not considered[37-39]. As salivary flow rates were not affected by HBOT either given before or after RT, this study is not conclusive on the preferable timing of HBOT. On the basis of our results in the short/medium term, and the fact that HBOT application after RT approaches the clinical situation more closely, only the HBOT after RT group was used in the long-term experiment. Also, caution should be taken when giving HBOT before RT in patients, as the effects of HBOT on tumour tissue are not yet fully known. Some studies claim that HBOT does not promote growth or recurrence of tumours[40, 41], whereas tumour growth after HBOT has also been reported[42].

Hyperbaric oxygen therapy (HBOT) did affect irradiated salivary gland tissue at the cellular level, in terms of proliferation and blood vessel density. However, in our model, these changes did not result in an increased salivary flow rate. The radiation dose of 15 Gy used in our mouse model has been used in various animal studies and has proven to cause significant gland impairment[43-47]. Clinically, fractionated radiation schemes are used with fractions of maximal 2 Gy day^{-1} with a cumulative dose of 24–26 Gy[48], while most animal studies use single-dose radiation for practical and ethical reasons. The single dose of 15 Gy used in our study is biologically equivalent to a clinically relevant scheme of 16 fractions of 2 Gy; however, Coppes

et al[49] showed higher radiosensitivity of the submandibular gland for the late effects after fractionated irradiation compared with single-dose irradiation. Possibly, our radiation dose was too high for the translation of the cellular effects of HBOT into functional differences.

In the current experimental study, it appeared that HBOT stimulates the viability of radiation-injured tissues, by an enhanced protection and/or a stimulation of the regeneration process. A better understanding of the cellular changes that take place after HBOT in different tissues may contribute to an improved implementation of the therapy in humans.

Acknowledgements

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Chapter 6

The effects of heparan sulphate mimetic RGTA-OTR4120 on irradiated murine salivary glands

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ABSTRACT

Background: This study focuses on the potential of ReGeneraTing Agent OTR4120 (RGTA-OTR4120) to treat radiation-induced damage of salivary glands. RGTAs are biopolymers designed to mimic the effects of heparan sulphate, thereby stimulation tissue repair and regeneration.

Methods: C3H mice were irradiated with a single dose of 15 Gy in the head and neck region. RGTA-OTR4120 was injected 24 h after radiotherapy, followed by weekly injections. At 2, 6 and 10 weeks after radiotherapy, salivary flow rates were measured and animals were sacrificed to obtain parotid and submandibular glands for histology. Periodic acid Schiff stain was performed to visualize mucins that are produced by acinar cells. Amylase and total protein content were measured in saliva samples.

Results: Salivary flow rates were increased at 2 weeks, but not at 6 and 10 weeks after radiotherapy with RGTAOTR4120 administration, compared to irradiated controls. Two and 10 weeks after radiotherapy, the mucin production activity of acinar cells was increased under influence of RGTA administration. RGTA-OTR4120 did not influence amylase or total protein secretion.

Conclusion: RGTA-OTR4120 administration has a positive effect on salivary flow rates in irradiated mice on the short term. The effect was absent 10 weeks after radiotherapy, while at that time point, mucin producing activity of acinar cells was elevated by RGTA-OTR4120 administration. Given these results and the advantages of RGTA use in irradiated patients, further investigation on the potential of this drug to treat radiation-induced salivary gland damage, alone or in combination with other drugs, such as amifostine, is suggested.

INTRODUCTION

Radiotherapy is an important treatment modality for patients with head and neck tumours. It uses high energy radiation to kill cancer cells by damaging their DNA, either directly or indirectly by the creation of free radicals. Unfortunately, healthy tissues surrounding the tumour are also affected. In the head and neck region, salivary glands are prone to experience such unwanted side-effects. In recent years, administration forms of radiotherapy have been improved in order to minimize damage to surrounding tissues, examples of which are intensity modulated radiation radiotherapy, hyperfractionated radiotherapy and accelerated fractionated radiotherapy[1]. However, because of the close proximity of the salivary glands to the treatment field, patients still suffer from significant side-effects. Due to radiation, the composition and amount of saliva changes, which often leads to xerostomia, a situation in which patients subjectively complain about a dry mouth[2]. This has negative effects on the quality of life of patients as they suffer from trouble with speech, mastication, swallowing, an impairment of taste and disturbed sleep patterns[3-5]. The problems tend to be life-long, with little or no improvement despite current treatments, which include the stimulation of the residual secretory capacity of the glands by sialogogues and the administration of salivary replacements[2]. However, these treatment modalities have considerable side-effects and, at maximum, may only partly improve patient's salivary condition.

Consequently, the need for therapies that can prevent or treat the damage to salivary glands caused by radiotherapy is apparent. Amifostine is the only currently available preventive therapy and is based on the free radical scavenging properties of the drug. Side-effects include hypotension, vomiting and allergic reactions[6], further indicating the necessity for new therapies.

This study focuses on the potential of ReGeneraTing Agent OTR4120 (RGTA-OTR4120) to treat radiation-induced damage of the salivary glands. RGTAs are biopolymers engineered to mimic heparan sulphate, stimulating tissue repair and regeneration[7]. Heparan sulphate is present in the extracellular matrix (ECM) and binds heparan-binding growth factors (HBGFs). These factors, which include fibroblast growth factors-1 and -2 and vascular endothelial growth factor (VEGF), are important in tissue regeneration[8]. However, in injury, heparan sulphate is degraded and loses its ability to bind growth factors. RGTAs can take over the task of heparan sulphate, enhancing the bioavailability of HBGFs and promoting tissue repair. This has been shown in various experimental models including bone[9], muscle[10, 11], skin[12, 13] and mucosa[14, 15].

We examined the effects of weekly RGTA-OTR4120 administration on salivary flow rate, saliva composition and salivary gland histology in mice irradiated in the head and neck region, for up to 10 weeks following radiotherapy.

MATERIALS AND METHODS

Animals

Fifty-two female C3H mice, 7–9 weeks old at the time of experimentation, were purchased from Harlan (Harlan Netherlands BV, Horst, The Netherlands) and were kept under standard housing conditions with free access to food pellets and acidified water. They were allowed to acclimatize for at least 1 week before experimentation started. Mice were randomly divided into the following groups: (A) control (n = 7), (B) radiotherapy only (n = 21) and (C) radiotherapy with RGTA administration (n = 21). At 2, 6 and 10 weeks after radiotherapy, mice were sacrificed by CO2-asphyxiation (n = 7 for each time point). Animals were weighed frequently, and after irradiation, food pellets were crushed and mixed with water daily to allow sufficient food intake. A normal control-group of three mice was used to measure the effect of weekly administration of RGTA alone on the salivary flow rate of healthy (non-irradiated) mice after 2, 6 and 10 weeks of treatment. The experimental protocol was approved by the Animal Experiments Committee under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of 'Guidelines on the protection of experimental animals' by the Council of Europe (1986), Directive 86/609/EC.

Irradiation

The mice were anesthetized intraperitoneally (i.p.) with a mixture of ketamine and xylazine (120 and 6 kg/g body weight, respectively). The head and neck region was locally irradiated with a single dose of 15 Gy by a 250 kV orthovoltage irradiator (Philips RT250) using a Cu filter and a dose rate of 1.9 Gy/min. The rest of the body was shielded by a 0.5 cm lead plate.

RGTA administration

The heparan mimetic RGTA-OTR4120 was obtained from the Tissue Repair Laboratory (OTR3, Paris, France) and prepared as previously described (12). RGTA-OTR4120, with a concentration of 0.1 mg/ml in sterile physiological salt solution (B. BraunMelsungen AG, Germany), was administered i.p. (1 kg/g body weight) 24 h after radiotherapy, followed by weekly injections during the course of the experiment.

Saliva collection

Whole saliva was collected before animals were sacrificed. Mice were injected i.p. with pilocarpine (2 kg/g body weight). Animals were manually fixed and saliva was collected for 10 min, starting 2 min after pilocarpine injection. Saliva waspipetted directly from the mouth and collected in pre-weighed Eppendorf tubes. Saliva

volume was determined gravimetrically, assuming a density of 1 g/ml of saliva[16], and flow rates (µl/min/100 g body weight) were calculated.

Biochemical analysis

After collection, saliva samples were diluted 10 times and 100 times with demineralised water for total protein and amylase measurements, respectively. Samples were then stored at 80˚C until analysis. Total protein and amylase activity were measured using a Roche Modular P800 and two different assays that were executed fully automatically (Roche Diagnostics, Mannheim, Germany). In the total protein assay, samples were preincubated in an alkaline solution containing EDTA, which denatures protein. Benzethonium chloride, which reacts with protein, was then added to produce a turbidity that was read at 505 nm. Values were expressed as g/l. The principle of the assay for amylase activity is that the a-amylase in the sample cleaves 4,6-ethylidene-(G7)- 1,4-notrophenyl-(G1)-a,D-maltoheptaoside and the degradation products are subsequently hydrolyzed to p-nitrophenol (PNP) with the aid of a-glucosidase. The colour intensity of PNP is measured photometrically and is directly proportional to the a-amylase activity expressed as U/l.

Histology

Immediately after sacrifice, parotid and submandibular glands were excised and stored in 10% buffered formalin for 36 h. Tissues were then dehydrated, embedded in paraffin blocks and 5 µm slides were cut. One slide per animal was used for Periodic Acid Schiff (PAS) staining, which detects mucins. In short, slides were rehydrated, quenched in 0.5% periodic acid solution for 5 min, followed by 15 min in Schiff's reagent, before dehydration and embedding with Permount. Slides were scanned and the entire glands were then analyzed with Celld Imaging Software for Life Science Microscopy (Olympus Life Science Europe GmbH, Hamburg, Germany). For the first slide, the purple PAS staining was manually selected and automatically assigned an accompanying Red Green Blue-value that was subsequently used for all slides. The total area that contained this RGB-value (which represents positive PAS staining) was calculated by the program. In this way acinar staining was expressed as a percentage of the total acinar area.

Statistical analysis

Data are expressed as mean values ± standard error of the mean (SEM), and were analyzed using SPSS PASW RGTA-effects on irradiated murine salivary glands 17.0 for Windows (SPSS Inc., Chicago, IL, USA). The Student's t-test was used to identify significant differences ($P < 0.05$).

RESULTS

Salivary flow rate

A single dose of 15 Gy irradiation of the head and neck region caused a decrease in salivary flow rate compared to controls, which is statistically significant at 6 and 10 weeks after radiotherapy (Fig. 1; $P < 0.05$ at 6 weeks, $P < 0.01$ at 10 weeks). The administration of RGTA-OTR4120 to irradiated mice caused an increased flow rate at 2 weeks compared to the radiation only group ($P < 0.01$). This increased flow rate was comparable to that of the non-irradiated control. Furthermore, no drug-alone effect on salivary flow rates was seen when RGTA-OTR4120 was administrated to healthy controls (data not shown).

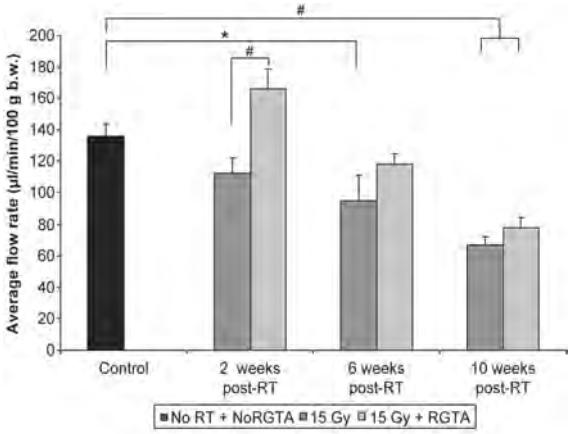

Figure 1 Salivary flow rates expressed as µl⁄min⁄100 g body weight (mean ± SEM). RT, radiotherapy. *Statistical difference of $P < 0.05$; #statistical difference of $P < 0.01$.

Histology

The PAS staining was used to identify mucins which are produced in acinar cells. Figure 2 shows examples of this staining in the submandibular gland. Control tissue (Fig. 2A and B) is compared with tissue that has been irradiated 10 weeks earlier, with (Fig. 2E and F) or without (Fig. 2C and D) the weekly administration of RGTA-OTR4120. The amount of staining, expressed as the percentage of PAS-positive area, was quantified (Fig. 3A and B for submandibular- and parotid gland, respectively). Radiotherapy caused a significant decrease of this percentage at all time-points ($P < 0.05$ at 2 and 6 weeks, $P < 0.01$ at 10 weeks). At 2 and 10 weeks after radiation, RGTA-OTR4120 administration increased the percentage of functionally active acinar cells significantly, towards a value comparable to non-irradiated controls ($P < 0.01$).

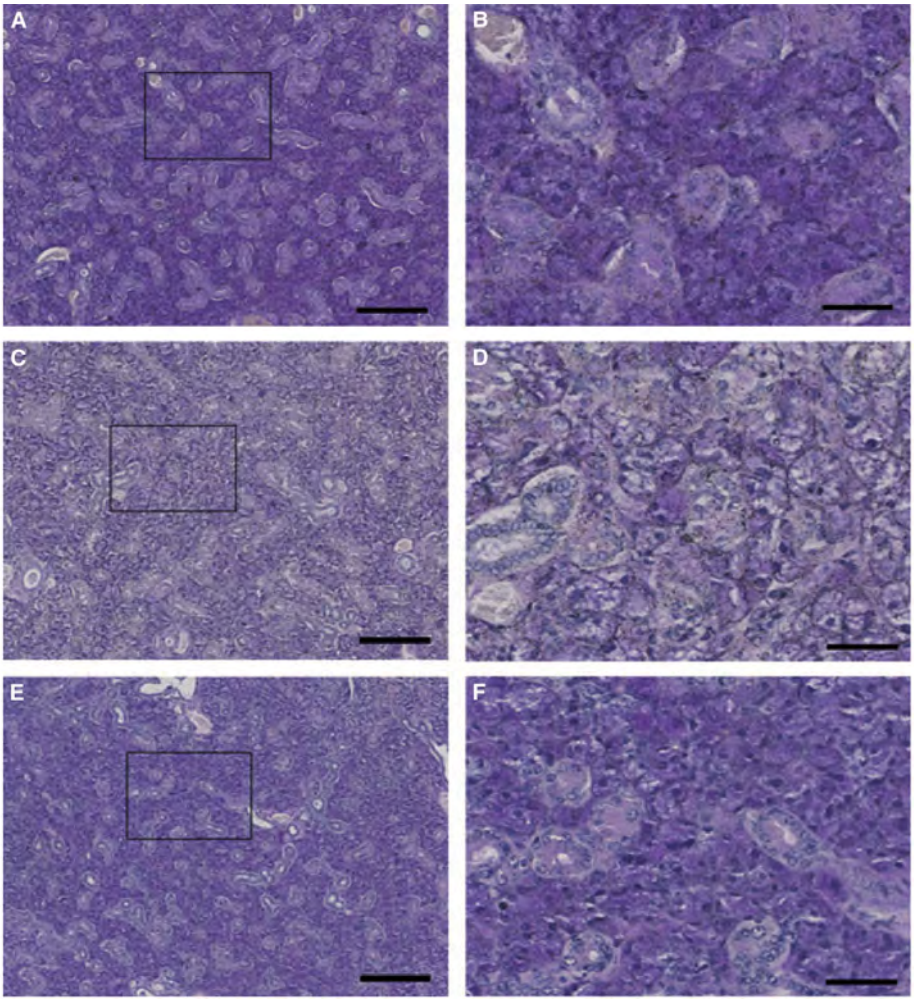

Figure 2 PAS staining in submandibular tissue. (A, B) Control; (C, D) 10 weeks after 15 Gy single dose irradiation; (E, F) 10 weeks after 15 Gy single dose irradiation with weekly administration of RGTA-OTR4120 (i.p., 1 µg/g body weight). A, C and E give an overview (scale bar 200 µm). Enlargements are shown in B, D and F (scale bar 50 µm). Control (A, B) and RGTA-treated (E, F) groups gave a strong purple staining of mucins, while in the radiated group (C, D) this staining was almost absent.

Biochemical analysis

Amylase and total protein secretion are expressed in Fig. 4A and B, respectively. The amount of amylase and total protein which was measured in saliva samples was multiplied by the salivary flow rate to generate the actual secretion. Both amylase and total protein secretion decreased in response to radiation. For amylase, this decrease was significant at 10 weeks after irradiation ($P < 0.01$), and for total protein at all time-points ($P < 0.01$ at 2 and 10 weeks, $P < 0.05$ at 6 weeks). RGTA-OTR4120 did not induce any significant differences.

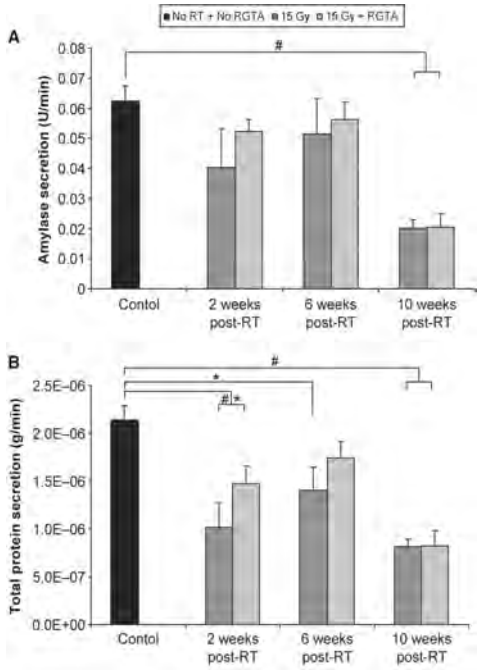

Figure 3 Percentage of PAS positive area of the total acinar area in the submandibular gland (A) and the parotid gland (B) (mean ± SEM). RT, radiotherapy. * Statistical difference of P<0.05; # statistical difference of P<0.01.

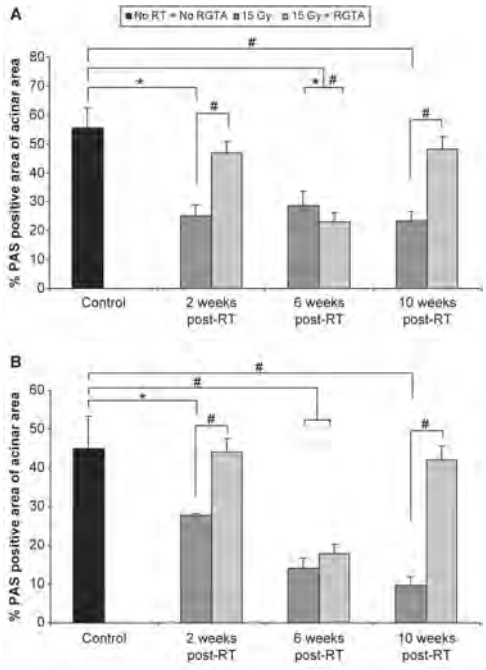

Figure 4 Amylase (A) and total protein (B) secretion (mean ± SEM). RT, radiotherapy. * Statistical difference of P<0.05; # statistical difference of P<0.01.

DISCUSSION

Radiotherapy in the head and neck region causes damage to healthy surrounding tissue, of which especially the salivary glands are at risk. Damage to these glands can result in chronic hyposalivation, a condition that greatly affects quality of life with no treatment currently available. In this study we investigated the effects of RGTA-OTR4120, a heparan mimetic, on radiation-induced salivary gland dysfunction in a mouse model. Mice were irradiated by a single dose of 15 Gy in the head and neck region, a dose biologically equivalent to the clinically relevant fractionation dose of 16 Gy x 2 Gy[17, 18], used in various animal studies[19-23] and proven to cause significant gland impairment[24-26]. Chronic radiation damage to salivary glands in rodents is fully developed within 60–90 days after therapy[25, 27]. Our endpoints of 2, 6 and 10 weeks after radiotherapy, therefore, give insight on the progress of the effects of RGTA-OTR4120 up until the chronic phase of radiation injury.

