

Research Paper  
Head and Neck Oncology

# An experimental study of the use of hyperbaric oxygen to reduce the side effects of radiation treatment for malignant disease

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**Abstract.** Hyperbaric oxygen (HBO) has been used for more than 20 years to assist wound healing in the treatment of the more severe complications associated with the side effects of therapeutic radiation treatment. A prospective study was performed in an irradiated rat model to determine whether HBO is effective in reducing the long-term side effects of therapeutic radiation treatment on normal tissue, when given 1 week after the completion of the radiation treatment. The experimental model was designed to simulate a fractionated course of therapeutic radiation that is commonly used in the treatment of cancer of the mandible. One week following completion of the radiotherapy, the animals underwent a 4-week course of HBO treatment, and two animals from each group were killed at 8-week intervals until the end of the experiment at 36 weeks. Histological sections of tissue clearly showed continued growth of teeth and maintenance of specialized tissues, such as salivary gland and bone, in the treated group compared to the non-treated group. This experimental model demonstrated that HBO is effective in reducing the long-term side effects of therapeutic radiation treatment in normal tissue, when given 1 week after the completion of the radiation treatment.

**Key words:** hyperbaric oxygen; therapeutic radiation; tooth growth; salivary gland; bone.

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Therapeutic radiation has been used for the treatment of cancer and other diseases for nearly a century. Over the past 20 years, hyperbaric oxygen (HBO) has been used to assist wound healing in the prevention and treatment of the more severe complications associated with the side

effects of therapeutic radiation treatment (TRT). The use of HBO treatment (HBOT) is based on the premise that increased oxygen tissue tension aids wound healing by increasing the hypoxic gradient and stimulating angiogenesis and fibroblast differentiation. As it takes up to

6 months for a hypoxic state to develop in treated tissue, following radiation treatment, current recommendations for HBO state that it is not effective until after this time. During this 6-month period, immediately following TRT, many specialized tissues in or adjacent to the field of irra-

diation, such as salivary glands and bone, are damaged due to a progressive thickening of arteries and fibrosis, and these tissues are never replaced. Currently, HBO is used to treat the complications of TRT, but it would be far better if they could be prevented. With this in mind a prospective study was performed in an irradiated rat model to determine if HBO is effective in reducing the long-term side effects of therapeutic radiation treatment on normal tissue, when given 1 week after the completion of the radiation treatment.

## Materials and methods

### Experimental animals

For the purpose of this research project, the Animal House at Royal Perth Hospital supplied and supervised the well being of 30 male Wistar rats (250–300 g). The rats were held under a 12-h light–dark cycle at  $22 \pm 2^\circ\text{C}$ . All experimental surgical procedures were carried out under licence from the Animal Ethics Committees of Royal Perth Hospital and The University of Western Australia in accordance with National Health & Medical Research Council of Australia guidelines.

The rats were divided into four groups:

- (1) air-breathing control group (Control) – no treatment (six rats);
- (2) HBOT only group (HBO) – (six rats);
- (3) TRT only group ( $R_x$ ) – (eight rats);
- (4) TRT plus HBOT group ( $R_x + \text{HBO}$ ) – (10 rats).

### Experimental time table

The experiment ran for 253 days (36 weeks). Day 1 was nominated as the first day of radiation treatment. The experimental time table was as follows.

- (a) A simulated therapeutic course of radiotherapy was given to the mandible of Groups 3 and 4 animals over 15 days.
- (b) One week recovery time for animals.
- (c) Four weeks of HBO for Groups 2 and 4.
- (d) Two animals from each group were then killed at 85 days, 141 days and 253 days.

### Irradiation protocol

A 42-Gy fractionated course of simulated therapeutic radiotherapy was given to the animals in Groups 3 and 4 over 15 days with 6 Gy being given on each of days 1, 3,

5, 8, 10, 12, 15 of the experiment. A 250-kVp Siemens Stabilipan DXRT Orthovoltage Therapy machine was used with the following specifications:

- distance from source to animal skin surface – 25 cm;
- kVp – 250;
- mA – 10;
- filter – half value layer of 0.5-cm copper;
- measured dose rate – 1.30;
- prescribed dose – 6.0 Gy;
- time to achieve dose – 4 min 37 s.

### Hyperbaric oxygenation

One week post radiotherapy, to allow recovery time for the experimental animals, Groups 2 and 4 underwent a 4-week course of HBO, similar to that used by MARX<sup>19,20</sup> to treat human subjects with osteoradionecrosis. This treatment course was carried out in a specially built HBO chamber, supplied by the Royal Australian Navy, for experimental HBO treatment of small animals. The course involved the rats undergoing 20 treatments of HBO. A hyperbaric treatment is defined as breathing 100% oxygen for 90 min at 2.4 ATA<sup>19,20</sup>. A HBOT, also called a dive, consists of:

- (1) descent time – 15 min;
- (2) bottom time – 90 min of HBO under 2.4 ATA pressure;
- (3) ascent time – 15 min.

As the animals in this experiment did not undergo a surgical procedure and therefore did not need postoperative HBO, only 20 treatments were given to

initiate a wound healing response, as suggested by DAVIDSON & MUSTOE<sup>7</sup>.

### Tetracycline

The use of low-dose tetracycline as a histological hard-tissue marker has been well established and allows clear evidence of continued hard-tissue growth in experimental animals<sup>10</sup>. A veterinarian-approved tetracycline was administered to all rats as a marker of tooth growth. The tetracycline is taken up by growing teeth and can be seen histologically as bands of growth, like growth rings in a tree section. Tetracycline is available, as a Veterinary Approved Drug (Alamycin), in vials of 200 mg/ml for intravenous/intramuscular/intraperitoneal use in Australia. Three treatments of 5 mg/rat/day (20 mg/kg)<sup>10</sup> of Alamycin (oxytetracycline) were administered as an i.p. dose every 4 weeks, beginning on Day 22, the same day as HBOT commenced, and continued for the length of the experiment. The dosage of 20 mg/kg of tetracycline for animal use has been reported by FROST<sup>10</sup>.

### Removal of mandible and preparation of samples

At necropsy, the mandibles were resected en bloc, then hemi-sectioned through the fibrous symphysis, and the lower incisor teeth removed. The specimens were stored in formalin and then embedded in wax blocks, prior to sectioning and then staining. Two slides of consecutive tissue slices from each specimen group, which best represented each tissue type, were selected for specific analysis. One of the slides had been stained with haematoxylin and eosin

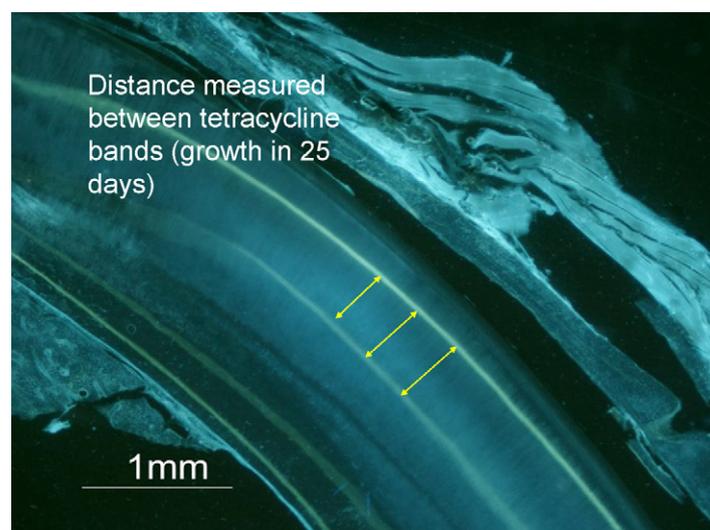


Fig. 1. Tetracycline staining (magnification 17.5 $\times$ ).

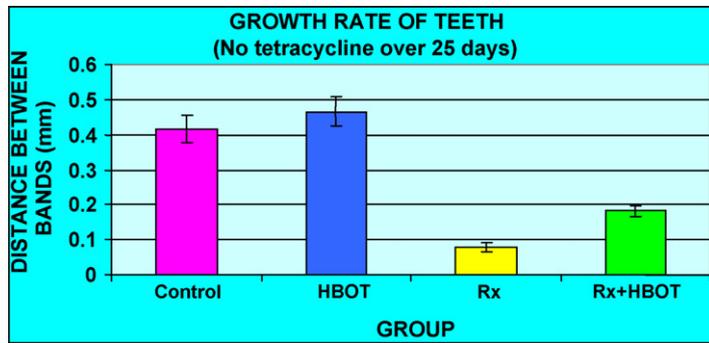


Fig. 2. Growth rate of teeth comparing the four groups. SDs are shown. The growth rate of Group 4 is more than twice that of Group 3.