For histology we used the two major salivary glands, the parotid and submandibular gland, and determined the presence of mucins, that are produced by acinar cells, by using the PAS staining. Parotid glands produce the majority of saliva in the stimulated

state, whereas submandibular glands produce more than two-thirds of the saliva in the unstimulated state[1, 28]. Both glands showed a similar response to radiotherapy and to the administration of RGTA-OTR4120 as determined by the PAS staining. This is in line with the study of Konings et al[29], that described a similar course of damage development for parotid and submandibular glands, consisting of an acute phase where water excretion is impaired while there is no cell loss and amylase secretion is not affected, followed by a reduction in acinar cells and amylase secretion, and finally the period of late radiation damage which is marked by a lack of functional acinar cells and a further drop in amylase secretion.

The reduction in the mucin production by acinar cells after radiotherapy was somewhat more prominent in the parotid gland, but both the pattern of radiation damage and the scale of the effect following RGTA-OTR4120 administration were the same for both glands. The fact that the parotid gland seems to be more vulnerable to irradiation is not surprising since parotid glands consist of mainly serous cells, which have generally been found to be more radiosensitive than mucous cells[30, 31], whereas the submandibular gland consists of both serous and mucous cells. Animal experiments in which the whole head is irradiated confirm the higher sensitivity of the parotid gland[27, 32]. However, after more localized irradiation to salivary glands, this effect was not observed in various other studies[33-35]. Also, Coppes et al[17] concluded that, for the late effects, the submandibular gland may even be more radiosensitive than the parotid gland when fractionated irradiation is used. Taken together, both glands should be carefully considered when examining radiation-induced damage.

Salivary flow rates were reduced after radiotherapy, which is in accordance with various other studies, reviewed by Grundmann et al[1]. The magnitude of the reduction was less than is seen in other studies, which can be explained by differences in saliva collection, type of saliva stimulant and dose[17, 20, 24, 36, 37]. Still, at 6 and 10 weeks after radiotherapy there was a significant reduction in salivary flow rate. The positive effect of RGTA-OTR4120 administration on salivary flow rate faded in time and at 6 and 10 weeks, no significant effect was detectable. This suggests a temporary effect of RGTA-OTR4120 administration. Strikingly, the mucin production of acinar cells was significantly higher in the RGTA-treated group compared to irradiated controls at 10 weeks after radiotherapy. This indicates a positive effect of RGTA-OTR4120 administration on the number of saliva-producing acinar cells, which was not reflected in the salivary flow rate. In that case, not all acinar cells contribute equally to salivary production. This dissociation between structural changes observed after radiotherapy and functional changes in salivary output has been described earlier[36, 38, 39]. Furthermore, the loss of cells can affect the function of surrounding cells. Factors that are secreted in the process of activating a cell death program could create an adverse environment[40].

To assess the quality of the saliva we measured amylase and total protein content. Amylase is an enzyme that has multiple functions. Besides its enzymatic activity, it coats oral tissues, binds to streptococci and is involved in the selective clearance and adherence of microorganisms[41]. Amylase activity is found to be reduced after radiotherapy[31, 41, 42], a finding that is confirmed by our results. The administration of RGTA-OTR4120 did not have a stimulatory effect on amylase activity. Total protein content was also not altered significantly by RGTA-OTR4120 administration. The increase in activity of acinar cells 2 and 10 weeks after radiotherapy did not lead to an increase in the secretion of either amylase or total protein secretion. A possible explanation could be that the acinar cells cannot function properly, due to intracellular damage or to an unfavourable environment (e.g. damage to ducts, blood vessels) [33]. Studies that measure salivary flow rate, as well as amylase and total protein after irradiation are scarce. Sumita et al[43] concluded that bone marrow-derived cells can rescue salivary gland function in mice after head and neck irradiation. Salivary flow rate is indeed protected, but there was no difference in total protein concentration. Coppes et al[33] focused on the use of adrenergic and muscarinic receptor agonists and found positive effects on saliva quality and quantity. However, they stated in their discussion that the concentration necessary to exert the effect cannot be used clinically.

Growth factors have also been the subject of research in several studies that aim to protect tissues from radiation-induced damage[21, 36, 44, 45]. The way to deliver the potentially radioprotectant growth factors locally to the site of radiation damage forms an obstacle. The local delivery is important in order to avoid protection of cancer cells and because growth factors have a very short in vivo half-life[45]. The advantage of the use of RGTAs is that they are only bound at the site of injury, even when administered systemically, because they will bind to free heparan sulphate binding sites that are only available at sites of tissue damage where proteases have degraded the existing heparan sulphates. Additionally, they have a long in vivo half-life and cannot be degraded by heparanases[7, 15, 46]. Mangoni et al[14] stated that RGTA administration is most effective in protecting radiation-induced mucositis in rats when administered after the completion of radiotherapy.

To our knowledge the effect of RGTA administration on radiation-induced damage on the long term has not been studied yet. The results of studies in other fields of tissue repair and regeneration, and the fairly simple administration, with little or no side effects known, make RGTAs plausible candidates as radioprotectants. Our study showed a short term protective effect of RGTA administration on salivary gland dysfunction, while results on the longer term are mixed, making it necessary to further investigate the action of RGTA administration in irradiated tissues.

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Chapter 7

Gene expression analysis reveals inhibition of radiation induced TGFβ-signaling by hyperbaric oxygen therapy in mouse salivary glands

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ABSTRACT

A side effect of radiation therapy in the head and neck region is injury to surrounding healthy tissues such as irreversible impaired function of the salivary glands. Hyperbaric oxygen therapy (HBOT) is clinically used to treat radiation-induced damage but its mechanism of action is largely unknown. In this study, we investigated the molecular pathways that are affected by HBOT in mouse salivary glands two weeks after radiation therapy by microarray analysis. Interestingly, HBOT led to significant attenuation of the radiation-induced expression of a set of genes and upstream regulators that are involved in processes such as fibrosis and tissue regeneration. Our data suggest that the TGFβ-pathway, which is involved in radiation-induced fibrosis and chronic loss of function after radiation therapy, is affected by HBOT. On the longer term, HBOT reduced the expression of the fibrosis-associated factor α-smooth muscle actin in irradiated salivary glands. This study highlights the potential of HBOT to inhibit the TGFβ-pathway in irradiated salivary glands and to restrain consequential radiation induced tissue injury.

INTRODUCTION

Treatment of head and neck cancer routinely involves radiation therapy (RT), which not only affects tumor tissue, but also the surrounding healthy tissues. Because of their position, salivary glands are often in the radiation portal. Radiation-induced damage to salivary glands is irreversible and results in chronic hyposalivation and a change in saliva composition, leading to a subjective feeling of a dry mouth called xerostomia which greatly affects quality of life. Despite salivary gland sparing techniques such as intensity modulated radiation therapy (IMRT), the surgical transfer of major salivary glands outside the radiation field and the use of cytoprotectants, xerostomia remains a significant problem after radiotherapeutic treatment of malignancies in the head and neck area[1].

Unlike other slowly dividing tissues, salivary glands respond acutely to radiation treatment. Whereas acinar cell number remains unaltered, salivary flow rates drop dramatically at early time points after RT (~0–10 d). It has been proposed that this is due to radiation-induceddamage to the plasma membranes, since no cell loss is visible yet[2, 3]. In the chronic stage of radiation damage (~120–240 d), a lack of functional acinar cells and replacement by connective tissue and fibrosis causes the diminished salivary flow[4]. In this phase, some generation of acinar cells does take place, but it is suggested that the new cells cannot function properly due to damage of ducts, blood vessels and nerves[5].

Hyperbaric oxygen therapy (HBOT), in which patients breathe 100% oxygen under elevated pressure, has been used for almost 40 years to treat radiation injuries. Increased oxygen concentration in combination with elevated pressure raises tissue oxygen tension up to ten times. As oxygen under pressure is dissolved in plasma, it can reach otherwise hypoxic areas with obstructed blood flow, like radiation-injured tissues. In the case of the prevention or treatment of xerostomia, some clinical trials report positive effects of HBOT[6-8], mostly measured by quality of life questionnaires. Experimental evidence on the beneficial effects of HBOT on irradiated salivary glands is however scarce[9]. In a previous study we showed an increased blood vessel density in irradiated mouse salivary glands in response to HBOT[10]. In other tissues and cells, it has been shown that vascular endothelial growth factor (VEGF) levels can rise in response to HBOT[11, 12], and angiogenesis can be promoted[13, 14]. Besides influencing angiogenesis, oxygen also is involved in other key processes associated with wound healing, such as modulating cytokine release, accelerating microbial oxidative killing, modulating leukocyte activation and adhesion, and reducing apoptosis[15]. The effects of HBOT on gene expression have been analyzed *in vitro* in neurons, osteoblasts and endothelial cells, maximally 24 h after a single HBO treatment[16-18]. In all three cell types, an upregulation of the oxidative stress response was reported. In an *in vivo* model of rat ischemic brain, genes of the neurotrophin

system and inflammatory immune response were affected after five consecutive HBO treatments[19]. In patients with nonhealing wounds, an upregulation of genes involved in extracellular matrix remodelling and angiogenesis was reported after HBOT[19, 20].

Thus far, the effects of HBOT on gene expression in irradiated tissues have not been studied in an *in vivo* model. In this study, we explore the molecular pathways that are influenced by HBOT in irradiated salivary glands of mice by means of microarray analysis. By understanding basic HBOT mechanisms, the clinical implementation of HBOT for accepted indications can be improved.

MATERIALS AND METHODS

Animals

Female C3H mice, 7–9 wks old, were treated with radiotherapy (RT) and/or hyperbaric oxygen therapy (HBOT) as described before[10]. The experimental protocol was approved by the Animal Care Committee of Erasmus MC, Rotterdam, the Netherlands (protocol IDs 133-08-09 and 133-11-04), under the national Experiments on Animals Act and adhered to the rules laid down in this national law that serves the implementation of the guidelines on the protection of experimental animals from the Council of Europe (1986), Directive 86/609/EEC[21].

Radiation Therapy (RT)

Radiation therapy was performed as described previously[10]. In short, mice were anesthetized intraperitoneally with a mixture of ketamine and xylazine (120 mg/kg and 6 mg/kg body weight, respectively) and irradiated locally in the head and neck area with a single dose of 15 Gy by a 250 kV orthovoltage irradiator (Philips RT250) using a Cu filter and a dose rate of 1.9 Gy/min (Philips Medical Systems, Brussels, Belgium). The rest of the body was shielded by a 0.5-cm lead plate.

Hyperbaric Oxygen Therapy

Hyperbaric oxygen therapy was performed in a custom-built hyperbaric oxygen chamber for small laboratory animals (Hytech BV, Raamsdonksveer, the Netherlands)[22]. HBOT was given once a day for five consecutive days a week, with a maximum of 20 sessions. Each session consisted of compression to 2.4 atmospheres absolute (ATA) and 100% oxygen during 30 min, isopression for 60 min, in which pressure and oxygen levels were kept constant and decompression to 1 ATA during 15 min. For animals that were treated with RT, HBOT started the day after.

RNA Isolation

Mice were euthanized by CO2- asphyxiation and submandibular salivary glands

were removed, snap frozen in liquid nitrogen and stored at –80°C until total RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Microarray Analysis

Microarray analysis was performed on RNA samples of submandibular glands of untreated mice (control), mice treated with 10 sessions of HBOT (HBOT), mice treated with RT at 2 wks after RT (RT) and mice treated with RT and HBOT at 2 wks after RT (RT + HBOT; n = 4 for each group). Assessment of total RNA quality and purity was performed with the RNA 6000 Nano assay on the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). cDNA was synthesized from total RNA using the IVT Express Labeling kit (Affymetrix, Santa Clara, CA, USA). Subsequent biotin- labelled cRNA synthesis, purification and fragmentation were performed according to the manufacturer's recommendations. A total of 12.5 µg fragmented biotinylated cRNA was subsequently hybridized onto Affymetrix Mouse Genome 430 2.0 Array chips. Image analysis was performed using GeneChip Operating Software with the Affymetrix GeneChip Scanner 3000 according to the manufacturer's protocol. Microarray Suite software (Affymetrix) was used to generate .dat and .cel files. To examine the quality of the various arrays, several R packages (including affyQCreport[23]) were run starting from the .cel files. All created plots, including the percentage of present calls, RNA degradation, NUSE and RLE indicated a high quality of all samples and an overall comparability. Raw intensity values of all samples were normalized by RMA normalization (Robust Multichip Analysis) (background correction and quantile normalization) using Partek version 6.4 (Partek Inc., St. Louis, MO, USA).

The normalized datafile was transposed and imported into OmniViz version 6.0.1 (BioWisdom Ltd., Cambridge, UK) for further analysis. For each probe set, the geometric mean of the hybridization intensity of all samples was calculated. The level of expression of each probe set was determined relative to this geometric mean and log2-transformed. The geometric mean of the hybridization signal of all samples was used to ascribe equal weight to gene expression levels with similar relative distances to the geometric mean. Differentially expressed genes were identified using statistical analysis of microarrays (SAM). Cutoff values for significantly expressed genes were a false discovery rate (FDR) of 0.1 or less and a fold change of ≥1.5.

Functional Annotation

Functional annotation of the statistical analysis of microarrays results was done using Ingenuity Pathway Analysis (Ingenuity, Mountain View, CA, USA). The results are shown for biological processes, which are significantly ($P < 0.05$) enriched after

multiple testing.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA from submandibular glands of four animals per experimental group was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The resulting cDNA was amplified in 40 cycles (enzyme activation at 95°C for 20 s, denaturation at 95°C for 3 s, annealing/ extension at 60°C for 30 s) with a Bio-Rad CFX 96 Real-Time Detection System (software version Bio-Rad CFX Manager 2.0) using Fast SYBR Green Master Mix (Applied Biosystems, Life Technologies Europe BV, Bleiswijk, the Netherlands) and primers for: α smooth muscle actin (α-*Sma*), B-cell translocation gene 2 (*Btg2*), Cd83 antigen (*Cd83*), connective tissue growth factor (*Ctgf*/*Ccn2)*, cysteine rich protein 61 (*Cyr61*/*Ccn1*), early growth response 1 and 2 (*Egr1* and *Egr2*), glyceraldehyde-3- phosphate dehydrogenase (*Gapdh*), serpin peptidase inhibitor, calde E, member 1 (*Serpine1*/*Pai1*), SRY-box containing gene 2 (*Sox2*), transferring receptor (*Tfrc*), transforming growth factor β 1 (*Tgf*β*1*) and thrombospondin 1 (*Thbs1*). For primer sequences see Supplementary Table S1). Each PCR reaction was performed in duplicate and the average threshold cycle (Ct) value was used for relative quantification of gene expression compared with the housekeeping gene Gapdh, with the comparative Ct method (ΔΔCT).

(Immuno-)Histochemistry

Immediately after euthanization, submandibular glands were excised and stored in 10% buffered formalin for 24 to 36 h. Tissues were then dehydrated, embedded in paraffin blocks and 5-µm slides were cut. Standard hematoxylin and eosin (H&E) and picrosirius red stainings were performed to visualize fibrosis and collagen content. For the detection of TGFβ1, Serpine1 and α-SMA, sections blocked with 5% nonfat milk powder and then probed with a primary antibody against TGFβ1 (1:50, Santa Cruz Biotechnology Inc, Dallas, USA), Serpine1 or α-SMA (1:100, Novus Biologicals Ltd., Cambridge, UK) overnight at 4°C. Biotinylated goat anti-rabbit IgG (Dako, Carpinteria, CA, USA) was used as secondary antibody (30 min at room temperature). Detection of the antibody complex was performed with streptavidin–peroxidase (R&D Systems, Oxon, UK) and 3,3'- diaminobenzidine (Dako). Hematoxylin served as counterstain.

Quantification

Slides stained for α-SMA were scanned using a slide scanner (Hamamatsu Photonics KK, Japan). A representative 10× magnified picture was taken for each gland and α-SMA staining was analysed by Celld (Olympus Life Science Europe GmbH) to detect the percentage of α-SMA positive staining.

Statistical Analysis

All data are expressed as mean values with standard deviation (SD), and were analyzed using SPSS PASW 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Univariate tests with *post hoc* Bonferroni correction were used to identify statistical differences ($P < 0.05$) between groups.

RESULTS

Gene Expression Analysis of Submandibular Glands

To investigate the effect of hyperbaric oxygen therapy on molecular pathways in irradiated and nonirradiated salivary glands, gene expression analysis was performed on submandibular salivary glands of control mice, irradiated mice two weeks after radiotherapy (RT), mice treated with daily hyperbaric oxygen therapy for two weeks (HBOT) and irradiated mice treated with hyperbaric oxygen therapy for two weeks (RT + HBOT) (n = 4/group). Principal component analysis (PCA) indicates a clear clustering of groups, with samples of both irradiated groups closest to each other (Figure 1A).

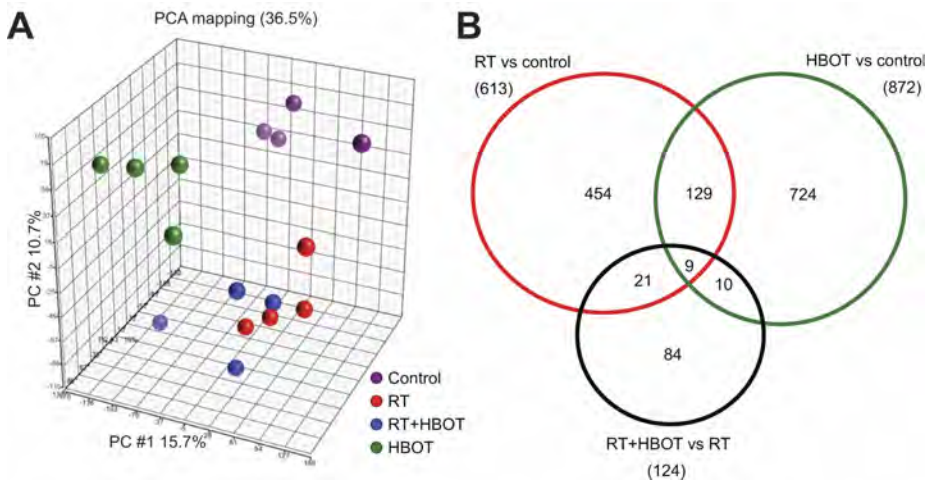

Figure 1 Summary of microarray data by PCA and Venn diagrams. (A) PCA-mapped scatter plot. The global gene expression profiles of the submandibular glands for different treatment groups and control analyzed by PCA. The figure represents the first three principal components of microarray analysis data (PC1, PC2 and PC3) in x, y and z axes, respectively. (B) Venn diagram that represents the number of differentially expressed Affymetrix probe sets in RT versus control (red circle), HBOT versus control (green circle) and RT + HBOT versus RT (black circle), with the number overlapping probe sets inside the circles.

Differentially expressed probe sets were identified in salivary glands of all experimental groups using statistical analysis of microarrays (SAM, ≥ 1.5-fold

change, FDR 0.1). Treatment with RT or HBOT resulted in a change in expression levels of 613 probe sets and 872 probe sets, respectively. Treatment of irradiated glands with HBOT led to the identification of 124 differentially expressed probe sets, of which 84 were unique to this group (Figure 1B), indicating that HBOT has a different effect on irradiated and healthy glands. The lists of significantly up- and downregulated genes of the different groups are shown in Supplementary Tables S2–S5. These sets of up- and downregulated genes were used for further functional annotation of pathways and functional categorization using Ingenuity Pathway Analysis (IPA). The canonical pathways that were affected by RT were anticipated for on the basis of existing knowledge and included the P53 and ATM signaling, NRF2-mediated oxidative response and the acute phase response. Table 1 shows cellular and physiological functions that are most significantly influenced by RT, HBOT or RT + HBOT.