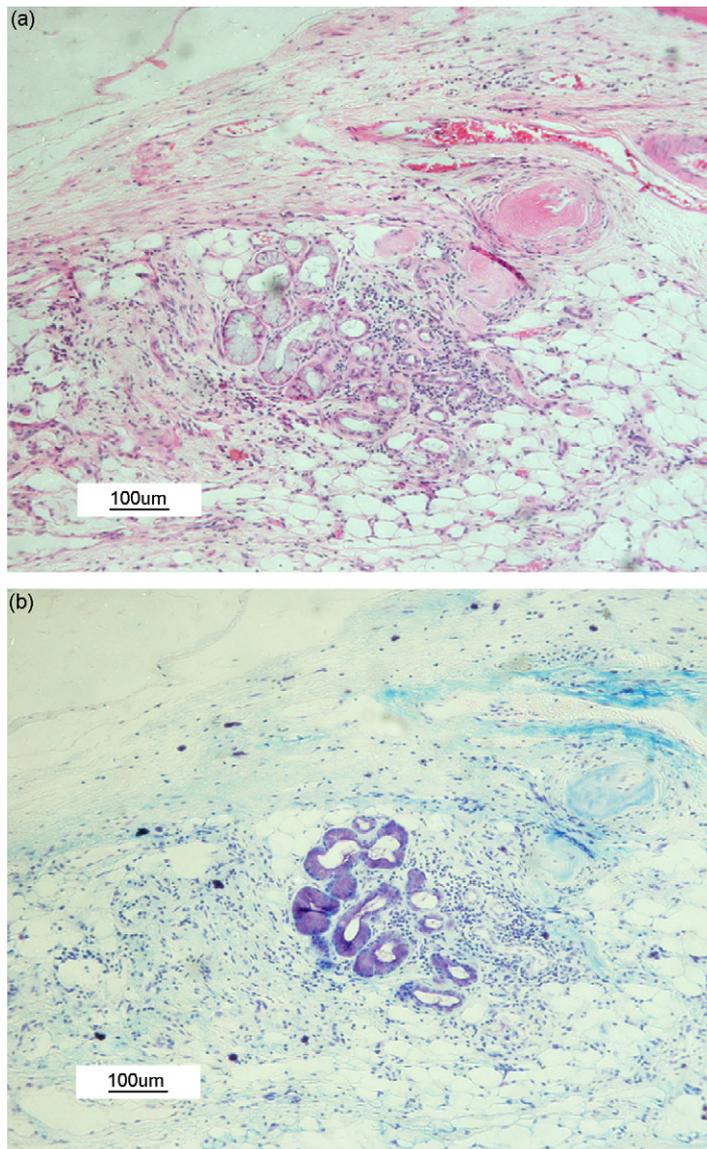


Fig. 3. Examples of salivary gland tissue from Group 3 to 6 ( $R_x$ ), stained with (a) H&E and (b) LFB, showing the reduction in volume of salivary gland tissue and thickening of arterial wall, 253 days after TRT. Note that (a) the H&E stain does not demonstrate fibrosed salivary gland clearly; (b) the LFB stain clearly demonstrates the residual fibrosed salivary gland.

(H&E) and a second consecutive slice of tissue was stained with Luxol Fast Blue/Cresyl Fast Violet (LFB). The LFB was particularly useful in demonstrating the presence of salivary gland tissue, especially in those sections where the salivary tissue was undergoing radiation fibrosis and was very difficult to see in H&E sections.

An incisor tooth from each dead animal was imbedded in an epoxy resin. Each embedded tooth was then ground to half its thickness and the ground side of the tooth glued to a glass slide. Each slide-mounted half tooth was then further ground down to achieve a tooth slice 30  $\mu\text{m}$  thick. The mounted tooth sections were viewed at 17.5 magnification using fluorescence microscopy with a narrow-band, blue-light filter block (450–490 nm). After image acquisition, the files were analysed by Metamorph Imaging 6.0 Software System by the IAAF, and used to carry out both histomorphological analysis and histomorphometric quantification of the images.

## Results

### Teeth

Sections of teeth were viewed under UV light and clearly showed continued growth rings of tetracycline in Groups 1, 2 and 4, with little growth shown in Group 3. Figure 1 shows an example of the tetracycline staining and measurement of the banding. As the tetracycline was given over 3 days, every 4 weeks, the distance between the bands represents 25 days' tooth growth. Figure 2 compares the growth rate of teeth between the four groups. The positive effect of HBO on growth rate can be seen in Group 4 ( $R_x + \text{HBOT}$ ), being twice the growth rate of the non-treated Group 3 ( $R_x$ ) teeth.

### Salivary gland

Histological sections of tissue taken from the angle of the mandible clearly showed maintenance of specialized tissues, such as salivary glands and osteoblasts, in the treated group compared to the non-treated group of animals at the end of the experiment. Figure 3a shows an example of salivary gland tissue from Group 3 ( $R_x$ ) stained with H&E taken from a rat killed at Day 253 (36 weeks), and Fig. 3b shows a consecutive slice from the same animal stained with LFB. Figure 4a shows an example of salivary gland tissue from Group 4 ( $R_x + \text{HBOT}$ ) stained with H&E taken from a rat killed at Day 253

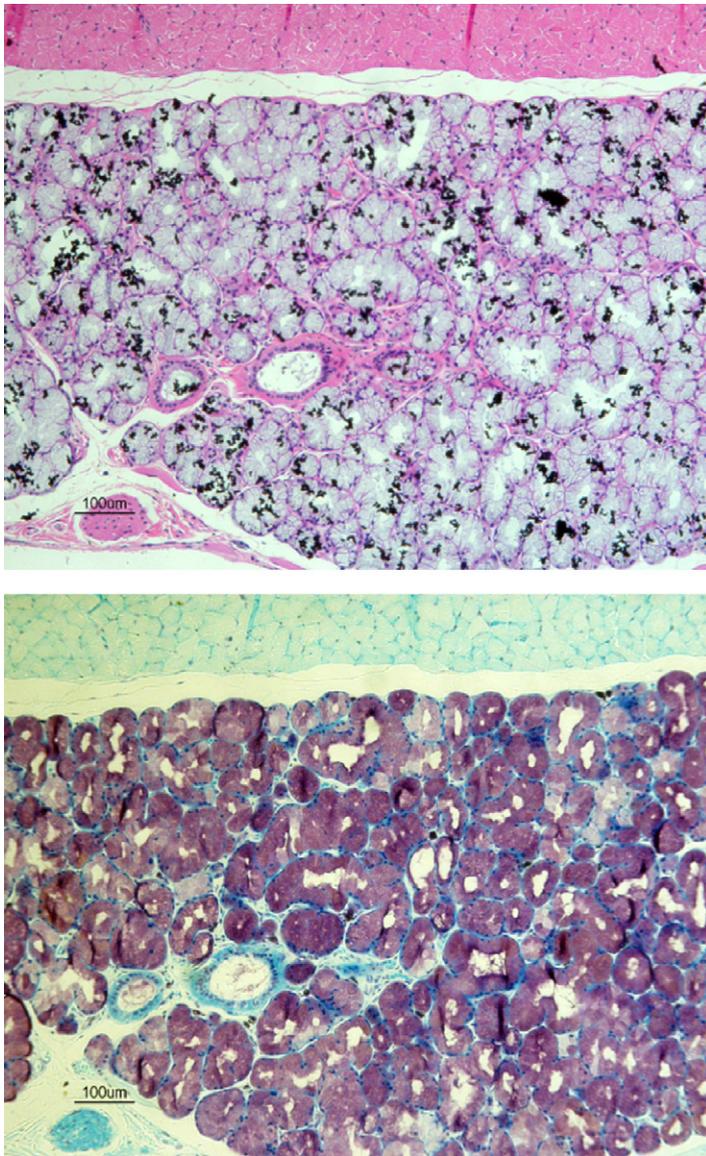


Fig. 4. Examples of salivary gland tissue from Group 4 to 9 ( $R_x + \text{HBOT}$ ), stained with (a) H&E and (b) LFB, showing the retention in volume of salivary gland tissue, 253 days after TRT and HBOT. H&E and LFB clearly demonstrate healthy salivary gland tissue.

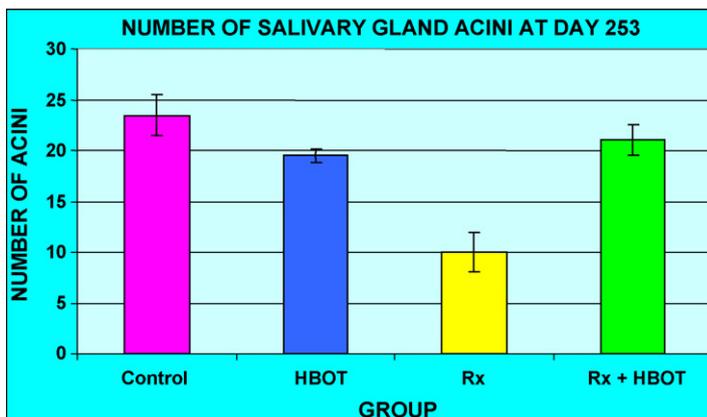


Fig. 5. Number of salivary gland acini at Day 253. SDs are shown. Group 4 is comparable to the controls, in contrast to Group 3.

(36 weeks), and Fig. 4b shows a consecutive slice from the same animal stained with LFB. To analyse the salivary gland tissue, this study employed a point-counting technique called the Multipurpose Test System, which is based on the stereological principles originally described by BOLENDER & WEIBEL<sup>4</sup>. Figure 5 tables the number of salivary gland acini at Day 253 (36 weeks). The presence and quantity of salivary gland acini in Group 4 ( $R_x + \text{HBO}$ ) were seen to be comparable to those of Groups 1 (Control) and 2 (HBO), whereas Group 3 ( $R_x$ ) had less than 50% of the quantity of salivary gland acini. Group 3 ( $R_x$ ) had significantly less than the other three groups ( $p < 0.05$ , ANOVA).