Table 1 Cellular and physiological functions.[a]

	Category	Functional Annotation	p Value	Activation z score
RT	Cell death and survival	apoptosis	4.14E-12	-1.285
		necrosis	8.00E-12	-0.863
		cell death	4.68E-10	-1.104
		cell death of connective tissue cells	6.76E-10	0.515
		cell survival	9.86E-08	2.742
	Cell cycle	arrest in interphase	5.19E-11	
		interphase	2.26E-10	0.512
		cell cycle progression	6.63E-09	2.092
		G1 phase	3.74E-07	-0.018
	Gene expression	binding of DNA	4.58E-10	-0.274
		transcription of RNA	1.01E-07	0.441
		binding of protein binding site	3.47E-07	0.839
		expression of RNA	5.59E-07	0.914
		expression of DNA	5.96E-06	1.485
	Cellular growth, development and proliferation	proliferation of cells	7.07E-09	0.710
		proliferation of connective tissue cells	4.59E-08	1.905
		differentiation of connective tissue cells	6.38E-08	2.668
		differentiation of cells	2.20E-07	1.256
HBOT	Cellular function and maintenance	function of leukocytes	2.57E-14	-0.751
		function of blood cells	3.58E-13	-0.751
		function of mononuclear leukocytes	1.20E-09	-0.684
		function of lymphocytes	3.20E-09	-0.684
	Cellular movement	chemotaxis of leukocytes	1.46E-11	-3.641
		cell movement of leukocytes	1.68E-11	-4.942
		homing of leukocytes	4.30E-11	-3.753
		chemotaxis of myeloid cells	6.64E-11	-3.110
	Cellular development	proliferation of lymphocytes	2.00E-10	-2.036
		proliferation of immune cells	7.99E-10	-2.268
		proliferation of blood cells	1.45E-09	-2.083
	Cell-to-cell signaling and interaction	activation of cells	2.32E-10	-3.952
		activation of leukocytes	3.17E-10	-4.119
		activation of blood cells	2.04E-09	-3.932
RT + HBOT	Cellular growth, development and proliferation	proliferation of cells	6.44E-08	2.676
		proliferation of fibroblast cell lines	4.57E-05	0.873
		differentiation of connective tissue cells	1.52E-04	2.074
		formation of cells	1.95E-04	0.528
		proliferation of connective tissue cells	4.71E-04	1.728
	Cell death and survival	necrosis	3.13E-06	-0.182
		cell death	7.26E-06	-0.101
		apoptosis	8.46E-06	-0.521
		cell viability of fibroblasts	3.66E-04	0.647
	Cell cycle	arrest in G1 phase	6.09E-05	
		polyploidization of cells	1.30E-04	-0.640
		arrest in interphase	1.77E-04	
	Cellular function and maintenance	transport of D-glucose	3.38E-05	2.419
		concentration of D-glucose	8.41E-05	-0.491
		quantity of carbohydrate	2.69E-04	0.585

[a]Cellular and physiological functions that are affected by RT, HBOT and RT+HBOT, divided into categories. *z* Scores of <2 (inhibition) or >2 (activation) are considered statistically significant..

Irradiated salivary glands of the groups with and without HBOT showed clear differential expression of genes that enhance survival, cell proliferation and differentiation of connective tissue cells. Strikingly, apoptosis was reduced and cell survival enhanced. HBOT resulted in a change in a remarkable number of functions associated with the immune response and inflammation in the salivary glands of nonirradiated mice. No such strong immunological response of HBOT was detected in the irradiated tissue.

To elaborate on the influence of HBOT on irradiated tissues, the expression levels of the differentially expressed genes of the RT + HBOT versus the RT group were analysed by treescape and revealed a group of genes that was upregulated after RT, but significantly downregulated if HBOT was applied after RT (Figure 2). This group consisted mostly of immediate early response genes like *Fos*, *Jun* and members of the *Egr* and *Ier* family, indicating that HBOT can prevent or inhibit the radiation-induced expression of these genes.

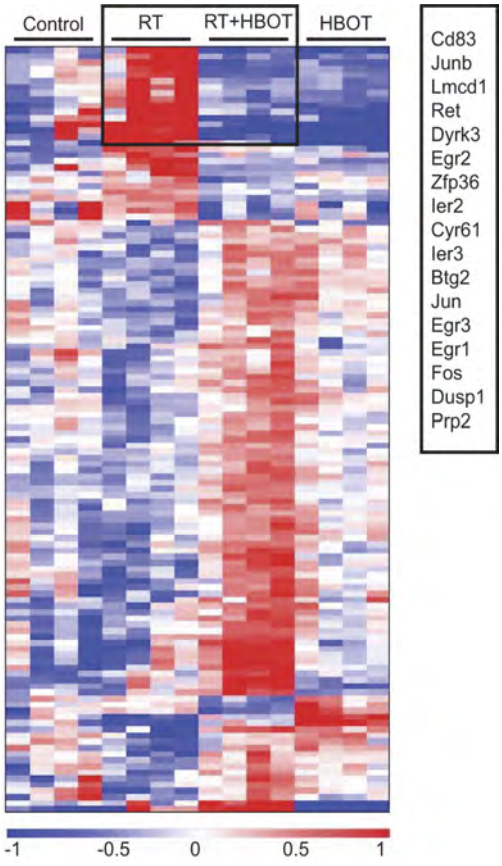

Figure 2 Differentially expressed probe sets between the RT– and RT + HBOT group. OmniViz treescape showing the hierarchical clustering of differentially expressed Affymetrix probe sets between the submandibular glands of the RT and RT + HBOT group (middle groups). Expression of these probe sets for the control and HBOT-group is shown on the outside. Red indicates upregulated probe sets compared with the geometric mean and blue indicates downregulated probe sets compared with the geometric mean. The color intensity correlates with the degree of change. Rectangle shows probe sets that are upregulated in the RT group, while downregulated in the RT + HBOT group. Genes within this rectangle are summarized.

By using the Ingenuity software, activation or inhibition of upstream regulators was predicted on the basis of upand downregulated genes in the dataset. In this way, regulatory cascades and biological activities occurring in the tissue were determined. Table 2 shows the list of RT-activated regulators that were significantly inhibited after HBOT treatment, a considerable amount of which are cytokines and growth factors known to play roles in radiation-induced processes such as fibrosis, apoptosis, tissue regeneration and inflammation.

Table 2 Upstream regulators.[a]

	RT versus control		RT + HBOT versus RT	
Upstream regulator	Activation *z* score	*p* Value	Activation *z* score	*p* Value
PDGF BB	5.039	6.79E-23	-3.449	1.91E-09
EGF	4.835	6.21E-20	-3.378	6.88E-12
TGFB1	3.632	4.02E-14	-3.175	1.60E-05
IL1B	2.506	5.82E-22	-2.829	2.42E-05
IL4	3.040	1.18E-06	-2.771	9.84E-04
TNFSF11	3.330	5.31E-05	-2.741	1.54E-05
P38 MAPK	3.598	2.71E-14	-2.731	3.68E-07
IL3	3.450	2.44E-07	-2.728	2.08E-05
FGF2	4.145	6.45E-10	-2.601	5.14E-04
CSF2	3.475	2.42E-07	-2.587	1.32E-04
Pkc(s)	3.703	1.73E-08	-2.503	6.37E-06
F2	4.361	1.01E-10	-2.442	1.60E-06
Ins1	3.030	2.57E-06	-2.439	1.04E-05
MAPK14	2.345	1.80E-06	-2.433	9.74E-07
CHRM1	2.423	1.09E-07	-2.423	6.17E-12
Jnk	3.647	8.55E-09	-2.414	4.30E-06
CSF3	3.109	2.81E-03	-2.401	6.07E-06
NfkB (complex)	3.823	4.77E-14	-2.396	3.32E-06
CSF1	2.964	4.82E-06	-2.375	8.40E-05
LEP	2.225	7.91E-08	-2.371	2.65E-03
ERK	3.772	1.41E-10	-2.258	2.58E-07
CREBBP	2.411	2.07E-09	-2.219	6.03E-07
EPHB1	2.425	5.06E-08	-2.216	7.11E-10
GNRH1	2.763	1.84E-05	-2.202	1.27E-06
IGF1	3.595	5.00E-19	-2.197	2.51E-06
Gm-csf	2.391	5.55E-04	-2.189	1.87E-06
POMC	2.404	1.36E-02	-2.187	3.77E-05
F7	2.934	2.63E-08	-2.183	3.11E-07
Il1A	2.391	4.43E-04	-2.168	1.63E-03
ELK1	2.542	7.79E-07	-2.146	9.07E-09
AGT	2.953	5.97E-07	-2.047	2.15E-05

[a]Upstream regulators that were predicted to be activated by RT, and inhibited when HBOT was applied after RT, on the basis of the microarray data of the submandibular glands. *z* Scores of <2 (inhibition) or >2 (activation) are considered significant.

qPCR Validation

On the basis of their expression profiles and putative roles in tissue repair, nine genes of interest were selected for qPCR-validation (Table 3). The first group consisted of genes that are linked to fibrosis and that were significantly downregulated by HBOT in irradiated salivary glands while upregulated by RT alone: *Egr1*, *Egr2* and

Cyr61. *Ctgf* was included because of its direct link to fibrosis and the fact that it was less upregulated in irradiated glands after HBOT treatment as well.

Table 3 Genes of interest[a]

Process	Gene	RT versus control (fold change)	RT + HBOT versus RT (fold change)
Fibrosis	*Cyr1*	+4.3	−4.6
	Ctgf	+3.4	
	Egr1	+6.5	−12.2
Regeneration	*Btg2*	+2.9	−4.9
	Sox2		+2.6
	Tfrc		+2.6
Immune response	*Cd83*	+2.3	−2.2

[a]Genes of interest that were selected for qPCR validation with fold changes from the microarray.

On the basis of their differential expression patterns, *Btg2* (antiproliferative capacities), *Sox2* (stem cell maintenance), *Tfrc* (control of cell proliferation and growth) and *Thbs1* (negative regulation of regeneration and angiogenesis) were selected because of their putative roles in regeneration. In addition, *Cd83* was included in the qPCR analysis as it is involved in the immune response and was affected considerably by HBOT according to the microarray analysis. By qPCR, differential expression was confirmed for all selected genes, although for *Egr2* and *Thbs1*, the differences were not statistically significant (Figure 3).

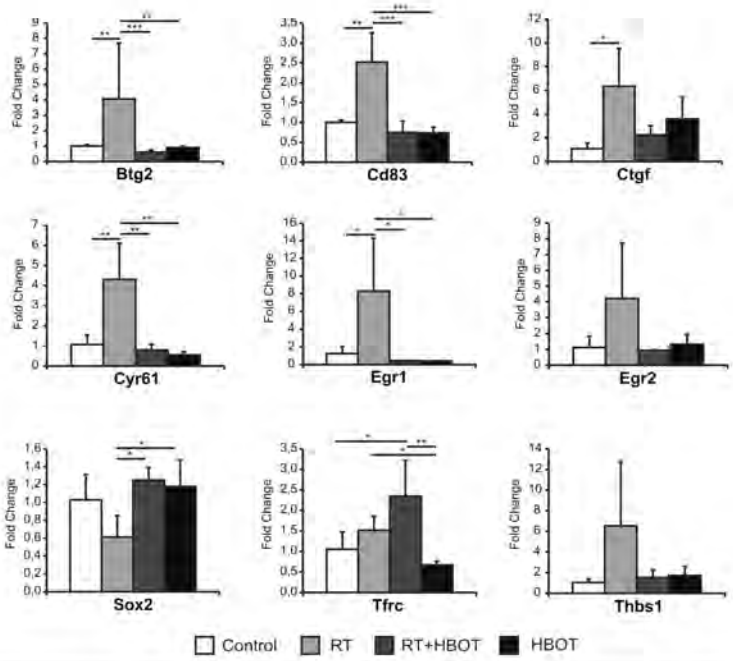

Figure 3 qPCR validation of genes of interest. qPCR validation of microarray results for the expression of genes of interest at 2 wks after RT in the submandibular glands. y Axis shows mean fold change relative to controls. Lines above bars represent statistically significant differences (*P < 0.05, **P < 0.01, ***P < 0.001).

TGFβ-Pathway

Because of its known role in (radiation- induced) fibrosis, we further analysed the TGFβ-pathway in our dataset using IPA. The TGFβ1 regulator pathway was predicted to be activated in the RT group and to be inhibited if HBOT was applied after RT (see Table 2). Figure 4 shows an upregulation of a set of target genes of the canonical TGFβ1-pathway in the RT group compared with controls (Figure 4A), and an inhibition when HBOT was given to irradiated tissue (Figure 4B). Expression analysis of the *Tgfβ1* gene and its effector gene *Serpine1* by qPCR at 2 wks after RT was performed to confirm the inhibitory effect of HBOT on the TGFβ-pathway (Figure 4C). Both genes showed lower expression 2 wks after RT if HBOT had been applied. Immunohistochemical staining for TGFβ1 and Serpine1 also showed an RT-induced upregulation that was partly counteracted by HBOT (Figure 4D).

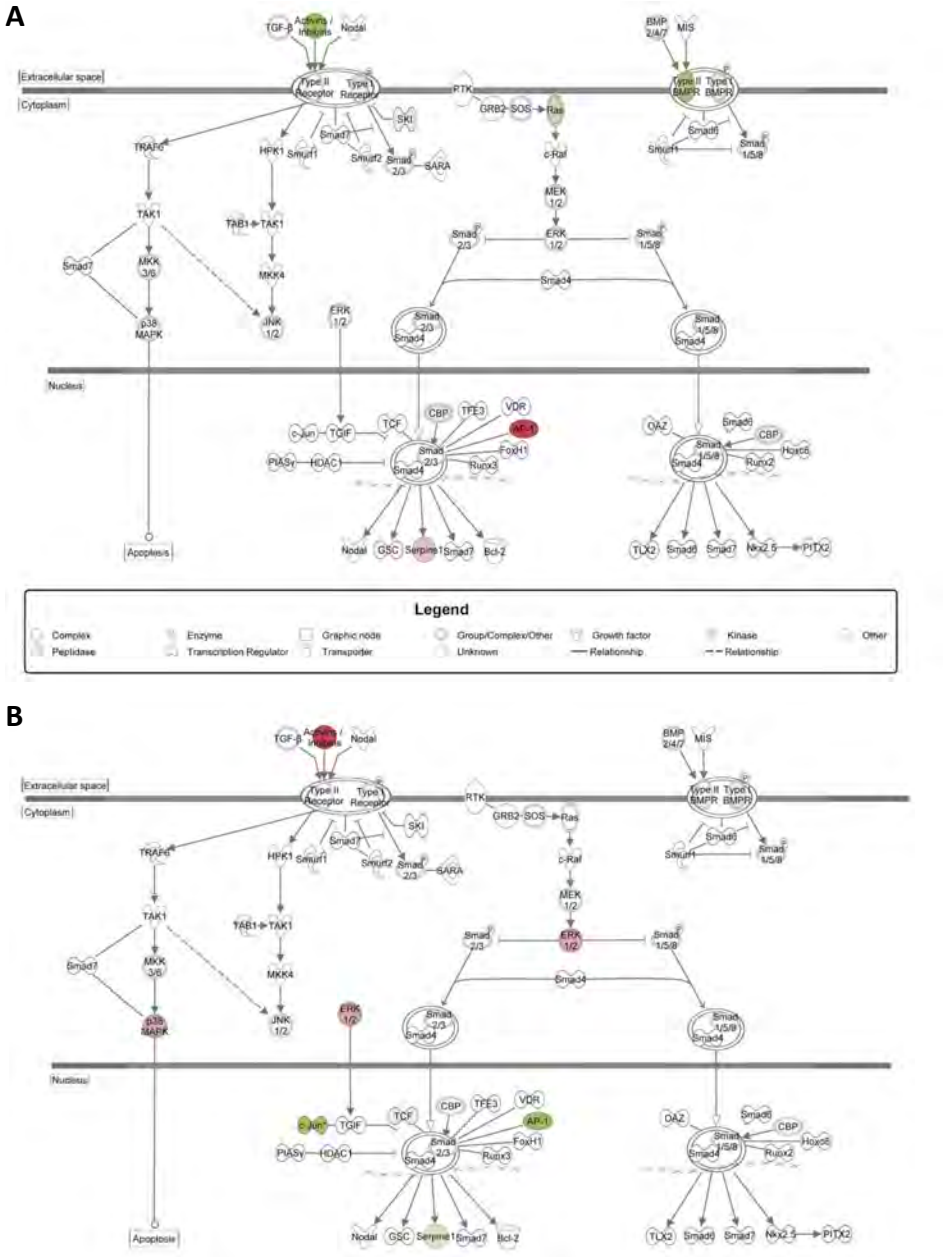

Figure 4 Influence of HBOT on the TGFβ-pathway. Differential expression of genes involved in the TGFβ-pathway in irradiated submandibular glands compared with control (A) and in irradiated glands that received HBOT compared with irradiated glands (B), by Ingenuity Pathway Analysis of microarray data. Colors show up- (red) and downregulated (green) genes (≥ 1.2-fold change, FDR 0.05). Notice the reverse expression of genes when HBOT is applied to irradiated glands. *Continued on next page*

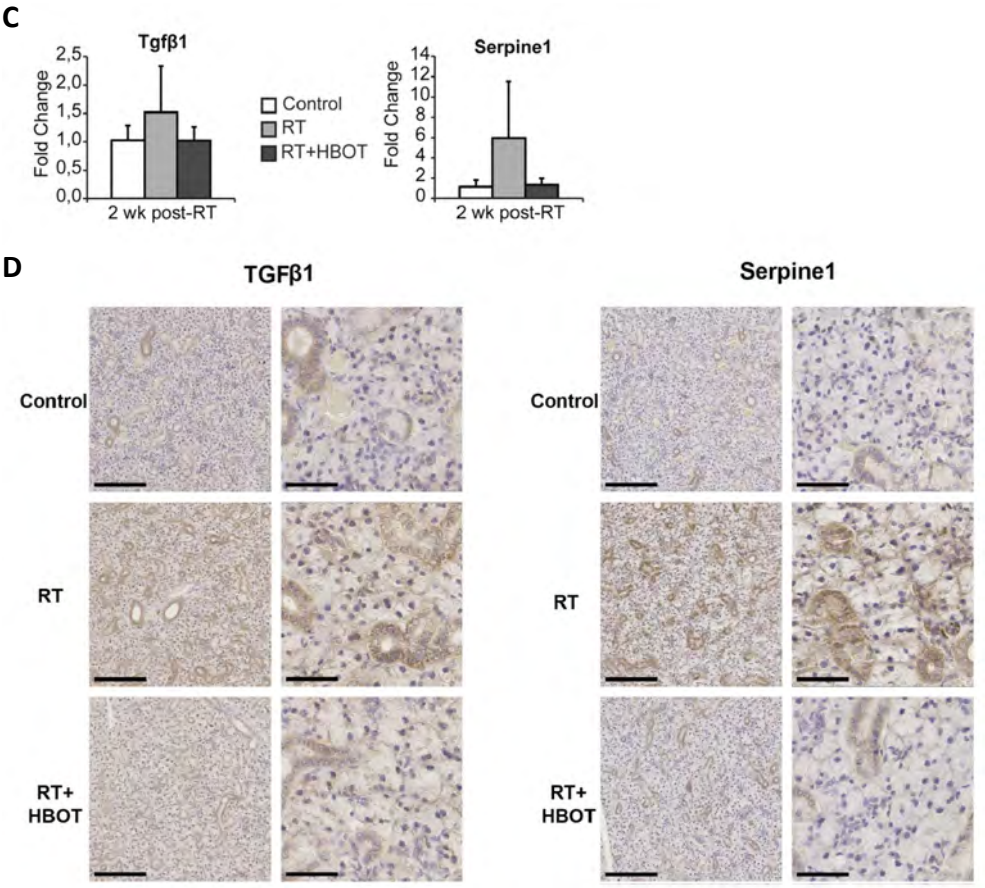

Figure 4 *Continued* qPCR validation (C) and immunohistochemical staining (D) of Tgfβ1 and Serpine1 at 2 wks after RT. Scale bars left pictures 200 μm, right pictures 50 μm.