**Bone**

Figures 6 and 7 show an example of bone from Group 3 ( $R_x$ ) and Group 4 ( $R_x + \text{HBO}$ ), respectively, taken from a rat killed at Day 253 (36 weeks) and stained with H&E. In this study the percentage of lacunae occupied by osteoblasts was used to quantify the vitality of the bone over the time frame of the experiment. It is immediately clear that there are far fewer lacunae occupied by osteoblasts in Group 3 ( $R_x$ ) compared to the other three groups. Group 4 ( $R_x + \text{HBOT}$ ) exhibits areas of new bone growth (reversal lines), whereas Group 3 ( $R_x$ ) has no areas of new bone growth. Figure 8 shows the percentage of lacunae occupied by osteoblasts in each group at Day 253. The percentage of lacunae occupied by osteoblasts in Group 4 ( $R_x + \text{HBOT}$ ) (87.5%) is comparable to that of Groups 1 (Control) (96.3%) and 2 (HBOT) (96.3%). In Group 3 ( $R_x$ ), the percentage of lacunae occupied by osteoblasts is only 47% of that of Group 4 ( $R_x + \text{HBO}$ ) and only 43% of the control group.

**Discussion**

Current HBO protocols are designed to manage some of the complications of TRT, particularly osteoradionecrosis (ORN). Prior to the introduction of HBOT, conditions such as ORN were difficult to treat surgically as operating on exposed bone lead to further soft-tissue breakdown and exposure of more irradiated bone. Most treatment regimes consisted of tetracycline mouthwash and a conservative approach to sequestrectomies. The introduction of HBOT to manage ORN by authors such as MARX<sup>19</sup> gave surgeons the ability to operate on patients with exposed ORN bone and to remove teeth

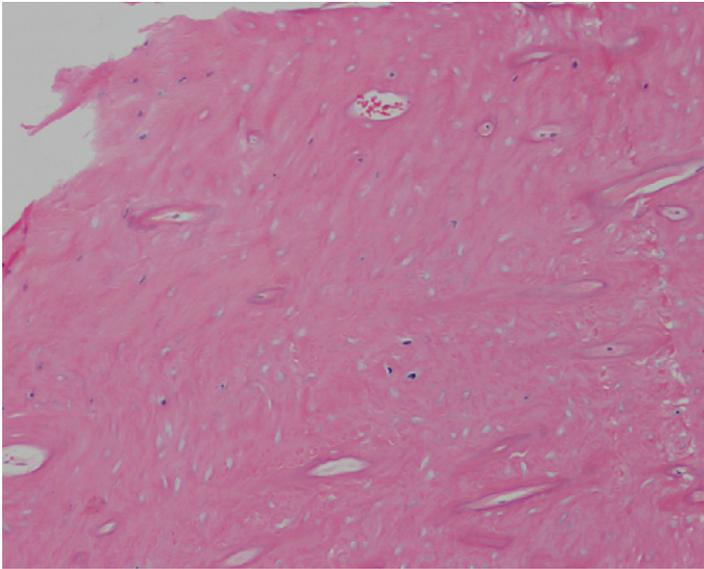


Fig. 6. Example of bone from Group 3 to 8 ( $R_x$ ) (H&E) showing most of the lacunae are empty, with few osteoblasts present, 253 days after TRT. Group 3 ( $R_x$ ) has no areas of new bone growth.

with a reduced risk of developing ORN. HBOT has been shown to induce angiogenesis and fibroblast differentiation<sup>17</sup> with resulting revascularization of the hypoxic tissues. But HBO does not retrieve dead or dying tissue such as salivary gland and bone that lies within the field of radiation; it simply revascularizes fibrous hypoxic, hypocellular and hypovascular tissue. Once the specialized tissues are lost, they are lost forever. While HBOT gives surgeons the ability to treat ORN, using current protocols where

patients wait at least 6 months before the HBOT, it does not prevent the complications associated with TRT and subsequent permanent loss of specialized tissues such as salivary gland and bone.

The MARX HBO<sup>18</sup> protocols for the treatment of ORN have been in use for over two decades. Although there have been many articles reporting the success of this treatment protocol over this time<sup>19</sup>, from the start there has been controversy over its efficacy, with a number of alternative treatment protocols offered for