The expression of α-*SMA*, which is a profibrotic factor expressed by myofibroblasts, but also by myoepithelial cells in the salivary gland, showed a similar pattern with higher expression in the RT-treated group at 2 and 10 wks after RT (Figure 5A). Immunohistochemistry showed a significantly higher expression in RT-treated glands at 24 wks after RT compared with irradiated glands that received HBOT (Figure 5B). Although expression analysis showed a potential inhibitory effect of HBOT on profibrotic markers, we were unable to identify major signs of fibrosis in H&E- and picrosirius red–stained tissue, at 2, 10 and 24 wks after RT.

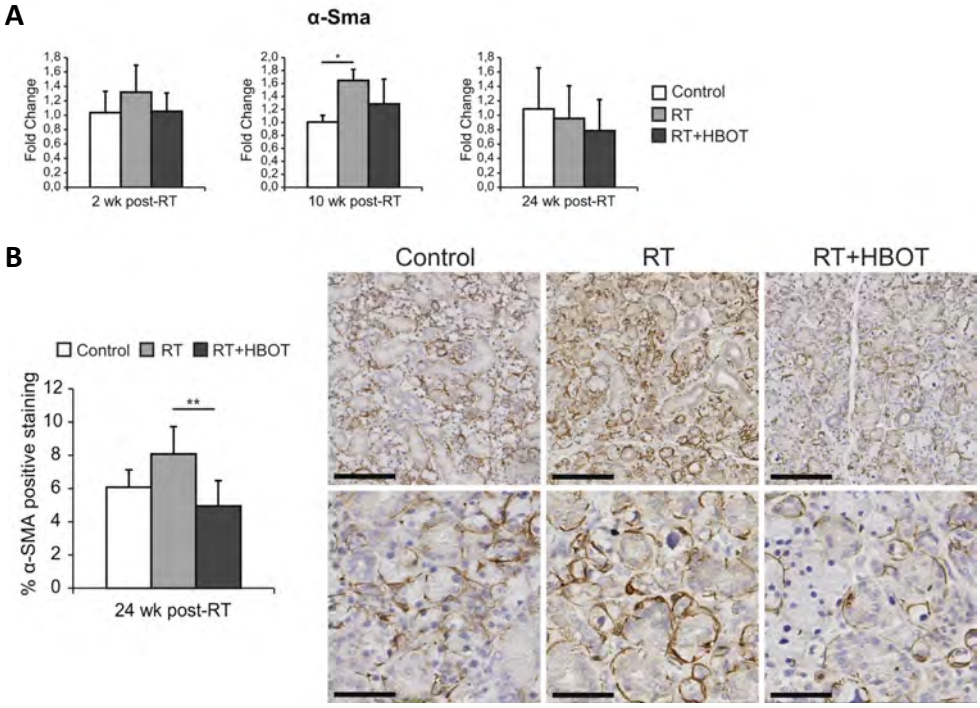

Figure 5 Expression of α smooth muscle actin. Relative expression (mean fold change relative to controls) of α smooth muscle actin (α-Sma) at 2, 10 and 24 wks after RT in the submandibular glands (A). Line above bars represents statistically significant difference (*P < 0.05). Immunohistochemical staining of α-SMA in submandibular glands of control and irradiated mice, either with hyperbaric oxygen therapy (RT + HBOT) or without (RT). Graph shows the percentage of positive α-SMA staining for the different groups (B). Line above bars represents statistically significant difference (**P < 0.01; P = 0.053 for RT versus control). Scale bars upper pictures 200 μm, lower pictures 50 μm.

DISCUSSION

Radiation-induced damage to salivary glands is a serious, irreversible complication of RT to the head and neck region. Hyperbaric oxygen therapy is used in the treatment of radiation-induced injury to normal tissues in the head and neck region while its mechanism of action remains poorly understood. Especially, little is known about the molecular pathways that are influenced by HBOT on irradiated salivary glands. Therefore, we investigated the effects of radiotherapy and HBOT on gene expression in submandibular glands of mice, 2 wks after a single dose of 15 Gy and after 10 consecutive HBO treatments.

As expected, RT resulted in the activation of pathways involved in cell cycle and DNA damage repair, such as the p53 pathway, the NRF2-mediated oxidative response and the acute phase response signaling. HBOT did not have an effect on these pathways. The microarray data predict that RT has an inhibiting effect on

apoptosis and increases cell survival 2 wks after irradiation. This apoptotic response may seem unexpected since it is known that p53 signaling after irradiation leads to apoptosis[24] and apoptosis of acinar cells after RT of salivary glands has been reported[25]. However, in response to RT, pro- as well as anti-apoptotic and cell survival signaling pathways are activated, which occurs in waves. For instance, to allow DNA repair to take place after RT-induced damage, specific signaling pathways stimulate cell cycle arrest and prevent apoptosis[26, 27]. The balance between these pathways will decide whether more pro- or anti-apoptotic proteins will be expressed[27]. Our microarray data don't reveal significant effects of HBOT on apoptosis or cell survival pathways, which corresponds to our previous histological study in which no profound effect of HBOT on apoptosis levels was detected in salivary glands after RT[10].

Radiation causes vascular damage and thereby hypoxia[28]. It is generally assumed that HBOT can positively influence angiogenesis (reviewed in [29]). In a previous study, we showed an increased blood vessel density due to HBOT in irradiated submandibular glands of mice at ten weeks after RT[10]. The microarray performed in the present study, at 2 wks after RT, did not show a discernible effect of HBOT on gene expression profiles associated with angiogenesis, possibly because the 2-wk time point is too early to detect these effects.

In healthy submandibular glands, HBOT led to a remarkable amount of differentially expressed probe sets, even more than RT alone (872 versus 613 probe sets) indicating that HBOT induces changes in healthy, nonhypoxic tissue. In particular biological functions that are associated with the immune system, such as the movement, activation and adhesion of different immunological cells, were decreased by HBOT. In a wound model, suppressive effects of HBOT on the expression of inflammatory genes have been described and are a basis of its use in treating chronic (diabetic) wounds[30-32].

Genes that were most upregulated by RT included the early response genes *Fos*, *Jun* and *Egr1*. This upregulation has been reported before in mammalian cells shortly after RT[33, 34] and our *in vivo* model shows that this upregulation still persists 2 wks after RT. Interestingly, HBOT significantly downregulated the expression of these early response genes, when applied in irradiated salivary glands, indicating a counteraction of HBOT on RT-induced mechanisms. Strikingly, the predicted activation of the TGFβ1 regulatory pathway by RT appears to be significantly attenuated if HBOT is applied. The TGFβ-pathway is strongly associated with radiation-induced fibrosis in different organs, and is the subject of investigation regarding possible antifibrotic therapies[35-38]. This pathway regulates the formation of extracellular matrix (ECM), and has been shown to be activated by RT[39-42]. The binding of TGFβ to its receptor can lead, via Smad dependent and independent pathways, to the expression of target genes such as *Ctgf* and *Serpine1*. CTGF

stimulates differentiation of fibroblasts to myofibroblasts, which express α-SMA and produce (components of the) ECM. Serpine1 is a protease inhibitor which suppresses matrix metalloproteases (MMPs) from breaking down ECM and is thus matrix preserving[43, 44]. Excessive matrix formation/preservation leads to fibrosis since functional cells are replaced by ECM leading to dysfunction of the tissue[37]. Conditional overexpression of *Tgfβ1* has been shown to induce fibrosis in salivary glands of mice as seen under pathological conditions[45].

Different genes that were upregulated by RT and downregulated when HBOT was applied to irradiated tissue are involved in the TGFβ-pathway. Increased *Egr1* and *Egr2* expression have been postulated as key mediators of TGFβ signalling and fibrosis and thereby as potential targets for antifibrotic therapy[46-49]. *Cyr61*, which is a member of the CTGF family, can be regulated by *TGF*β and has proinflammatory properties[50, 51]. THBS1 is an activator of latent TGFβ so it can bind to its receptor[52]. All together, our microarray results indicate that the TGFβ-pathway is repressed by HBOT in irradiated tissue, which was confirmed by immunohistochemical staining for TGFβ1 and Serpine1. Staining for α-SMA in the submandibular glands also showed a HBOT-induced decrease at 24 wks after RT; α-SMA is expressed by myofibroblasts, but also by myoepithelial cells that are abundant in the salivary glands. Myoepithelial cells can differentiate into myofibroblasts by epithelial-to-mesenchymal transition (EMT), a process that is induced by TGFβ and is known to contribute to fibrosis[53, 54]. Fibrosis or higher collagen content was not detected in the submandibular glands at any of the time points studied, but the higher expression level of α-SMA in the RT-group could precede fibrosis. Presumably in our model, fibrosis is not yet revealed at 24 wks after RT. It has been shown that irradiation-induced lung fibrosis in mice can take 30 wks to develop and is highly strain-dependent[55].

An effect of HBOT on TGFβ signalling *in vivo* has been observed before in nonirradiated tumor tissue by Moen *et al*[56]. They found reduced *Tgf*β expression after HBOT and stated that HBOT is able to induce mesenchymal-to-epithelial transition (MET), the opposite of EMT. *In vitro*, TGFβ expression and secretion by fibroblasts has been shown to be decreased in response to HBOT [11, 57]. A reduction of fibrosis by HBOT has been demonstrated in animal models of laminectomy, tracheal anastomosis and myocardial infarction[58-60], although in a tendon healing model, HBOT seemed to enhance fibrosis[61].

Except for TGFβ1, HBOT was predicted to inhibit other regulatory factors, such as platelet derived growth factor (PDGF). PDGF has been shown to influence the TGFβ-pathway and is implicated to be involved in fibrosis as well[62]. The suppression of TGFβ and PDGF as potential antifibrotic therapies is of interest and has been investigated in several studies[36, 42]. Fibrosis is a complex process involving several pathways that are interconnected and the concurrent suppression of more of these factors is more likely to have an effect on a biological process. Therefore,

multitargeted therapies may be more effective and HBOT could potentially be such a therapy.

We cannot distinguish whether 100% oxygen only or 100% oxygen delivery under pressure leads to the reported changes in gene expression. However, HBOT is an established therapy in the treatment of radiation-induced injury and the elevated pressure has been shown to increase oxygen tension in tissue more than elevated oxygen levels only[63]. Under pressure, oxygen concentrations are increased in the plasma, allowing better oxygenation of obstructed tissue areas, since these are not reached by the oxygen that is bound to the hemoglobin of the red blood cells[29]. Therefore it is thought that HBOT is more effective in oxygenation and restoration of hypoxic tissues.

CONCLUSION

We showed that HBOT influences a number of pathways and genes in irradiated salivary glands. With respect to radiation-induced damage, the TGFβ- pathway is of particular interest, since it is directly related to the pathogenesis of fibrosis. On the basis of our microarray data, HBOT seems to inhibit the radiation-induced activation of this pathway. In our model, at 24 wks after RT, lower levels of the profibrotic marker α-SMA were detected in the salivary glands of mice that underwent HBOT, indicating that this therapy could possibly affect the process of fibrosis. Therefore it is of interest to further investigate the influence of HBOT on the TGFβ-pathway and fibrosis, not only for salivary glands, but also for other tissues that are sensitive to radiation-induced fibrosis such as lung, kidney, heart and intestine.

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SUPPLEMENTARY DATA

Supplementary table S1 Primers used for qPCR.

Gene	Forward (5'-3')	Reverse (5'-3')
α-Sma	CCAGCTATGTGTGAAGAGGAAG	TCCAACCATTACTCCCTGATGTC
Btg2	AAGAGAACCGACATGCTCCC	GTAATGATCGGTCAGTGCGTC
Cd83	GGCCAAGGTCTCCGAGAGTG	CCTCGAAGGAGCTGTTTTGC
Ctgf	CCCTAGCTGCCTACCGACTG	TTAGAACAGGCGCTCCACTC
Cyr61	AAGAGGCTTCCTGTCTTTGGC	AGACGTGGTCTGAACGATGC
Egr1	TGAGCACCTGACCACAGAGTC	TAACTCGTCTCCACCATCGC
Egr2	TACCCGGTGGAAGACCTCG	ATGTTGATCATGCCATCTCC
Gapdh	AAGGGCTCATGACCACAGTC	TGCAGGGATGATGTTCTGGG
Serpine1	ACAACCCGACAGAGACAATCC	ATGAAGGCGTCTCTTCCCAC
Sox2	GCTGCCTCTTTAAGACTAGGGC	CGCCGCGATTGTTGTGATTAG
Tfrc	GAGGCGCTTCCTAGTACTCC	CTTGCCGAGCAAGGCTAAAC
Tgfβ1	CCCGAAGCGGACTACTATGC	CATAGATGGCGTTGTTGCGG
Thbs1	CGATGAGTGCAAAGAAGTGCC	TGAGCCAGTGAATCGTGGTG

Forward and reverse primers used for qPCR

Supplementary tables S2-S5 can be found online at:
http://static.smallworldlabs.com/molmedcommunity/content/pdfstore/14_003_Spiegelberg%20Suppl.pdf

Chapter 8

Optical imaging of tumor response to hyperbaric oxygen treatment and irradiation in an orthotopic mouse model of head and neck squamous cell carcinoma

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ABSTRACT

Hyperbaric oxygen therapy (HBOT) is used in the treatment of radiation induced tissue injury in cancer patients, but its effect on the growth and development of residual tumor tissue is unclear. In this study an orthotopic floor of the mouth mouse model of head and neck squamous cell carcinoma (HNSCC) was used in combination with optical imaging techniques to investigate the response of untreated and irradiated tumors to HBOT. HBOT treatment of mice resulted in accelerated growth (~19%) of the non-irradiated but not the irradiated tumors, as was assessed by in vivo bioluminescence imaging (BLI). Nevertheless HBOT appeared to be beneficial for mouse survival time. In vivo optical imaging using the fluorescent blood pool agent AngioSense revealed that HBOT leads to enhanced tumor vascular leakiness (~30%) while histological blood vessel parameters were not affected. Fluorescent imaging with the HypoxiSense probe showed increased tumor hypoxia after irradiation and HBOT. Histological tumor characteristics and epithelial-to-mesenchymal transition markers were not influenced by HBOT. The development of metastatic lesions in the mice could be detected by BLI, revealing that HBOT did not affect the incidence of cervical lymph node metastases. In conclusion, the current HNSCC mouse model allowed us to detect and monitor effects of HBOT and radiation therapy on tumor growth, vascular permeability, hypoxia and metastasis. The combined longitudinal imaging of tumor growth and (supra) molecular analysis of tumor characteristics highlight the versatility and potential of optical imaging methods in future oncological research.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer type worldwide and is associated with a poor prognosis. Treatment of these cancers often involves surgical resection followed by radiotherapy. Despite advances in radiation protocols that minimize the targeted tissue volume, radiation treatment often causes considerable damage to the surrounding healthy tissues including salivary glands, oral mucosa, vasculature, muscle and bone. Consequently, wound healing is impaired and osteoradionecrosis may occur, resulting in higher complication rates of reconstructive surgery in HNSCC patients that had been treated with radiotherapy. Hyperbaric oxygen therapy (HBOT) is frequently used in the management of radiation induced tissue injury, and has shown beneficial effects although its working mechanism has not completely been unraveled yet[1, 2]. In HBOT, patients inspire 100% oxygen at elevated air pressure, which enhances the amount of oxygen that is dissolved in the plasma leading to an increase of the oxygen tension in tissues. By creating an oxygen gradient, HBOT is thought to induce neovascularization by which the progressive loss of the microvasculature in hypoxic irradiated tissue may be overcome and tissue healing is improved[3-5].

The use of HBOT in patients with a history of cancer has often raised concerns about the promoting effect this therapy might have on the growth of (residual) tumor tissue. Poor oxygenation and abnormal vasculature is a common feature of solid tumors and reduces the ability of cells to divide. It was anticipated that by its pro-angiogenic effect, HBOT would stimulate cancer growth and recurrence[6-8]. On the other hand, tumor hypoxia is known to be essential for the progression of cancer and is related to increased cell survival, induction of angiogenesis, metastasis and therapy resistance[9, 10]. Enhanced oxygenation of tumors by HBOT could therefore lead to less aggressive cancer growth and a better prognosis. Based on clinical and experimental studies it was recently adopted that there is no evidence that HBOT has a cancer promoting effect[11, 12]. Even more, on certain cancer subtypes like gliomas and mammary tumors, an anti-angiogenic and growth-inhibitory effect of HBOT was reported[13-15] .

The effect of HBOT on squamous cell carcinoma has been investigated in several experimental tumor models and reached controversial results. Inhibition of tumor-growth and lymph node metastasis was observed in a carcinogen-induced hamster cheek pouch model [16]. No differences in growth between control and HBOT groups were seen in five other studies in which subcutaneously implanted squamous carcinoma cell lines in mice were used[17-21]. However, in a recent study, Paniello et al[22] reported enhanced growth of HNSCC tumor cells in C3H mice after HBOT. These divergent outcomes suggest that the choice of the experimental model, regarding cancer cell type, tumor location or HBOT protocol, is critical for the proper

determination of tumor responses to HBOT.

One of the tumor conditions that is relevant to the clinical situation but has been scarcely investigated in experimental HBOT studies is the irradiated tumor. Radiation not only modifies the cancer cells but also the microenvironment of the tumor by affecting angiogenesis and the hypoxic state of the tissue[23]. Therefore previous irradiation might well influence the response of the residual tumor to HBOT.

To get more insight in the consequences and risks of HBOT for cancer patients, further investigations with improved tumor models and advanced analytical methods are required. In the present study we used bioluminescent imaging (BLI) to non-invasively and adequately monitor the growth of a human squamous cell carcinoma line (FaDu) in the floor of the mouth of immunodeficient mice. Near infrared fluorescence (NIRF) optical imaging was applied to detect and quantify the effects of HBOT and irradiation on specific tumor characteristics in vivo. The fluorescent blood pool agent AngioSense was used to analyze tumor blood vessel quality and the NIRF targeting probe HypoxiSense was applied to study hypoxia in the tumors. Furthermore, this orthotopic mouse model allowed us to investigate the effects of HBOT on the development of regional and distant metastases, which are likewise frequently seen in patients with HNSCC.

MATERIALS AND METHODS

Mice

All animal experiments of this study were approved by the Animal Experiments Committee of the Erasmus Medical Center (DEC 2645). The Dutch Experiments on Animal Act is established under European guidelines (EU Directive No. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes). BALB/c nu/nu female mice (Charles River Laboratories), aged 8 to 11 weeks were kept in filter-top cages with autoclaved pellet food and sterilized water without restriction. Mice with tumors in the floor of the mouth were given soft food and were monitored daily. Animals were euthanized when they had lost more than 20% of their initial body weight or had reached day 35 after tumor implantation.