ORN. In 1989, BALOGH & SUTHERLAND<sup>3</sup> reviewed the then current treatment regimes for ORN and reported that the role of hyperbaric oxygen remained controversial, with this treatment being complex and time-consuming with results that are confounded by the concurrent use of local antiseptic/antibiotic measures and surgery. Although the pathogenesis of ORN may often involve vascular hypoxia<sup>20</sup>, ORN was recently suggested to be triggered by a predominantly fibro-atrophic mechanism<sup>1,6,8</sup>. DELANIAN & LEFAIX<sup>8</sup> reported the complete healing of severe ORN for the first time using an antifibrotic treatment with combined pentoxifylline, tocopherol and clodronate in 12 patients. They considered that the reduction of microscopic radiation-induced fibrosis, which is always associated with the necrotic process, may allow tissue restoration. This, in turn, may reverse defective osteoblastic healing, with clodronate, a well known bisphosphonate, inhibiting osteoclastic bone destruction<sup>22</sup>. GAL et al.<sup>11</sup> reported that microvascular reconstruction of mandibular ORN can be successfully achieved without the use of perioperative HBO therapy. In 2004, in a double-blind trial which had to be stopped for potentially worse outcomes in the HBO arm, ANNANE et al.<sup>2</sup> concluded that patients with overt mandibular ORN did not benefit from hyperbaric oxygenation. Clearly, further studies, such as this one, are needed to assess the efficacy and safety of HBO in ORN.

Since we know that HBOT induces endothelial proliferation and angiogenesis, there has been a real concern that it may accelerate the development or growth rate of mitotic cells. The possibility of tumour cell stimulation has been raised but has never been proven. Well controlled studies involving animals and humans with various malignancies, including cancer models, have shown no difference between HBOT and non-HBOT groups with respect to tumour size, metastasis and long-term survival<sup>14,16,21</sup>. The most comprehensive review of this subject was given in a presentation by Professor FELDMEIERS, in 2001<sup>9</sup>. He listed 73 papers from the literature, dating from 1966 to 2001, including both animal studies and human follow-up studies. He concluded that 'The available published evidence strongly suggests that intermittent HBOT has no enhancing effect on cancer primary or metastatic growth. Likewise, there is no credible evidence that HBOT is an initiator or promoter of cancer de novo.'

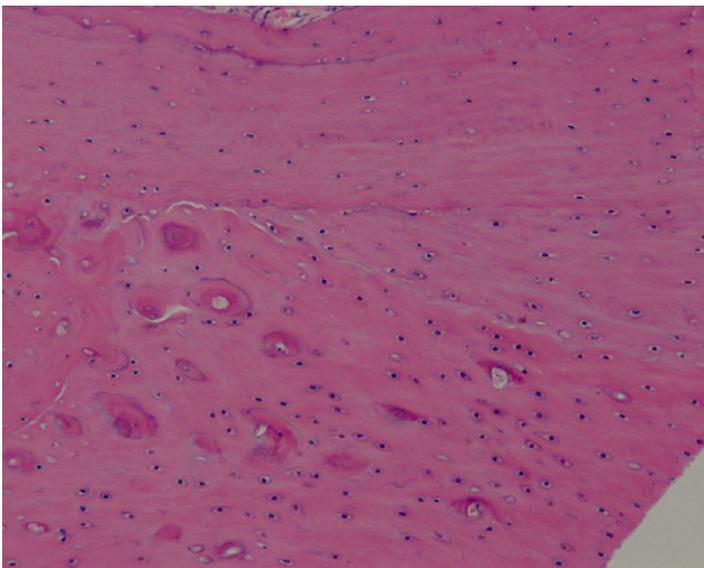


Fig. 7. Example of bone from Group 4 to 9 ( $R_x + \text{HBOT}$ ) (H&E) showing that all the lacunae are occupied by osteoblasts, 253 days after TRT and HBOT. Group 4 ( $R_x + \text{HBOT}$ ) exhibits areas of new bone growth (reversal lines) compared to Fig. 6, Group 3 ( $R_x$ ), which has no areas of new bone growth.

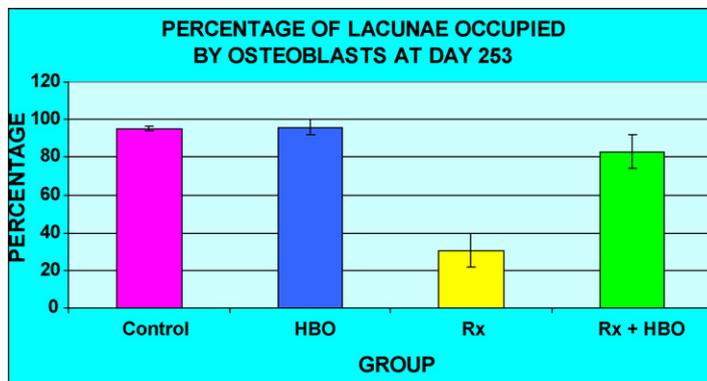


Fig. 8. Percentage of lacunae occupied by osteoblasts at Day 253. SDs are shown. Group 4 is comparable to the controls, in contrast to Group 3.

The mechanism by which HBO achieves angiogenesis and fibroplasia in irradiated tissue has now been elucidated<sup>18,19</sup>, and found to be stimulated by a similar oxygen-gradient phenomenon to that which KNIGHTON et al. and SILVER reported to be central in the angiogenesis and fibroplasia of normal wound healing<sup>17,25</sup>. The HBO creates an oxygen gradient that is steep across a short distance between irradiated and normal tissue. Such steep oxygen gradients are the physio-chemotactic factor attracting wound-regulating macrophages to a wound<sup>17,25</sup>. Steep oxygen gradients, along with the lactate, iron and low pH inherent in wounds, stimulate macrophage-derived angiogenesis factor and macrophage-derived growth factor, which in turn promote the capillary budding and collagen synthesis of wound healing<sup>6</sup>. Some other possible suggestions for the mechanism of HBO in wound healing were made by ZHAO et al.<sup>26</sup> in 1994, who noted a 100% reversal of the healing deficit induced by ischaemia in experimental animals when wounds were treated with HBO and growth factors simultaneously. Clearly, this synergistic response suggested a direct interaction between oxygen and growth factors rather than the response expected from restoring adequate nutrition to compromised cells. The nature of this interaction was deduced by BONOMO et al.<sup>5</sup> who reported a statistically significant rise in the production of platelet-derived growth factor (PDGF) receptor protein I in ischaemic wounds that were treated simultaneously with both HBO and PDGF. DAVIDSON & MUSTOE<sup>7</sup> have stated that this finding leads strong support to the concept that HBO functions as an intracellular signalling transducer and thus is a modulator of gene function. SIDDIQUI et al.<sup>24</sup>, who documented that HBO treatment produces hyperoxic (not physiologic) concentrations in ischaemic

wounds and that oxygen concentration falls promptly to pre-treatment levels in ischaemic tissue, also demonstrated a progressive increase in the real oxygen concentration in ischaemic tissue when it was challenged with 100% oxygen at 1 atm as well as a more rapid washout of oxygen from tissue after serial HBO treatments. The magnitude of these changes was proportional to the number of HBO treatments. DAVIDSON & MUSTOE<sup>7</sup> attributed these acute changes in the responsiveness of ischaemic tissue to serial HBO treatments, and suggested prompt but transient production of a potent local vasodilator.

It is now becoming increasingly apparent that hyperoxia, as well as hypoxia, induces a distinct set of cellular responses. As HBO has been shown to modify the expression of vascular endothelial growth factor (VEGF) and PDGF receptors, it has been suggested by DAVIDSON & MUSTOE<sup>7</sup>, and HUNT et al.<sup>15</sup> that hyperoxia may act via an oxygen-sensing transduction pathway to impact on other important regulators of cell growth and metabolism. The recent papers by HUNT et al.<sup>15</sup> and GORDILLO & SEN<sup>13</sup> suggest further mechanisms by which oxygen exerts its vital actions in wound healing. They state that reactive oxygen species are a constructive force in wound healing. In the right quantity and place, they promote angiogenesis and collagen synthesis. GIBSON et al.<sup>12</sup> confirmed that HBO can be angiogenic and added, because the effect was inhibited by anti-VEGF antibody, that it is mediated by VEGF. Since current doctrine is that hypoxia is a key trigger of VEGF expression, and the data that hypoxia does instigate VEGF production must be accepted, it fell to SHEIKH et al.<sup>23</sup> to show that VEGF protein levels in actual wounds are raised by HBO. HUNT et al.<sup>15</sup> have therefore concluded that both hypoxia and hyperoxia can promote VEGF activity or release. It logically follows that HBO in

normal tissues produces hyperoxia which in turn promotes VEGF. The experimental data mentioned above support the hypothesis that transient hyperoxia, when given 1 week after completion of TRT, functions as an intracellular signal transduction agent or modulator of gene function via more than one signalling pathway, in addition to supplying the critical element for cellular respiration, and reduces some of the side effects of radiotherapy. This hypothesis is summarized in Fig. 9. The implications of being able to prevent the complications of TRT, rather than treating them as is currently practiced, are far reaching. Prevention of the complications of TRT will go a long way to improving the quality of life of patients who undergo this form of cancer treatment. These patients will therefore not lose specialized tissues such as salivary gland and bone, and this will impact on their ability to retain their teeth and salivary gland function. The patients may then be treated in a normal fashion by dentists and not have to undergo removal of all teeth as a preventive measure. In addition, the implication of the continued growth of teeth in irradiated animals, when treated early with HBO, as found in this study, suggests a protective mechanism which may be extrapolated to other tissues such as muscle, and will need further investigation. Children receiving TRT, for conditions such as rhabdomyosarcoma, may be cured by the treatment but are left with loss of continued growth of all tissues in the treatment area. Early treatment with HBO, as found in this study with teeth, may well benefit these children with continued growth of normal tissues in the treated area. Obviously much further research needs to be done in this area, but this study has certainly indicated that treatment with HBO reduces the acute inflammatory damage caused by TRT and thereby increases the preservation of specialized tissues, which in turn leads to a reduction in the long-term complications associated with TRT.