Tumor generation

The human hypopharyngeal squamous cell carcinoma line FaDu-luc2 was kindly received from the laboratory of Prof. C.W. Löwik, PhD (Leiden University Medical Center, Leiden, the Netherlands). This cell line had been transfected with a luciferase-expressing vector (pCAGGS- Luc-2) allowing the monitoring of the tumor growth by bioluminescence imaging (BLI)[24]. FaDu-luc2 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% (v/v)

fetal bovine serum (Hyclone) and antibiotics (50 units/ml of penicillin and 50 µg/ml streptomycin) at 37°C in a humidified atmosphere of 5% CO_2 in air. Orthotopic tumors were established by transcervical injection of $1x10^5$ cultured FaDu-luc2 cells, suspended in 20µl serum-free DMEM into the floor of the mouth of anesthetized (2-4% isoflurane) nude mice.

Hyperbaric oxygen treatment (HBOT)

Treatment with hyperbaric oxygen started at day 5 after tumor implantation and consisted of daily sessions, until the end of the experiment with a maximum of 30 sessions. The hyperbaric oxygen chamber used in this study was custom-built for small laboratory animals (Hytech BV, Raamsdonksveer, the Netherlands)[25]. Each session started with a compression phase of 15 min, during which the pressure in the chamber was elevated to 2.4 atmospheres absolute (ATA) and the oxygen level to 100%. After 90 min of isopression, decompression to 1 ATA took place in 15 min.

Radiation therapy (RT)

Mice were locally irradiated at day 5 after tumor implantation with a single dose of 5 Gy using a Gammacell 40 Exactor ^{137}Cs γ-source. For tumor irradiation mice were anesthetized by intraperitoneal (i.p.) injection of a mixture of ketamine and xylazine (65 mg/kg and 10 mg/kg respectively) and shielded using a Gammacell 40 Collimator centering the head and neck region in a 3 cm radiation field.

Bioluminescence imaging (BLI)

Tumor growth was monitored twice a week by bioluminescence imaging using an IVIS Spectrum Imaging System (Xenogen). An aqueous solution of luciferin (Caliper Life Sciences) at 150 mg/kg was injected intraperitoneally 10-20 minutes before imaging. During imaging animals were anesthetized with 2-4% isoflurane and placed in a dorsal position. Using the Living Image software 3.2 (Xenogen) photon flux was quantified within a circular region of interest encompassing the head and neck region of each mouse. For 3D reconstruction, BLI images were coregistered with computed tomography (CT) images.

Fluorescence molecular tomography (FMT) imaging

One day prior to their endpoint and at least 20 hours after the last HBOT session, mice were intravenously injected with 1.3 nmol of the fluorescent blood pool imaging agent AngioSense750 (PerkinElmer) and/or 1.3 nmol of the carbonic anhydrase IX (CAIX) targeted fluorescent imaging agent HypoxiSense680 (PerkinElmer) or MMPSense680, a probe that is activated after cleavage by matrix metalloproteinases (MMPs). For quantitative fluorescence molecular tomography imaging (FMT 2500, PerkinElmer), mice were anesthetized (isoflurane, 2-4%) and

fixed in a definite position in an animal imaging cassette. The FMT 2500 tomography software was used to quantitate fluorochrome concentration distribution of AngioSense in a region of interest (ROI) of 750 mm^3 in the tumor area (floor of the mouth). In vivo imaging sessions were performed 2 h and 24 h post-injection and immediately hereafter, the mice were euthanized with an overdose of isoflurane, the tumors were dissected and used for ex vivo imaging. For multi-modality imaging, image data from FMT were fused with CT using markers in the multimodal mouse bed.

Histology

Mouse tumors were fixed in 10% formalin, embedded in paraffin and 5 µm slides were cut. Routine hematoxylin and eosin (H&E) staining was performed and assessed by a pathologist. For immunohistochemistry slides were probed with primary antibodies against Ki67 (Novus Biologicals Ltd.) and CD-31 (Abcam) to assess proliferation and blood vessel density and diameter, respectively. Biotinylated goat antirabbit IgG (Dako) was used as secondary antibody. Detection of the antibody complex was performed with streptavidin–peroxidase (R&D Systems) and 3,3'-diaminobenzidine (Dako). Hematoxylin served as counterstain.

Slides were scanned using a slide scanner (Hamamatsu Photonics). To measure proliferation the percentage of Ki67 positive cells per tumor area was determined by using Cell[d] (Olympus Life Science Europe GmbH). Apoptosis levels were determined by counting the number of apoptic cells in proliferating tumor areas (20x) in H&E stained slides. To determine vascular density, CD31-positive blood vessels were counted in 20 representative fields (40x) for each tumor. The vascular diameter of 30 vessels for each tumor was measured in 63x high power fields.

Metastasis

During the course of the experiment the development of metastases was monitored by BLI of the total mouse body. Imaging was performed using unmixed emission spectra, allowing signal detection at particular tissue depths, which prevented outshining of the signal of the regional metastasis by the primary tumor. To establish the incidence of lymph node metastases, 2 superficial cervical lymph nodes were resected from each mouse, incubated for 10 minutes in luciferin solution (30 µg/ml) and ex vivo BLI was performed. To confirm the metastatic lesions, lymph nodes were embedded in paraffin, sectioned and H&E-stained.

Quantitative real-time reverse transcription polymerase chain reaction (qPCR)

Mouse tumors were dissected, rapidly frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). The resulting cDNA was

amplified in 40 cycles (enzyme activation at 95°C for 20 sec, denaturation at 95°C for 3 sec, annealing/extension at 60°C for 30 sec) with a Bio-Rad cycler using Fast SYBR Green Master Mix (Applied Biosystems). Specific primers (supplematary table S1) were used to amplify cDNA from human vascular endothelial growth factor A (VEGF), carbonic anhydrase IX (CAIX), E-cadherin (CDH1), Vimentin (Vim), snail family zinc finger 1 (Snail), transforming growth factor beta 1 (TGFβ1) and the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each PCR reaction was performed in duplicate and the average threshold cycle (Ct) value was used for relative quantification of gene expression with the comparative Ct method (ΔΔCT).

Statistical analysis

Data are expressed as mean values with standard error of the mean (SEM), and were analyzed using SPSS PASW 21.0 for Windows (SPSS Inc., Chicago, USA). The Shapiro-Wilk test was used to test for normality, followed by the Mann-Whitney U test for the comparison of non-normally distributed data, while Student's t-test was used for normally distributed data. $P<0.05$ indicated significant differences. Survival data was analyzed by the Kaplan-Meier and logrank tests for survival distribution. The Fisher's exact test was used to analyze differences in the incidence of lymph node metastasis between groups.

RESULTS

Effect of HBOT on tumor growth and mouse survival

To examine the effect of HBOT on the growth of orthotopic tumors FaDu-luciferase cells were implanted in the floor of the mouth of nude mice and the growth was monitored by bioluminescence imaging until they met criteria for euthanasia, mostly due to weight loss. As shown in Figure 1 the increase of the bioluminescent (BLI) signal was significantly higher ($P=0.023$) in the group of mice that had undergone daily treatments of HBOT compared with the untreated group on day 18 after tumor cell inoculation (Fig. 1A, B). The mean doubling times for the BLI signals of the individual tumors were determined and were significantly lower in the HBOT group versus the control (2.15 vs 2.47 days, $p=0.006$) (Fig. 1C). In irradiated tumors the increase of the BLI signal was delayed compared to control tumors, but no significant effect of HBOT on the tumor growth rate was observed here (doubling times 3.46 and 3.65 days) (Fig. 1B, C).

The median survival periods for mice in the control, HBOT, RT and RT+HBOT groups were 23, 21, 27 and 36 days respectively. There was no significant effect of HBOT on the survival of non-irradiated mice, but mice with irradiated tumors had an increased survival time if HBOT had been applied ($p=0.003$) (Fig. 1D). The

maximal BLI values measured at the time of euthanasia were higher in the HBOT group as compared to the control for both non-irradiated (p=0.020) and irradiated tumors (not significant, p=0.176)(Fig. 1E)

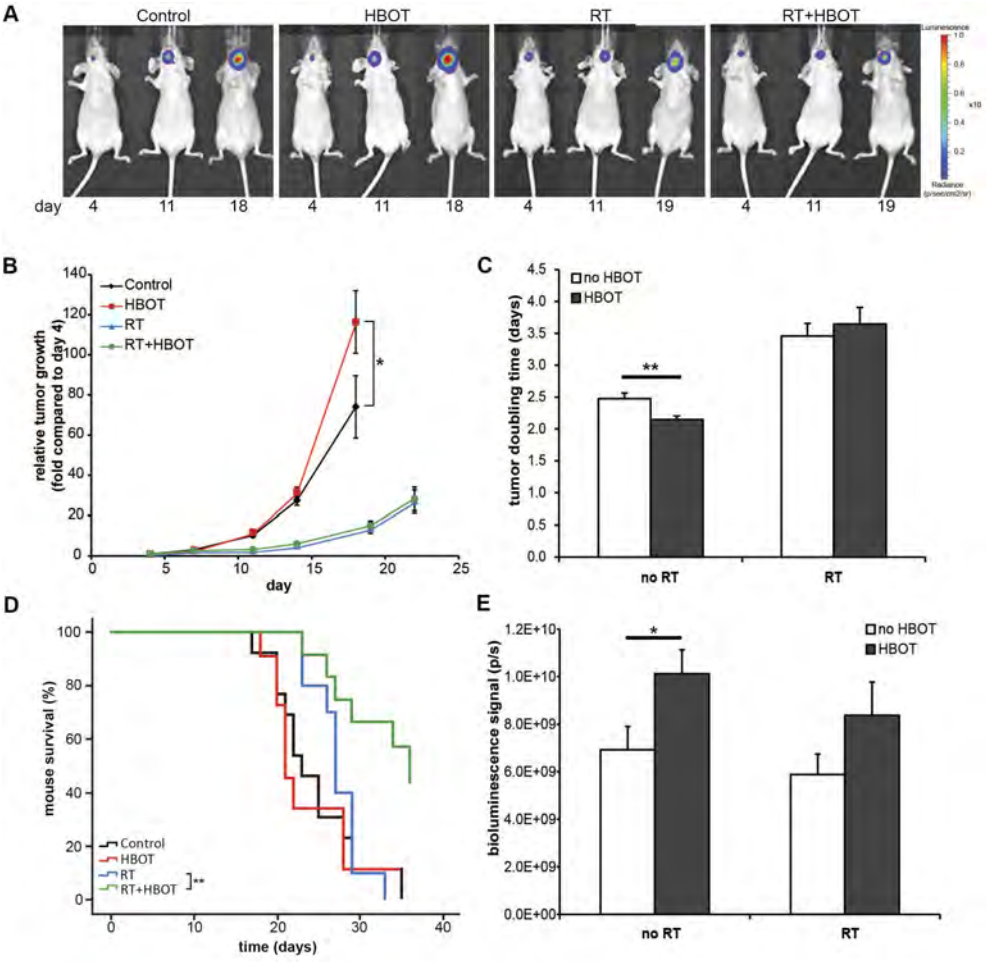

Figure 1 In vivo effects of HBOT on tumor growth and survival time in FaDu-luc tumor bearing mice. **A**. Bioluminescence imaging (BLI) of representative mice of the different treatment groups on day 4, 11 and 18 or 19 after xenografting tumor cells in the floor of the mouth. **B.** BLI was measured twice a week and data points indicate the increase in tumor signals compared to day 4 after xenografting tumor cells (9-12 mice per group). Error bars indicate SEM. *p<0.05 **C**. Mean doubling times of the tumors based on the BLI signals measured between day 7 and 18 (ctrl and HBOT) or day 11-22 (RT and RT+HBOT). Error bars indicate SEM. **p<0.01. indicate SEM. *p<0.05. **D**. Mouse survival time analysis using the Kaplan-Meier method and comparisons using log rank tests. **E**. Mean BLI signals of the tumors at the endpoint. Error bars RT: radiation therapy, HBOT: hyperbaric oxygen therapy.

Effect of HBOT on tumor vascularization and vascular permeability

Vascularization of the tumors was analyzed by investigating the CD31 positive blood vessels in tumor sections (Fig. 2A). The mean blood vessel density was slightly increased in irradiated tumors (1.2 fold, p=0.014) but no significant effect of HBOT was observed (Fig. 2B). The mean tumor blood vessel diameter did not differ between the groups (Fig. 2C). Expression of VEGF, a key factor involved in angiogenesis, was quantified in the tumors of the different experimental groups by qPCR. VEGF mRNA levels were significantly increased in the irradiated tumors (1.3 fold, p=0.000), but not affected by HBOT (Fig. 2D).

Tumor blood vessel quality was analyzed in vivo with FMT using AngioSense750 as a blood pool marker. AngioSense remains in the vasculature for 0-4 hours and therefore the signal detected in the tumor area 2 h after probe injection is a measure for the tumor vascular volume. The degree of AngioSense retention in the tumor area after 24 h is indicative for vascular leakiness[26, 27]. In Fig. 2E the site of accumulation of AngioSense750 in and around the tumor is shown and coregistered with the signal of the probe MMPSense680 which indicates the tumor margins. Mean AngioSense concentrations in the tumor areas were determined shortly after injection and after 24 h (Fig. 2F), but differences between the groups did not reach statistical significance (results not shown). Because variations in tumor size might have obscured the differences, the mean ratio between the 24 h and 2 h AngioSense signals for each individual mouse was determined. Probe accumulation appeared to be higher in the HBOT groups of the non-irradiated (1.3 fold, p=0.042) as well as the irradiated animals (1.3 fold, p=0.078), indicating an increase in tumor vascular permeability after HBOT (Fig. 2G).

Effect of HBOT on tumor hypoxia

Expression of the hypoxia inducible marker carbonic anhydrase IX (CAIX) in the tumor was measured by quantitative PCR. A clear increase in CAIX mRNA levels (2.4 fold, p=0.000) was observed in tumors of irradiated animals but no significant effect of HBOT on CAIX expression was detected (Fig. 3A).

Tumor hypoxia was further analyzed by FMT using the CAIX targeted fluorescent imaging agent HypoxiSense680 as a probe. Because of the low fluorescence levels it was not possible to obtain in vivo data regarding the hypoxic state of the tumors. Mice were sacrificed 24 h after probe injection and analyzed ex vivo (Fig. 3B). HypoxiSense signals were detected in a subset of excised tumors. Strikingly, in none of the control tumors but half of the HBOT tumors fluorescence was detectable. Stronger HypoxiSense signals were observed in the tumors of the irradiated animals with again the highest fluorescent levels in the HBOT group (Fig. 3B, C), indicating that tumor hypoxia is increased after HBOT.

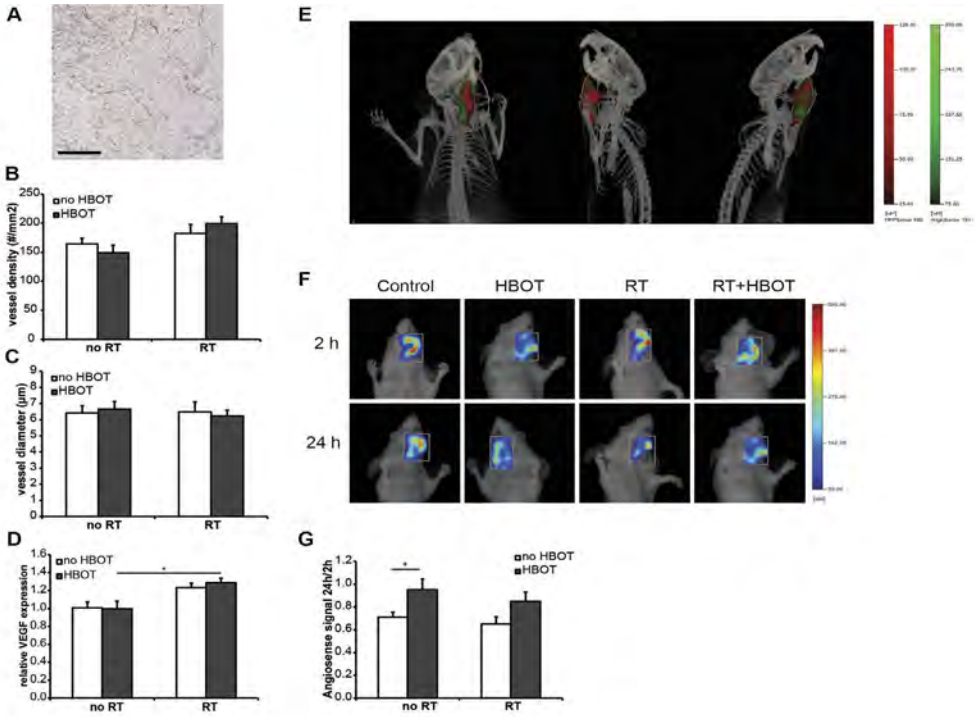

Figure 2 Effect of HBOT on tumor vasculature **A.** Representative immunohistochemical CD31 staining of blood vessels in FaDu-luc tumors. Scale bar represents 20 µm. **B.** Quantification of the tumor blood vessel density. **C.** Quantification of the tumor blood vessel diameter. **D.** Relative expression levels of VEGF mRNA in the tumors as determined by qPCR. **E.** Multimodal FMT/CT imaging of a FaDu-luc mouse 24h after i.v. injection of MMPSense680 (shown in green) and the blood pool agent AngioSense750 (shown in red) to detect tumor margins and region of tumor vascular leak, respectively. **F.** Representative FMT images of tumor regions in FaDu-luc mice of the different treatment groups, 2h and 24h after i.v. injection of AngioSense750. **G.** Quantification of blood vessel leakage in the tumor regions. For each animal the 24h/2h AngioSense signal ratio was determined (n=6). Error bars indicate SEM. *p<0.05. RT: radiation therapy, HBOT: hyperbaric oxygen therapy.

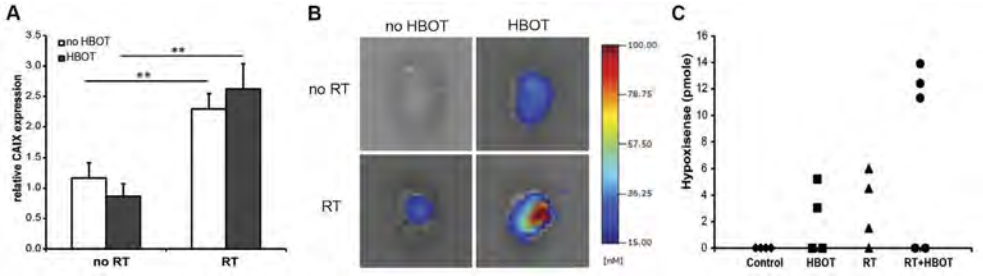

Figure 3 Effect of HBOT on tumor hypoxia **A.** Relative expression levels of CAIX mRNA in the tumors as determined by qPCR. Error bars indicate SEM. **p<0.01. **B.** Representative ex vivo FMT images of dissected FaDu-luc tumors from mice 24 hours after i.v. injection of the hypoxia probe HypoxiSense **C.** Quantification of HypoxiSense signals in individual dissected tumors of the different treatment groups. RT: radiation therapy, HBOT: hyperbaric oxygen therapy.

Effect of HBOT on tumor pathological features and metastasis

To investigate if HBOT influenced pathological features of the tumor, histologic sections of the FaDu-tumors were rated by a pathologist. For all experimental groups, the tumors were characterized as poorly to moderately differentiated squamous cell carcinoma with moderate infiltrative borders (Fig. 4A, B, C). Perineural growth and vascular invasion of tumor cells was evident in several tumor sections but no significant differences were observed between the HBOT and the control groups. The degree of necrosis (Table 1) was highly variable among the tumors (0-42.2% of the tumor area) and was related to tumor size, but no significant effect of HBOT on the level of tumor necrosis could be established. Furthermore, immunohistological staining of the proliferation marker Ki67 showed no effect of HBOT on cell proliferation levels (Table 1). Apoptosis levels were also not significantly different between the groups although a trend towards decreased cell death after HBOT was observed (Table 1).

Table 1 Proliferation, apoptosis, and necrosis in FaDu tumors.

	control	HBOT	RT	RT+HBOT
Metastatic incidence	8/12	8/11	9/10	10/11
Metastatic percentage	67%	72%	90%	91%
Fisher's exact test vs control		1.000	0.323	0.317

[1]Mean values ± SEM
[2]Median values [range]
RT: radiation therapy, HBOT: hyperbaric oxygen therapy

To investigate the impact of HBOT on EMT, mRNA expression levels of the malignancy markers E-cadherin, Vimentin, Snail and TGFβ were determined in tumors of the different experimental groups. Vimentin expression was slightly upregulated in the irradiated tumors (1.4 fold, p=0.039) but its levels were not significantly influenced by HBOT (Fig. 4E). On the tumor expression of E-cadherin, Snail and TGFβ, no significant effects of either RT or HBOT were observed (Fig. 4D, F, G).

The orthotopic tumor model allowed us to identify and monitor the development of regional or distant metastases in real time by in vivo BLI using spectral unmixing and 3D reconstruction to circumvent outshining of the signals by the strong total BLI signals of the primary tumor in the floor of the mouth (Fig. 4H). In the time frame of the experiment distant metastases were not detected but cervical lymph node metastases developed in the majority of the mice. To confirm and quantify the

locoregional metastases, two superficial cervical lymph nodes of each mouse were harvested immediately after euthanasia and analyzed by ex vivo BLI and histological methods (Fig. 4I, J). As shown in Table 2 the metastatic incidence was 67%, 72%, 90% and 91% for the control, HBOT, RT and HBO+RT groups respectively. A trend towards enhanced metastasis rates after irradiation was observed (p=0.137), but there was no effect of HBOT on the incidence and histological stage of the lymph node metastases.

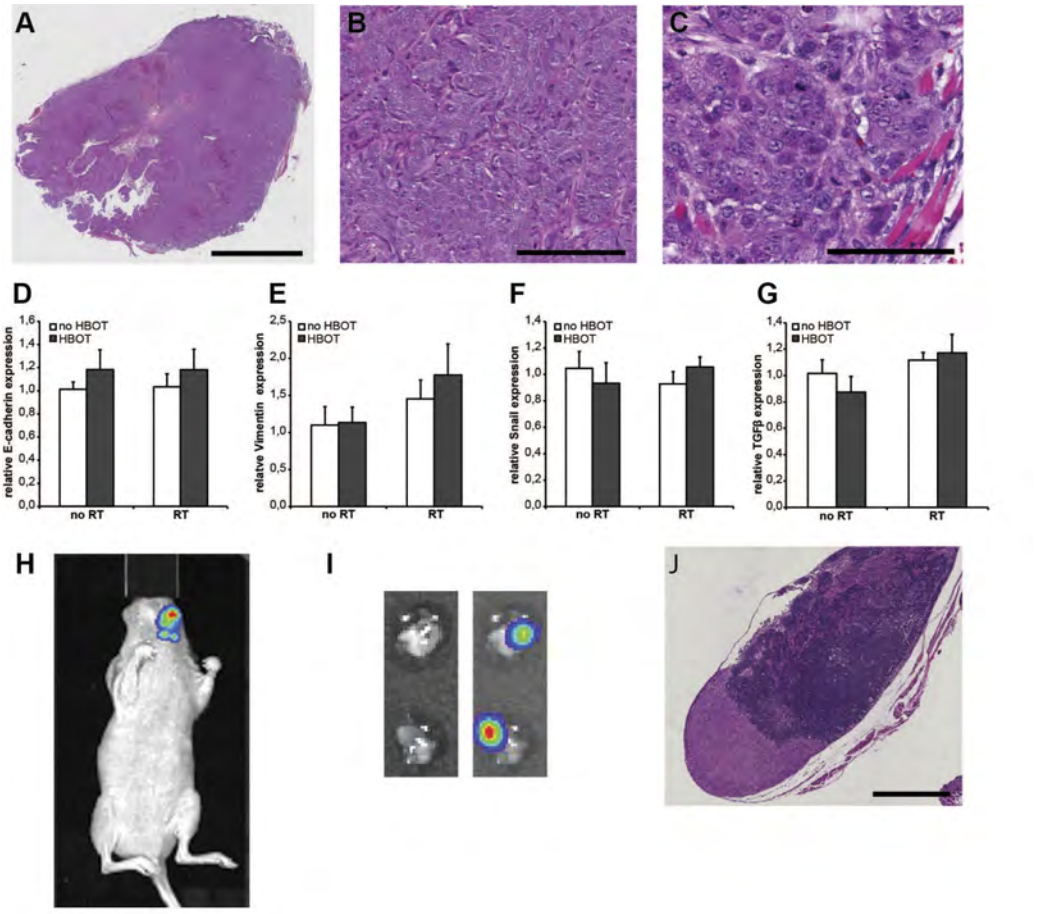

Figure 4 Effect of HBOT on tumor malignancy parameters **A.** H&E staining of a tissue section of a representative FaDu-luc tumor dissected from the floor of the mouth. All treatment groups show poorly to moderately differentiated squamous cell carcinoma. Scale bar represents 2 mm. **B.** Detail of A. Scale bar represents 20 µm. **C**. Detail of A. Scale bar represents 100 µm. **D-G.** Relative expression levels of E-cadherin, Vimentin, Snail and TGFβ1 mRNA in the tumors as determined by qPCR. RT: radiation therapy, HBOT: hyperbaric oxygen therapy. **H.** In vivo BLI at an emission wavelength of 560 nm of a tumor bearing mouse at day 20 showing lymph node metastases **I.** Ex vivo imaging of metastasized cervical lymph nodes. Photographic (left) and bioluminescent (right) images are shown **J.** H&E staining of a tissue section of a representative metastasized lymph node. Scale bar represent 0.5 mm.

Table 2 Incidence and rate of lymph node metastasis in mice with FaDu tumors.

	control	HBOT	RT	RT+HBOT
Proliferation (% Ki67 positive tumor cells)[1]	38.1±1.8	40.0±1.5	43.2±1.7	41.9±1.2
Apoptosis (# cells/mm^2) [1]	30.6±2.9	24.9±2.4	22.2±3.9	19.0±4.1
Necrosis (% area) [2]	2.9 [0-28.0]	6.9 [0.5-27.5]	5.0 [0-42.1]	6.9 [0-31.0]

DISCUSSION

In this study tumor responses to HBOT and irradiation were investigated in an orthotopic model of head and neck squamous cell carcinoma using optical imaging methods. By means of bioluminescence imaging, the growth of a human hypopharyngeal carcinoma cell line in the floor of the mouth of mice was accurately monitored and revealed a small but significant increase in tumor growth rate (19%) under the influence of HBOT. No effect of HBOT however was detected on the growth of tumors that had been irradiated before. The difference in response might lie in the fact that irradiation, except for killing tumor cells, also damages endothelial cells[28, 29], resulting in a tumor microenvironment that is less susceptible to HBOT-induced, growth promoting stimuli. Although tumors grew faster in HBOT- treated mice as compared to controls, the survival time of these animals was not affected. Interestingly, the bioluminescent signals of the tumors at the endpoints were higher in the HBOT group, indicating that these mice survived higher tumor loads. Also in the irradiated groups, in which the survival period was extended by HBOT but the tumor growth rate was not affected, a trend towards increased endpoint tumor sizes was noticed, suggesting a beneficial effect of HBOT on survival rate.

HBOT stimulates vessel development in normal tissue and in wounds[3, 5] but its effect on tumor vascularization is unclear. Tumors possess disorganized and leaky tumor vessels which block adequate tissue perfusion leading to the presence of hypoxic regions that are associated with poor prognosis and treatment outcome[30, 31]. Normalization of the tumor vasculature is thought to lead to less tumor hypoxia and is a goal of anti-angiogenic therapies[9, 32]. We evaluated the effect of HBOT on vascular function and tissue hypoxia in our HNSCC tumor model. Histological analyses revealed no significant effect of HBOT on tumor vascular density and diameter. Using in vivo optical molecular imaging with the blood pool agent AngioSense the effect of HBOT on tumor vascular permeability was investigated and disclosed a higher vascular leakiness in tumors of HBOT treated animals. This is the first study exploring the effects of HBOT on vascular permeability, revealing that HBOT does not lead to normalization of blood vessels and even appears to deteriorate tumor

vascular quality.

To compare the hypoxic states of the tumors using optical imaging, the recently developed HypoxiSense probe, which detects the protein CAIX on the tumor cell surface, was employed. Due to relatively low fluorescent signals, however, in vivo data could not be obtained. In previous studies this fluorescent agent was successfully used in subcutaneous xenograft tumors with volumes of 600-700 mm^3 [33]. In our orthotopic model the tumors in the floor of the mouth did not grow beyond 250 mm^3 and therefore signal detection was probably hampered by optical properties such as background absorption and scattering[34]. Nevertheless, ex vivo, hypoxic regions were detected in a subset of tumors and, although the numbers were small, the data suggest that irradiated tumors were more hypoxic than non-irradiated tumors and moreover, that HBOT increased tumor hypoxia as well. It has been shown that HBOT increases the oxygen concentration in tumor tissue during and shortly after treatment[35, 36] but this effect is transient. The drop in oxygen level following a HBOT session may lead to the induction of a hypoxic response in the tumor tissue, by which CAIX expression could be enhanced. This would correspond to previous studies in which exposure to HBOT resulted in increased levels of the hypoxia inducible factor HIF-1α in the liver and brain of rats[37-39]. The data indicate that enhanced oxygenation using an intensive HBOT protocol, does not lead to long term overall reduction of tumor hypoxia.

The presence of cervical lymph node metastasis is an important prognostic indicator for patients with HNSCC. The current bioluminescent orthotopic tumor model allowed us to monitor the effect of treatment on the development of lymph node metastases. Metastatic incidence was increased from approximately 70% to 90% in the irradiated animals, but was not affected by HBOT. This confirms previous experimental results obtained in different cancer and animal models, in which stimulation of metastasis by HBOT was not established either[16, 40-44]. Histopathological analysis of tumors taken from mice after the maximal growth period also revealed no significant HBOT-induced changes in malignant parameters, like differentiation grade and degree of infiltrative and invasive growth. Epithelial-to-mesenchymal transition (EMT) is a central mechanism of cancer metastasis whereby epithelial cells are reprogrammed, resulting in decreased adhesion and enhanced migration and invasion[45-47]. The expression of the hallmark molecules of EMT, the epithelial marker E-cadherin, the mesenchymal marker Vimentin, and the EMT-inducing factors Snail and TGFβ was not affected by HBOT, indicating that there was no switch to more aggressive tumors. Moen et al[15] reported induction of mesenchymal-to-epithelial transition (MET) by HBOT in a mammary tumor model, but thus far there are no indications for similar effects in squamous cell cancer.

Altogether, using an orthotopic mouse model for squamous cell carcinoma we found the following effects of HBOT on tumor growth and development: HBOT

stimulated the growth of non-irradiated tumors, enhanced tumor blood vessel leakiness and appeared to increase tumor hypoxia. These are factors known to promote aggressive tumor behavior and poorer treatment outcome[10, 31]. On the other hand HBOT was beneficial for animal survival and no effects of HBOT were detected on metastatic incidence, histological grade and malignancy markers, suggesting that the effects of HBOT on disease outcome are limited. Irradiated tumors differed from non-irradiated tumors in that they grew slower but showed more malignant features since more metastasis and hypoxia was observed and higher transcript levels of VEGF, CAIX, and Vimentin, factors that are negatively associated with patient prognosis in cancer, were detected. The response of irradiated tumors to HBOT was similar to that of non-irradiated tumors, except that no effect on growth rate was observed. Therefore, there might be no increased risk for negative effects of HBOT in patients that were previously subjected to radiation therapy. Previous experimental studies on the effects of HBOT on tumor behavior thus far revealed varying results[8, 11]. In mammary and glioma tumor models growth-inhibiting, anti-metastatic and anti-angiogenic effects of HBOT were reported[13, 14, 48], but studies using squamous cell cancer models in mice did not reveal effects of HBOT on tumor growth[16-21], except for a recent study of Paniello et al[22] who also observed enhanced growth of xenografted tumors in mice. Specific tumor characteristics and treatment conditions might underlie the different outcomes. The improved animal model and in vivo molecular imaging methods used in this study disclosed influences of HBOT on the growth rate, blood vessel quality and hypoxic state of squamous cell carcinoma and opens up possibilities to further investigate the circumstances and conditions in which HBOT can be safely used in cancer patients.

Acknowledgements

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SUPPLEMENTARY DATA

Table S1 Primers used for qPCR

mRNA	Forward (5'-3')	Reverse (5'-3')
VEGF	CGAAACCATGAACTTTCTGCTG	TCCATGAACTTCACCACTTCG
CAIX	AGGGGTCTCTGACTACACCG	GAGGGTGTGGAGCTGCTTAG
CDH1	AATCCCACCACGTACAAGGG	GTGTATACAGCCTCCCACGC
Vim	AGGAGGAAATGGCTCGTCAC	AGAAATCCTGCTCTCCTCGC
Snail	CCAGTGCCTCGACCACTATG	CTGCTGGAAGGTAAACTCTGG
TGFβ1	CGTGGAGGGGAAATTGAGGG	CCGTTGATGTCCACTTGCAG
GAPDH	CACCGTCAAGGCTGAGAACG	GAGGGATCTCGCTCCTGGAAG

Chapter 9

General Discussion

TREATMENT OF RADIATION INDUCED TISSUE DAMAGE

Normal tissue damage due to radiation treatment of head and neck cancers (HNC) remains an important complication that can greatly affect quality of life of these patients. Although different radiation techniques have improved the local targeting of tumor tissue, it is still inevitable that surrounding normal tissue will receive radiation, leading to impaired functionality. In the head and neck region, many different tissues lie in close proximity to each other, and are thus susceptible to radiation damage. This thesis focusses on radiation damage to salivary glands and bone. Damage to salivary glands can lead to chronic hyposalivation and xerostomia, which is the most common complication of radiation therapy for head and neck cancer. It also creates an oral environment in which bone damage is more prone to develop, which increases the risk of developing osteoradionecrosis (ORN). Both complications are irreversible and greatly compromise oral functions. In addition, reconstructive surgery, often needed in HNC patients, may be complicated.

Treatment options are scarce and do not provide full recovery, therefore, prevention of radiation-induced damage is of optimal interest. We investigated the potential of hyperbaric oxygen therapy (HBOT), a therapy that is used in the treatment of ORN, and of the heparan sulfate mimetic RGTA, which has shown promising results in the treatment of chronic non-healing wounds, to prevent radiation damage to salivary glands and bone. The main focus of this thesis is HBOT, since this is already clinically used.

HBOT is mostly used in the treatment of chronic (diabetic) wounds and delayed radiation injury, such as ORN. In chapter 2, the literature about HBOT on irradiated head and neck bone was surveyed. Experimental as well as clinical studies proved to be scarce. Experimental animal studies were mostly done in rodents and monitored the effects of HBOT on tissue regeneration after implant placement in hind legs or dealt with radiation therapy that was combined with distraction osteogenesis.

Most clinical studies were retrospective and therefore lacked controls. HBOT is used either as treatment for ORN, or as a prevention modality when surgery is needed in previously irradiated bone. None of the studies used HBOT as a prevention for bone damage by administering it shortly after radiation therapy. Roughly seventy-five percent of these studies report a positive effect of HBOT on prevention or treatment of ORN, although most conclusions are merely suggestions. Recent publications have questioned the efficacy of HBOT regarding treatment or prevention of ORN[1-4] and state that HBOT may not offer any appreciable clinical benefit, although positive effects, and thus recommendation for HBOT use, are also reported[5-8]. The consensus among virtually all clinical studies is that there is a need for large randomized controlled trials, which have not been performed up to

now. Schoen and colleagues[9] did perform a randomized clinical trial in which HBOT could not be shown to enhance implant survival, but they used a rather small study population. Another trial of Annane et al[10] was prematurely terminated because of possible worse outcomes in the HBOT group. However, the study design was heavily criticized by others[11-14]. The formation of a control group when studying effects of HBOT on ORN can be difficult. Since HBOT is regarded as a standard treatment for ORN, ethical committees can decline to withhold ORN patients from HBOT[15, 16].

ANIMAL MODELS FOR RADIATION INDUCED TISSUE DAMAGE

More insight into the efficacy and mechanisms of action of HBOT is needed on a functional, cellular and molecular level. Animal models provide an important tool for this purpose. Radiation damage to salivary glands and bone has been studied using different animal models, most commonly mice and rats. Various radiation doses and schedules have been used. The 15 Gy local radiation dose used in our model has been proven to cause significant salivary gland damage, without compromising the general health of the animals[17-21]. Animal studies regarding radiation damage to mandibular bone generally administer much higher doses, targeted at the mandible[22-25]. Also for salivary glands, it is proposed that radiation therapy should be targeted locally to the salivary glands in order to exclude indirect radiation effects caused by the irradiation of other tissues in the radiation portal[26]. In our model, however, the aim was to simultaneously investigate effects on bone and salivary glands and therefore the complete head and neck region was irradiated. Clinically, these tissues are often not direct targets of radiation therapy but the interplay between radiation effects on multiple head and neck tissues cause the side effects which we aim to address. Pilot experiments were performed to assess whether bone damage was more feasible with a 25 Gy single dose, compared to 15 Gy. The 25 Gy dose, however, resulted in severe mucositis, cataracts and significant loss of body weight within days after irradiation. Since we were mainly interested in the long-term effects, the 15 Gy single dose was chosen. Mice tended to lose some body weight in the first week after irradiation, but it never reached critical values. In the irradiated area, mice had some hair loss and because of somewhat loosened teeth, soft diet was provided in order to maintain sufficient food intake. None of the mice had to be prematurely taken out of the experiment because of weight loss or other detrimental health effects.

In clinical practise, fractionated radiation therapy is used instead of single dose radiation. DNA repair of normal tissue is allowed between fractions and tumor cells that were in a radio-resistant cell cycle phase during one fraction, might be in a more radiosensitive phase during the next fraction, allowing more tumor damage[27]. A single dose of 15 Gy has been reported to be biologically equivalent to a clinically

relevant scheme of 16 fractions of 2 Gy[28], however, according to Coppes et al. higher radio-sensitivity of the submandibular gland for late effects was achieved after fractionated irradiation[28]. The extrapolation of results of animal radiation experiments to the human situation should therefore be carefully considered. Since mice need to be anesthetized every time they receive radiation, which is not without risk and is an invasive procedure for the animals, we used single dose irradiation for practical and ethical reasons.

HYPERBARIC CHAMBER FOR ANIMAL EXPERIMENTATION

To be able to investigate the effects of HBOT in irradiated mice, a hyperbaric oxygen chamber was built especially for animal experimental purposes, which is described in chapter 3. HBO chambers that are clinically used are not suitable for animal experimentation for logistical, ethical and practical reasons. The custom built HBO chamber which is placed at the Animal Experimental Center of the Erasmus MC meets the strict safety regulations for the use of high pressure and pure oxygen and is easy to operate. It allows experimentation with small animal models such as mice, rats and rabbits. The most notable difference with chambers used clinically is that the whole chamber is flushed with 100% oxygen, rather than supplying the oxygen via masks that are used for patients but are not feasible for animals. Apart from that, the HBOT protocol in our mice study was designed to closely resemble clinically used treatment schedules, with a pressure of 2.4 atmospheres absolute and 100% oxygen during daily sessions of one hour.