In summary, this experimental model has fulfilled its prime objective of demonstrating that HBO is effective in reducing some of the long-term side effects of TRT in normal tissue, when given 1 week after the completion of the radiation treatment.

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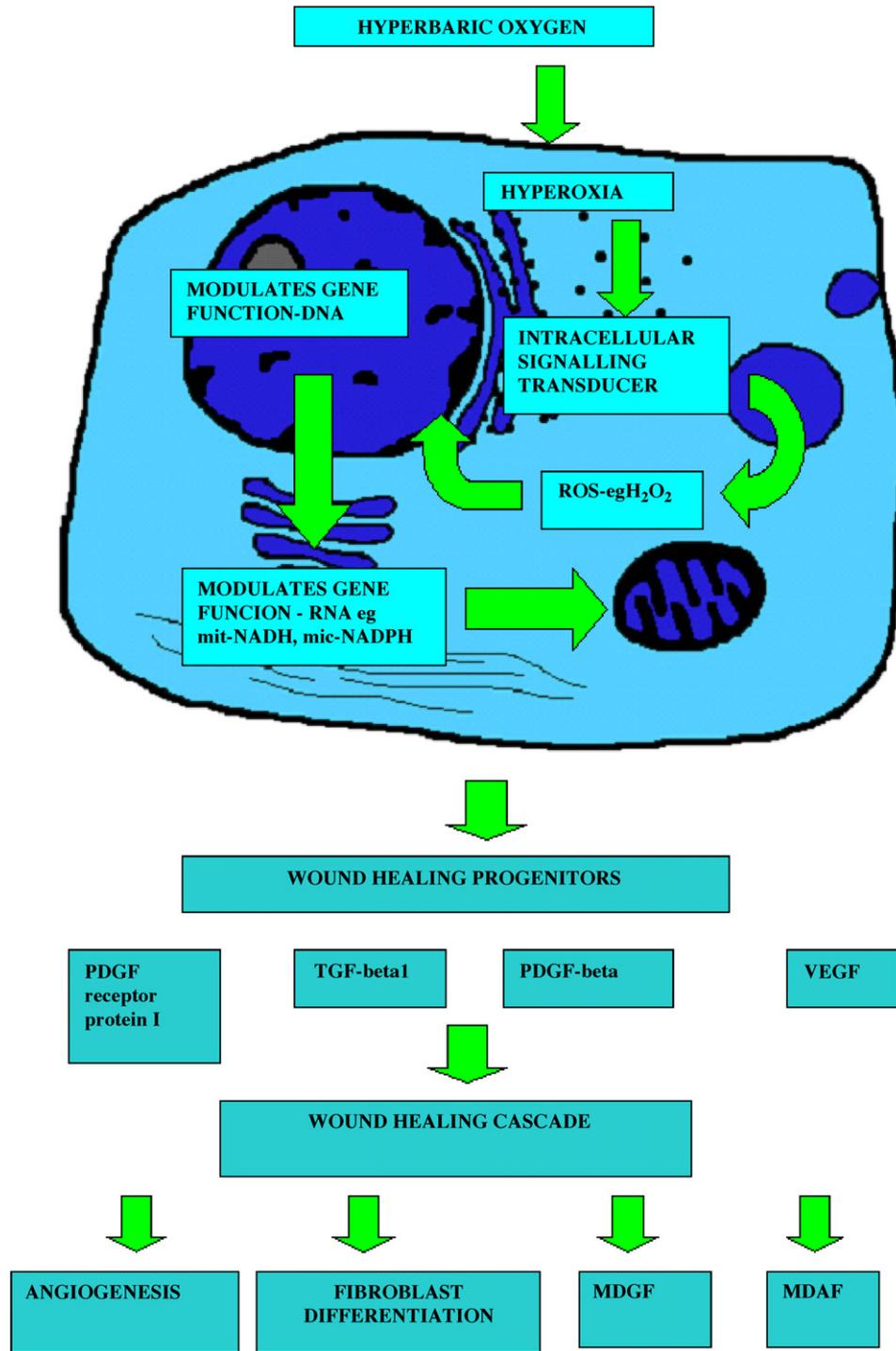


Fig. 9. Wound healing pathway elicited by HBO. This diagram outlines the author's hypothesis of the molecular pathway of wound healing elicited by HBOT, when given 1 week after TRT, in irradiated tissue. MDAF, macrophage-derived angiogenesis factor; MDGF, macrophage-derived growth factor; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

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