HBOT AND IRRADIATED MANDIBULAR BONE

In our study on the prevention of radiation-induced damage to mandibular bone (chapter 4), we showed a positive effect of HBOT on microstructural parameters such as bone volume and trabecular thickness, which were negatively affected by RT. On a histological level, the amount of osteoclasts and of empty lacunae was decreased by HBOT, reflecting less bone resorption and an increased bone viability. These changes were all apparent on the long term, i.e. 24 weeks after RT.

The pathophysiology of ORN remains a matter of debate, since different theories have been suggested. The so-called 3H-model proposed by Marx has long been the most accepted theory, on which the treatment with HBOT is based. Marx describes the irradiation-induced hypoxic, hypocellular and hypovascular (3H) environment caused by endarteritis as the basis for the development of ORN[29]. This underscores the potential of HBOT as a treatment for ORN since it could overcome the hypoxic environment[30]. Another theory describes radiation-induced fibrosis as the key event in the development of ORN, where a dysregulation of the fibroblastic activity leads

to atrophy of the tissue[31]. Furthermore, the direct effect of radiation on osteoclasts, resulting in higher bone resorption, has been suggested as an important factor[32]. The latter two theories do not substantiate the use of HBOT, since it is suggested that HBOT might not have an effect on these processes[31, 33]. Our study however shows that HBOT is able to reduce the amount of osteoclasts in irradiated tissue, corresponding to a recent study that reported suppressed osteoclast formation due to HBOT[34]. The mechanism behind the suppression of osteoclasts by HBOT is not elucidated and needs further research.

Green and colleagues report that bone loss is not solely dependent on the activity of osteoclasts, but that irradiation-induced effects on the stem cell pools in bone marrow are also of importance[24]. They showed radiation-induced stem cell depletion and compromised bone marrow at 2 and 10 days after irradiation, which recovered at 8 weeks after irradiation. Hyperbaric oxygen therapy has been shown to promote proliferation of neural stem cells[35], stimulate growth and differentiation of vasculogenic stem cells[36] and mobilize bone marrow-derived stem cells[37]. The improved bone quality by HBOT in terms of microstructure and viability seen in our study could therefore be caused by its effect on bone marrow stem cells that takes place shortly after RT and prevents bone damage on the long term.

HBOT AND RGTA AND IRRADIATED SALIVARY GLANDS

The measurement of salivary flow rate is an important tool in animal experimental studies regarding injury of salivary glands. Whole saliva measurements following pilocarpine injections are fairly easy to perform and reflect the functionality of salivary glands. The resulting stimulated salivary flow rate measured, which is also used in our study, is primarily indicative of parotid gland function, as the majority of stimulated saliva production takes place in this gland. Unstimulated salivary flow rates, predominantly the result of submandibular saliva production, are important as well since xerostomia complaints are also expressed in the unstimulated state (i.e. sleeping, speaking), but they are not easy measurable. Our morphological analysis showed a comparable pattern of radiation-induced damage to both parotid- and submandibular glands (chapters 5 and 6) suggesting that the stimulated salivary flow rate can be used as an indicative measurement for overall salivary gland damage.

While in our study salivary flow rates were significantly lower in irradiated mice at 6 weeks post-RT, no clear effect on the overall morphology of the glands was visible at this time-point. At ten weeks after irradiation, only a slight disorganization of the acinar cells was shown, which was severed at 24 weeks and accompanied with a reduction in the amount of acinar cells. This dissociation between structural and functional changes has been shown by others[38-40] and confirms the theory that in

salivary glands radiation does not cause a direct severe reduction of acinar cells by apoptosis. Instead, it is proposed that radiation-induced damage to the membrane of cells causes the early drop in saliva production by affecting signaling pathways that use cell surface receptors to facilitate excretion and by causing a dysfunction of the water channels in the membrane[26, 41].

Hyperbaric oxygen therapy

Salivary flow rates of irradiated mice were not influenced by HBOT in our study (chapter 5), which indicates that HBOT is not able to prevent or restore damage to the acinar cells and that it thus may not reduce xerostomia. In line with this finding, Schoen and colleagues reported no effect of HBOT on oral dryness in their prospective clinical study regarding prosthodontic rehabilitation in radiated head and neck cancer patients[9]. However, Bui et al would considerate the use of HBOT in xerostomia patients based on their study[16], and even more, clinical (pilot) studies regarding oral dryness have advocated a positive effect of HBOT[42-45].

In some clinical studies stimulated salivary flow rates were measured, but more often questionnaires were used to assess patient-scored xerostomia. Salivary flow rate and patient-scored xerostomia do not always correlate[43, 46, 47], indicating that other factors such as saliva composition may also contribute to the feeling of dry mouth.

In our study, cell proliferation in the salivary glands increased in response to HBOT. This has been shown in other tissues like pancreas, brain and liver[48-52]. An enhanced proliferation rate was primarily visible in acinar cells, and not in duct cells where stem cells reside[53]. This indicates that HBOT did not have a significant effect on the proliferation of salivary gland stem cells, but did affect homeostasis mechanisms in the acinar cells, that retain the ability to replicate, resulting in increased proliferation that potentially could overcome the radiation-induced damage. Other studies have proposed the recruitment of bone marrow derived stem/progenitor cells by HBOT[36, 37, 54, 55].

Regenerating Agent

RGTA-OTR4120 is designed to mimic the function of heparan sulfate at sites of injury. Heparan sulfate is normally present in the extracellular matrix, but degraded upon tissue damage. It is able to bind growth factors thereby stimulating tissue repair and regeneration. The administration of RGTA-OTR4120 to irradiated mice (chapter 6) positively influenced salivary flow rate on the short term, two weeks after irradiation, but not on the long term. Overall morphology was not influenced although the amount of PAS-positive, mucin producing acinar cells was increased at ten weeks post-RT. This suggests that some regeneration of the impaired functionality of the acinar salivary glands had taken place, albeit it was not translated in a higher

salivary flow rate. RGTA may be useful to ameliorate salivation shortly after RT, but more evidence of its efficacy will be needed. Whether RGTA is able to treat salivary gland damage on the longer term needs further investigation. The combination of RGTA with other therapies, like HBOT, might possibly increase functionality of acinar cells and thereby restore salivary flow rates.

HBOT AND MOLECULAR PATHWAYS IN IRRADIATED SALIVARY GLANDS

Little is known about the influence of HBOT on gene expression, and thereby on molecular pathways, in irradiated tissue. Some *in vitro* studies analyzed the effect of a single HBO treatment on different cell types such as neurons, osteoblasts and endothelial cells[49, 56, 57]. Results showed a great variety of responses between cell types, except for a shared upregulation of the oxidative stress response. An *in vivo* rat ischemic brain-model showed an influence on the neurotrophin system and inflammatory immune response after five consecutive HBO treatments[58]. HBOT effects seem to be highly cell type specific and dependent on the environment in which the cells reside. For a better understanding of the cellular and molecular processes that are affected by HBOT in irradiated tissue, analysis of the gene expression profiles can be of great significance. In our study whole genome gene expression was measured in salivary glands by microarray analysis two weeks after irradiation, and thus after two weeks of HBO-treatment (chapter 7). Subsequent functional pathway analysis gave more insight in pathways and processes influenced by HBOT.

Angiogenesis

The main action by which HBOT is believed to exert its positive effects is by promoting angiogenesis. In 1990, Marx demonstrated an eight- to nine fold increase in blood vessel density of irradiated rabbit tissue due to hyperbaric oxygen, compared to normobaric oxygen and air-breathing controls[59]. Later, more studies confirmed the angiogenic potential of HBOT[52, 60-63]. This angiogenic potential may be counterintuitive, since it is known that hypoxia triggers angiogenesis by increasing hypoxia inducible factor (HIF1α) which in turn increases the expression of vascular endothelial growth factor (VEGF), the main growth factor involved in angiogenesis[64]. Nevertheless, it has been shown that hyperoxia is also able to increase HIF1α and/or VEGF levels[52, 60-62, 65-67]. Hyperbaric oxygen increases tissue oxygen levels that reduce to normal within a few hours after a hyperbaric oxygen session. The tissue will repeatedly experience relative hypoxia, which may be the cause for HIF1α accumulation and VEGF production[52].

In our model, we observed an increased blood vessel density in salivary glands of hyperbaric oxygen-treated irradiated mice, confirming the angiogenic potential

of HBOT (chapter 5). However, from our microarray data, obtained two weeks after irradiation and after ten HBO sessions, no clear difference in the expression of angiogenic factors could be detected. Possibly the expression of these factors is transient and since the salivary gland tissue used for the microarray was obtained approximately twenty-four hours after HBO treatment, the increased expression could have been missed in our experiment. It is also possible that the two-week time-point is still too early to see effects. The blood vessel density measured immunohistochemically did not show an HBOT-induced difference at the two week time-point.

Finally, from our experiments it cannot be excluded that the increased blood vessel density observed after HBOT was a consequence of protection of the blood vessels from radiation-induced damage rather than of induced angiogenesis.

The TGFβ-pathway

Although HBOT not clearly altered the expression of angiogenic factors, our microarray data showed a remarkable HBOT-induced inhibition of genes and regulators that are activated by RT. We focused particularly on the TGFβ-pathway, which was attenuated by HBOT according to the differential expression of genes involved in this pathway. The TGFβ-pathway is involved in many biological processes, such as cell proliferation, epithelial-mesenchymal transition (EMT), extracellular matrix (ECM) regulation and immune-suppression and inflammation[68]. The TGFβ-affected extracellular matrix regulation is of particular interest regarding radiation-induced damage. It has been shown that radiation causes overexpression of TGFβ and stimulation of its pathway in various tissues[68-71]. Via different pathways and the consecutive expression of target genes, radiation-induced TGFβ activation leads to excessive matrix formation (by activated fibroblasts) and preservation. This ultimately leads to fibrosis of the tissue in which functional cells are replaced by ECM and thereby loss of tissue function. Indeed, Hall and colleagues showed induction of fibrosis in salivary glands of mice that conditionally overexpress TGFβ[72]. Although fibrosis presents itself as a late effect, it has been proposed that a cascade of cytokines, including TGFβ, is initiated early after irradiation and persists for a long time leading to the development of late damage. Early overexpression of TGFβ after RT has been shown in various tissues, such as skin, intestine, mammary gland and lung[71]. Our study showed that also in salivary glands radiation caused an activated TGFβ response two weeks after RT, which was suppressed by HBOT. Therefore, HBOT could have a potential to inhibit radiation-induced fibrosis. In our model however, fibrosis was not seen in irradiated salivary glands. Probably the endpoint of 24 weeks after RT was not long enough to reveal fibrosis, taken into account that fibrosis can take 30 weeks to develop and is highly strain dependent (the c3h mice used in our experiments are relatively radioresistant)[73]. Therefore, we cannot

conclude whether HBOT indeed is able to inhibit fibrosis. The lower expression of the profibrotic factor α smooth muscle actin-protein at 24 weeks after RT in HBO-treated salivary glands could indicate a reduced onset of fibrosis, but no conclusive evidence on the effect of HBOT on fibrosis was obtained. Despite the fact that fibrosis was not evident, salivary flow rates and thus salivary gland functionality, was greatly impaired. Radiation-induced fibrosis did therefore not play a major role in the damage to salivary glands in the timeframe used in our study. It is however interesting to elucidate the precise effect of HBOT on the TGFβ-pathway and the potential of HBOT to prevent radiation fibrosis in other tissues, in which radiation-induced fibrosis has a big impact on tissue functionality such as lung, kidney, heart and intestine.

HBOT AND TUMOR GROWTH AND DEVELOPMENT

Since patients that receive HBOT in order to prevent or treat radiation-induced tissue damage have a history of cancer, the effects of HBOT on tumor cells are important to decipher. Concerns have been raised on the potential stimulatory effect of HBOT on tumor tissue. While solid tumors are poorly oxygenated with a reduced ability of cells to divide, the pro-angiogenic effect of HBOT could stimulate cancer growth[74-76]. On the other hand, tumor hypoxia is a feature of aggressive tumors with a bad prognosis. HBOT could prove to be beneficial by overcoming hypoxia and thereby could lead to less aggressive tumor behaviour[77, 78].

Using an orthotopic mouse model for squamous cells carcinoma, effects of HBOT on irradiated and non-irradiated tumors were investigated (chapter 8). HBOT slightly stimulated the growth of non-irradiated tumors but not of irradiated tumors. Fluorescent imaging remarkably showed that HBOT did not normalize blood vessels in terms of leakiness, but even seems to deteriorate tumor vascular quality, and that HBOT increased tumor hypoxia. It has been shown previously that HBOT increases the oxygen concentration in tumor tissue during and shortly after treatment[79, 80]. The drop in oxygen level following a HBOT session may lead to the induction of a hypoxic response which was measured in our study by the fluorescent probe that detects carbonic anhydrase IX on the tumor cell surface.

Furthermore, animal survival was increased by HBOT, but there were no effects on metastatic incidence, histological grade, malignancy markers or blood vessel density. Altogether, our results suggest that there is no increased risk for negative effects of HBOT in patients previously subjected to RT with a history of squamous cell carcinoma. While in salivary glands HBOT positively affected proliferation and blood vessel density, no effects on these parameters were seen in tumor tissue. The complex and very different microenvironment of (irradiated) tumors, with divers oxygen concentrations, pH and distribution of nutrients amongst others[81, 82], is

possibly accountable for the difference in effect of HBOT.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, the main question is whether HBOT has the ability to protect head and neck tissues from radiation-induced damage, when given in a preventative manner directly after RT.

Mandibular bone tissue appeared to be positively affected by HBOT, although the clinical relevance of these results needs to be elucidated. It is of importance to know if the observed changes can result in stronger bone, if regeneration is improved when trauma is inflicted, and if ORN can thus be prevented. This could first be investigated using an animal model in which trauma, for example drilling a small hole in the mandibular bone, is inflicted and the subsequent regeneration monitored. Bone strength tests can be performed to see whether HBO-treated bone is indeed stronger and less likely to experience microfractures. For this purpose, the use of rats would be preferred, since the mandibles of mice are too small.

Salivary flow rates were not improved by HBO-treatment, implying that HBOT does not ameliorate xerostomia. However, proliferation and blood vessel density were positively affected, suggesting an effect on the regeneration of salivary glands. It is discussed that the radiation dose may have been too high to translate these cellular effects into functional difference. The use of fractionated radiation, as applied clinically, may lead to different results. Adapted timing of the HBO treatment may also improve the effects on salivary gland function. Maybe the cellular changes that are induced by HBOT are more desirable at later time-points after RT and thus have more impact on the regeneration of the tissue if applied then. Varying durations of HBOT, in terms of the total amount of HBOT sessions, might also cause different effects and it is clinically relevant to know whether fewer HBO treatments can cause the same effect. The most optimal HBOT-protocol should be identified in animal models, and subsequently tested in randomized controlled clinical trials. The protocols used in practise today are not (fully) evidence-based and a reconsideration of these protocols might lead to improved treatment and outcome.

The inhibitory influence that HBOT showed to have on the TGFβ-pathway in salivary glands is an interesting factor to further investigate. The TGFβ-pathway is activated in many other tissues that suffer from radiation-induced fibrosis, and HBOT could prove to be a valuable tool to prevent radiation-induced fibrosis and thus impaired functionality of irradiated tissues. For this, the most suitable timing

and duration of HBO-treatment needs to be investigated and will probably vary between tissues and conditions.

Lastly, HBOT did not seem to have strong adverse effects on the growth and development of irradiated squamous cell carcinomas located in the floor of the mouth of mice. This suggests that HBOT is safe to use in patients that previously received radiation therapy as treatment for cancer. However, literature reports various effects of HBOT in various types of cancer[76, 83]. Further investigation of the circumstances and conditions in which HBOT can be safely used in cancer patients is necessary. The animal model and imaging techniques used in our study can contribute herein.

This thesis has shown that HBOT does affect molecular pathways, cells and tissues in irradiated mice. Effects are cell type- and tissue dependent. For example, proliferation rate and blood vessel density were increased in irradiated salivary gland tissue, but not in irradiated tumor tissue. Additional experimental research with adapted animal models will be necessary to further unravel the working mechanism of HBOT for different conditions. Furthermore, it is of importance to investigate whether the molecular and cellular effects of HBOT are clinically relevant.

Currently, too many patients might receive HBOT while there is only little evidence for its effectivity. On the other hand, other patient groups might exist that potentially could benefit from HBOT. Preclinical- as well as clinical research, in the form of randomized controlled trials, is necessary to obtain a more evidence-based referral of patients to HBOT.

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Appendices

SUMMARY

For the treatment of tumors in the head and neck region, radiation therapy (RT) is often used. Apart from the irradiation of tumor cells, it is inevitable that surrounding normal tissues will also receive radiation. Salivary glands and bone frequently lie in the radiation portal and their functionality can be impaired after RT, leading to serious health problems like chronic hyposalivation and osteoradionecrosis (ORN; bone death due to radiation). Quality of life is highly impacted in these patients, since they experience trouble with eating, speaking and swallowing, suffer from taste loss, and moreover ORN proves to be a very painful condition. The effects are irreversible and definite treatment or prevention options are scarce.

Hyperbaric oxygen therapy (HBOT) has been used to treat delayed radiation injury since the 1970s. Patients take place in a chamber that is pressurized to 2.4 atmospheres absolute (normal pressure at sea level is 1.0 ATA) and breath 100% oxygen during 1,5 hour, with a total of approximately 20-30 daily sessions. This will raise the oxygen tension in the tissues, which is supposed to facilitate repair of the hypoxic irradiated tissues, especially by means of the induction of angiogenesis. Although ORN is treated in this way, the efficacy of HBOT remains a matter of debate. In addition, the working mechanism is not fully understood.

This thesis aims to give more insight in the effects of hyperbaric oxygen therapy on irradiated head and neck tissues and on tumor tissue. For this purpose we used a mouse model and analysed the different tissues at a cellular and molecular level (**Chapter 1**).

In **chapter 2**, the existing literature regarding HBOT in the irradiated head and neck region is discussed. Animal experimental studies were especially scarce, and clinical studies lacked randomized controlled trials. Research outcomes varied, but in general beneficial effects of HBOT were suggested although strong conclusions could not be made.

Chapter 3 describes the development of a custom-built hyperbaric oxygen chamber that allowed us to perform HBOT experimentation with small laboratory animals, in a clinically relevant manner.

In **chapter 4** the effects of HBOT on irradiated murine mandibles are investigated. Mice were irradiated with a single dose of 15 Gy aimed at the head and neck region. HBOT started the day after RT and followed clinical protocols with 20 daily sessions (excluding weekends). At 10 and 24 weeks after RT, mice were sacrificed and mandibles were examined by microCT and histology. Radiation-induced changes were mostly apparent after 24 weeks. MicroCT data proved that the negative effect

of RT on different microstructural parameters (i.e. reduction of bone volume and trabecular thickness and increase of trabecular separation) was positively affected if HBOT had been administered directly after RT. Furthermore, HBOT decreased the amount of empty lacunae and bone resorbing osteoclasts in irradiated tissue, indicating increased bone viability.

Chapter 5 focusses on immunohistochemical changes due to HBOT in irradiated salivary glands at different time-points (i.e. 2, 6, 10 and 24 weeks after RT) using the same mouse model as mentioned in chapter 4. An enhanced proliferation rate and blood vessel density was observed in mice that received HBOT after RT, at ten weeks post-RT. Salivary flow rates, which dropped soon after RT, were not recovered by HBOT. The results indicate that although HBOT in our experimental setting was not capable to improve the overall functionality of the salivary glands, it positively influenced regenerative processes.

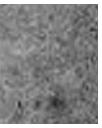
In **chapter 6** we made a small detour and addressed the effects of the heparan sulfate mimetic RGTA (ReGeneraTing Agent) on the regeneration of irradiated salivary glands. RGTAs are designed to mimic the effect of heparan sulfates that are normally present in the extracellular matrix, but are degraded upon tissue injury. RGTAs can bind growth factors and are thereby believed to facilitate regeneration. In the mouse model used, with weekly RGTA injections in irradiated mice, RGTA was able to increase the salivary flow rate, but only at two weeks after RT and not at later time-points. Although histology showed an increase of mucin production activity of acinar cells at ten weeks post-RT, the salivary flow rate was decreased. It was concluded that RGTA may be useful to ameliorate salivation shortly after RT and that further research into its application, alone or in combination with other therapies like HBOT, is needed.

In **chapter 7**, the molecular pathways that are influenced by HBOT in irradiated salivary glands are investigated by means of whole genome microarrays, which were performed two weeks after RT. We showed that HBOT was able to significantly attenuate the radiation-induced expression of a set of genes and upstream regulators including mostly immediate early response genes like Fos, Jun and members of the Egr- and Ier family. This indicates a counteraction of HBOT on certain RT-induced mechanisms. Functional analysis revealed that stimulation of the TGFβ-pathway by RT was attenuated by HBOT. This pathway plays, amongst others, a crucial role in radiation-induced fibrosis. Fibrosis did not develop in our mouse model in the used time frame, so we were not able to establish effects of HBOT on radiation-induced fibrosis. However, the potential of HBOT to inhibit the TGFβ-pathway is a very interesting finding, not only regarding tissue regeneration of salivary glands,

but also of other tissues that suffer from radiation-induced injury.

Finally, in **chapter 8** the effects of HBOT on the growth and development of tumor tissue is investigated, since there is concern whether HBOT can promote tumor growth. For this purpose, a mouse model was set-up in which a luciferase expressing human squamous cell carcinoma cell line was injected into the floor of the mouth of nude mice. *In vivo* monitoring by bioluminescence imaging revealed that the growth of non-irradiated tumors was slightly accelerated by HBOT, whereas the growth of irradiated tumors was not influenced. Fluorescent imaging with a blood pool agent and a hypoxia probe showed increased leakiness of blood vessels and increased hypoxia due to HBOT, respectively. Furthermore, no effect of HBOT was seen on metastatic incidence, histological grade, malignancy markers and blood vessel density and -diameter. The results suggest that there is no increased risk of HBOT for previously irradiated patients with a history of squamous cell carcinoma.

In conclusion, it was shown that HBOT has the ability to induce cellular and molecular changes in irradiated tissues that are cell type and tissue dependent. Proliferation rate and blood vessel density in salivary glands were increased and it was suggested that induction of the TGFβ-pathway was attenuated by HBOT. Despite these auspicious signs, improvement of salivary gland function, by means of a measurable increase in salivary flow rate, could not be established in our experiments. In mandibular bone, certain radiation-induced microstructural and histological deteriorations were prevented by HBOT. Squamous cell carcinoma tissue responded to HBOT to some extent, but the results do not lead to concern for the application of this therapy in irradiated cancer patients. The clinical relevance of these results remains to be elucidated in order to achieve a better implementation of the therapy.



SAMENVATTING

Bij de behandeling van tumoren in het hoofd-halsgebied wordt vaak gebruik gemaakt van radiotherapie (RT). De bestraling is erop gericht de tumorcellen te doden, maar het is onvermijdelijk dat ook omliggende gezonde weefsels straling ontvangen. Speekselklieren en kaakbot liggen dikwijls in het bestralingsgebied waardoor de functie kan verminderen, wat kan leiden tot chronische hyposalivatie en osteoradionecrosis (ORN; het doodgaan van bot door bestraling). Dit heeft een grote impact op de kwaliteit van leven van deze patiënten; ze krijgen moeite met eten, praten en slikken, ervaren een vermindering van smaak en daarbij is ORN een erg pijnlijke aandoening. De effecten zijn irreversibel en opties voor behandeling of preventie zijn schaars.

Hyperbare zuurstoftherapie (HBOT) wordt al sinds de jaren ’70 gebruikt bij de behandeling van late radiatieschade. Patiënten nemen plaats in een cabine die op druk wordt gebracht tot 2.4 atmosfeer (normale druk op zeeniveau bedraagt 1.0 atmosfeer) en ademen 100% zuurstof in gedurende 1,5 uur. In totaal worden op deze manier ongeveer 20-30 dagelijkse sessies ondergaan. Dit zorgt ervoor dat de zuurstofdruk in de weefsels toeneemt, wat het herstel van het bestraalde, hypoxische weefsels zou bevorderen, vooral door de inductie van angiogenese. ORN wordt op deze manier behandeld, maar de effectiviteit van de therapie blijft onderwerp van discussie. Daarnaast is het precieze werkingsmechanisme nog niet volledig doorgrond.

Dit proefschrift heeft als doel een beter inzicht te geven in de effecten van hyperbare zuurstoftherapie op bestraalde weefsels van het hoofd-halsgebied alsmede op tumorweefsel. Hiervoor is een muismodel gebruikt en zijn weefsels op cellulair en moleculair niveau geanalyseerd.

In **hoofdstuk 2** wordt de bestaande literatuur over HBOT in eerder bestraald hoofd-halsgebied bediscussieerd. Vooral proefdierexperimenten op dit gebied zijn schaars en bij de klinische studies is er een gebrek aan gerandomiseerde gecontroleerde onderzoeken. De resultaten varieërden, maar in het algemeen werden er gunstige effecten van HBOT gesuggereerd, hoewel harde conclusies niet getrokken konden worden.

Hoofdstuk 3 beschrijft de ontwikkeling van een hyperbare zuurstoftank waarmee HBOT experimenten met kleine proefdieren gedaan konden worden in een klinisch relevante setting.

In **hoofdstuk 4** worden de effecten van HBOT op bestraalde onderkaken van de muis onderzocht. Muizen werden bestraald met een éénmalige dosis van 15

Gy, gericht op het gehele hoofd-halsgebied. HBOT startte de dag na bestraling en was gelijk aan de klinische protocollen met 20 dagelijkse sessies (met uitzondering van het weekend). Tien en 24 weken na bestraling werden de muizen geofferd, het kaakbot geoogst en door middel van microCT en histologie onderzocht. Stralingsgeïnduceerde schade werd vooral waargenomen na 24 weken. MicroCT data toonde aan dat de negatieve effecten van RT op verschillende microstructurele parameters (reductie in botvolume en dikte van botbalkjes en een vergrote afstand tussen botbalkjes) positief beïnvloed werden als HBOT direct na RT gegeven werd. Tevens verlaagde HBOT de hoeveelheid lege holtes en osteoclasten (die bot resorberen) in bestraald weefsel, wat duidt op een verhoogde levensvatbaarheid van het bot.

Hoofdstuk 5 is gericht op de immunohistochemische veranderingen ten gevolge van HBOT in bestraalde speekselklieren op verschillende tijdspunten (2, 6, 10 en 24 weken na RT). Er werd gebruik gemaakt van het muismodel zoals uitgelegd in hoofdstuk 4. Verhoogde proliferatie en bloedvatdichtheid werd waargenomen in speekselklieren van muizen die na bestraling HBOT kregen, op tien weken na bestraling. De speekselproductie, die al snel na RT drastisch daalde, werd niet hersteld door HBOT. Deze resultaten duiden erop dat hoewel HBOT in onze experimentele setting de algemene functionaliteit van de speekselklieren niet kon verbeteren, regeneratieve processen weldegelijk positief beïnvloed kunnen worden.

In **hoofdstuk 6** wordt kort uitgeweid en worden de effecten van het heparan sulfaat mimeticum RGTA (ReGeneraTing Agent) op de regeneratie van bestraalde speekselklieren onderzocht. RGTAs zijn ontworpen om de effecten van heparan sulfaten na te boosten. Normaal gesproken zijn deze aanwezig in de extracelullaire matrix, maar ze worden afgebroken wanneer het weefsel schade ondervindt. RGTAs kunnen groeifactoren binden, waardoor ze mogelijk regeneratie kunnen bevorderen. In het muismodel dat in deze studie werd gebruikt bevorderde de wekelijkse toediening van RGTA de speekselproductie in bestraalde muizen. Dit effect werd alleen twee weken na bestraling gezien. De histologie liet wel een verhoging van de activiteit van de mucine productie in de acinaire cellen zien op tien weken na bestraling, terwijl op dat tijdspunt de speekselproductie niet werd bevorderd. Concluderend kan RGTA bruikbaar zijn om de speekselproductie kort na RT te verbeteren en is verder onderzoek naar de mogelijkheden van RGTA, alleen of juist in combinatie met andere therapieën zoals HBOT, nodig.

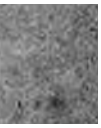
In **hoofdstuk 7** wordt gekeken naar de moleculaire pathways die door HBOT beïnvloed kunnen worden in bestraalde speekselklieren, door middel van microarrays die twee weken na RT werden uitgevoerd. HBOT kon de expressie

van een set genen en regulatoren die door bestraling geïnduceerd werden, vooral early response genen als Fos, Jun en leden van de Egr- en Ier familie, significant verzwakken. Dit duidt op een tegengestelde actie van HBOT op bepaalde stralingsgeïnduceerde mechanismen. Functionele analyse liet zien dat de stimulatie van de TGFβ-pathway door RT kon worden verzwakt door HBOT. Deze pathway speelt onder andere een belangrijke rol bij stralingsgeïnduceerde fibrose. In het gebruikte muismodel werd geen fibrose waargenomen, waardoor we de effecten van HBOT op stralingsgeïnduceerde fibrose niet konden vaststellen. Echter, het vermogen van HBOT om de TGFβ-pathway te remmen is een zeer interessante bevinding, niet alleen als het gaat om weefselregeneratie van speekselklieren, maar zeker ook voor andere weefsels die stralingsschade ondervinden.

Tot slot worden in **hoofdstuk 8** de effecten van HBOT op de groei en ontwikkeling van tumorweefsel onderzocht. Er bestaat enigszins bezorgdheid over het gebruik van HBOT bij patiënten die kanker hebben gehad, omdat het tumorgroei zou kunnen bevorderen. Hiertoe werd een muismodel opgezet waarbij humane plaveiselcarcinoomcellen die luciferase tot expressie brengen, werden ingespoten in de mondbodem van naakte muizen. Met behulp van bioluminiscente beeldvorming konden de cellen *in vivo* gevolgd worden en werd gezien dat de groei van niet-bestraalde tumoren lichtelijk werd versneld door HBOT, terwijl de groei van bestraalde tumoren niet werd beïnvloed. Fluorescente beeldvorming met een middel om de bloedpoel in beeld te brengen en een probe voor hypoxie lieten respectievelijk meer lekkende vaten en verhoogde hypoxie in de tumoren onder invloed van HBOT zien. Verder was er geen effect van HBOT op de metastase index, de histologische stadiëring, maligniteitmarkers en bloedvatdichtheid en -diameter. Er werd derhalve geconcludeerd dat op basis van deze studie geen verhoogd risico voor HBOT bestaat bij patiënten die bestraald zijn en een voorgeschiedenis met plaveiselcelcarcinoom hebben.

Concluderend hebben we aangetoond dat HBOT cellulaire en moleculaire veranderingen kan bewerkstelligen in bestraalde weefsels, die celtype- en weefsel-afhankelijk zijn. Proliferatie en bloedvatdichtheid werden in speekselklieren verhoogd na bestraling en er werd gesuggereerd dat de activiteit van de TGFβ-pathway verzwakt kon worden door HBOT. Ondanks deze veelbelovende bevindingen, kon er geen verbetering van de speekselklierfunctie, gemeten aan de hand van speekselproductie, vastgesteld worden. In het bot van de onderkaak werden bepaalde stralingsgeïnduceerde microstructurele en histologische veranderingen voorkomen door HBOT. Plaveiselcelcarcinoom reageerde in enige mate op HBOT, maar de resultaten leiden niet tot bezorgdheid als het gaat om het gebruik van HBOT bij patiënten met hoofd-halskanker die bestraald zijn. De klinische relevantie

van deze resultaten zal onderzocht moeten worden zodat de therapie uiteindelijk beter geïmplementeerd kan worden.

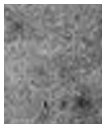


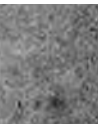
CURRICULUM VITAE

Linda Spiegelberg was born at the 22nd of June, 1984 in Eindhoven, and lived there with her parents and brother until her early twenties. She graduated at the Pleincollege Eckart (Atheneum, pre-university education) in Eindhoven in 2002. Later that year, she started to study Biomedical Sciences at the Radboud University Nijmegen and chose the major Pathobiology and minor Neuroscience.

After her graduation in 2007, she worked shortly as a safety data specialist at Organon NV, after which she travelled around the world for half a year with two of her friends.

In April 2009, she started her PhD at the department of Oral and Maxillofacial Surgery & Special Dental Care at the Erasmus MC in Rotterdam under the supervision of Prof.dr K.G.H. van der Wal and later Prof.dr. E.B. Wolvius. The topic was hyperbaric oxygen therapy in irradiated tissues of the head and neck and the results are bundled in this thesis.





LIST OF PUBLICATIONS

Hyperbaric oxygen therapy as a prevention modality for radiation damage in the mandibles of mice (2014). **Spiegelberg L**, Braks JAM, Groeneveldt LC, Djasim UM, van der Wal KGH, Wolvius EB. J Craniomaxillofac Surg. *Accepted*.

Gene expression analysis reveals inhibition of radiation induced TGFβ-signaling by hyperbaric oxygen therapy in mouse salivary glands (2014). **Spiegelberg L**, Swagemakers SM, Van Ijcken WF, Oole E, Wolvius EB, Essers J, Braks JAM. Mol Med. Jul 10;20(1):257-69.

Hyperbaric oxygen treatment of tissue-engineered mucosa enhances secretion of angiogenic factors in vitro (2014). Tra WM, **Spiegelberg L**, Tuk B, Hovius SE, Perez-Amodio S. Tissue Eng Part A. May;20(9-10):1523-30.

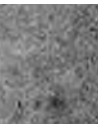
Effects of hyperbaric oxygen therapy on the viability of irradiated soft head and neck tissues in mice (2014). **Spiegelberg L**, Braks J, Djasim U, Farrell E, van der Wal K, Wolvius E. Oral Dis. Apr;20(3):e111-9.

The effects of heparan sulphate mimetic RGTA-OTR4120 on irradiated murine salivary glands (2012). **Spiegelberg L**, Djasim UM, van Neck JW, Wolvius EB, van der Wal KG. J Oral Pathol Med. Jul;41(6):477-83.

A hyperbaric oxygen chamber for animal experimental purposes (2012). Djasim UM, **Spiegelberg L**, Wolvius EB, van der Wal KG. Int J Oral Maxillofac Surg. Feb;41(2):271-4.

Hyperbaric oxygen therapy in the management of radiation-induced injury in the head and neck region: a review of the literature (2012). **Spiegelberg L**, Djasim UM, van Neck HW, Wolvius EB, van der Wal KG. J Oral Maxillofac Surg. Aug;68(8):1732-9.

Ultrastructural and immunocytochemical characterization of the rat non-preganglionic Edinger-Westphal nucleus (2009). Van Wijk DC, Xu L, **Spiegelberg L**, Struik RF, Meijer KH, Gaszner B, Kozicz T, Roubos EW. Gen Comp Endocrinol. Oct;164(1):32-9.



PHD PORTFOLIO SUMMARY

Name PhD student:	Linda Spiegelberg
Erasmus MC Department:	Oral and Maxillofacial Surgery & Special Dental Care
PhD period:	April 2009 - April 2014
Promotors:	Prof.dr. EB Wolvius Prof.dr. KGH van der Wal
Co-promotor:	dr. JAM Braks

Phd training	**Year**	**Workload (ECTS)**
Courses		
• Biomedical Research Techniques	2009	1.5
• Handelingen met dieren gehuisvest in IVCs	2013	0.15
• Introduction to Adobe Photoshop and Illustrator	2013	0.3
• Introduction to Adobe Indesign	2013	0.15
Conferences – poster presentations		
• Effects of hyperbaric oxygen therapy on the regeneration of irradiated murine salivary glands. 2nd symposium of the Dutch Society for Radiobiology (NVRB), Noordwijkerhout.	2012	1.0
• Effects of hyperbaric oxygen therapy on radiation-induced damage in the salivary glands of mice. 18th Molecular Medicine Day, Rotterdam.	2014	1.0
Conferences – podium presentations		
• Hyperbaric oxygen therapy in the management of radiation-induced injury in the head and neck region. 13th European Congres of Scientists and Plastic Surgeons (ECSAPS), Rotterdam.	2009	1.5
• Hyperbaric oxygen therapy in the management of radiation-induced injury in the head and neck region. Vergadering Rotterdamse Werkgroep Hoofd-Hals tumoren (RWHHT), Rotterdam.	2010	1.5
• Exploration of the efficacy of hyperbaric oxygen therapy on the repair and regeneration of irradiated head and neck tissues in a murine model. Vergadering Rotterdamse Werkgroep Hoofd-Hals Tumoren (RWHHT), Rotterdam.	2011	1.5

• Het effect van hyperbare zuurstoftherapie op de regeneratie van bestraalde speekselklieren. Wetenschappelijke vergadering van de Nederlandse Werkgroep Hoofd-Hals Tumoren (NWHHT), Nijmegen.	2011	1.5
• Hyperbare zuurstoftherapie in de regeneratie van bestraalde speekselklieren van de muis. 55e Najaarsvergadering van de Nederlandse Vereniging voor Mondziekten, Kaak- en Aangezichtschirurgie (NVMKA), Leiden.	2011	1.5
• Hyperbare zuurstoftherapie in de regeneratie van bestraald weefsel in het hoofd-halsgebied. 3e Jonge Onderzoeksdag van de Nederlandse Werkgroep Hoofd-Hals Tumoren (NWHHT), Utrecht.	2013	1.5

Conferences – attendance

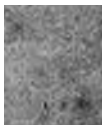
• 53e Najaarsvergadering Nederlandse Vereniging voor Mondziekten, Kaak- en Aangezichtschirurgie (NVMKA), Groenekan.	2009	0.6
• Congress of the European Association for Cranio-Maxillo-Facial Surgery(EACMFS), Brugge.	2010	0.3
• Symposium van de Nederlandse Vereniging voor Radiobiologie (NVRB) en de Nederlandse Vereniging voor Radiotherapie en Oncologie (NVRO), Utrecht.	2010	0.3
• Scientific Meeting Nederlandse Vereniging voor Radiobiologie (NVRB), Utrecht.	2014	0.3

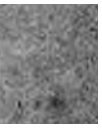
Grant

• BOOA Research Grant (5000,-) van de Stichting BOOA van de Nederlandse Vereniging voor Mondziekten, Kaak- en Aangezichtchirurgie (NVMKA).	2009	3.0

Teaching activities

• Supervision Master student Molecular Medicine	2012	3.0





DANKWOORD

Mijn proefschrift is af! En dan is het nu tijd om iedereen te bedanken die in welke mate dan ook hieraan heeft bijgedragen.

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Prof.dr. E.B. Wolvius, beste Eppo, bedankt voor de supervisie over mijn promotie. Ondanks je vaak overvolle agenda was er altijd wel een gaatje te vinden om de voortgang van mijn promotie te bespreken en kwamen al mijn artikelen snel weer van commentaar voorzien terug.
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Dr. Femke Verseijden, hoewel kort, heb ik met plezier met je samengewerkt en van je geleerd, met name als het gaat om het hebben van een kritische onderzoekersblik.
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