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Hyperbaric Oxygen Stimulates the Proliferation and Differentiation of Raw264.7 Cells

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Abstract

Background: Hyperbaric Oxygen (HBO) Therapy improves the outcome of various types of sugery, such as postoperative bone grafts, fixation of jaw fractures, and osteoplasty of jaw deformities. Therefore, it is important to determine the effects of HBO on bone regeneration. The purpose of this study was to clarify the influence of a hyperbaric oxygen environment on bone regeneration at the osteoclastogenic cytokines level. Sample & Methods: RAW264.7 cells were stimulated under the various conditions by using a hyperbaric oxygen chamber. We evaluated the ability of the RAW264.7 macrophages to proliferate, differentiate and produce various osteoclastogenic cytokines. Results: A hyperbaric oxygen (HBO) environment and high concentration oxygen (HCO) environment increase cellular proliferation in a time-dependent manner. On the other hand, a HCO environment and a hyperbaric with room air (HBA) environment enhanced the differentiation of RAW264.7 cells. In addition, NFATc1 and c-Fos were increased by both the HBA environment and HCO environment. However, the effects of HBA and HCO environments were in contrast with each other with regard to RANK, TNF-α, C-FMS and TRAP. Conclusions: It was suggested that a HBO environment influenced the bone regeneration by altering osteoclastogenic cytokines level, and that a HCO environment and HBA environment acted independently on the proliferation and differentiation of macrophages and the secretion of osteoclastogenic cytokines, and that they acted in concert in a hyperbaric oxygen environment to induce conditions favoring regeneration.

Keywords

Hyperbaric Oxygen Therapy, Bone Regeneration, Osteoclast, RAW264.7

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1. Introduction

Hyperbaric Oxygen (HBO) Therapy improves the outcome of various types of surgery, such as the repair of bone fractures [1]-[3], osteoplasty of jaw deformities, the treatment of osteoradionecrosis [4]-[6] and distraction osteogenesis [7] [8], as well as postoperative bone grafting [1] [9] and the use of dental implants [10]. The reason was generally assumed that HBO stimulates the growth of blood vessels, thus resulting in an increased blood supply and enhanced bone formation [11]. This is partly because HBO is believed to generate reactive oxygen species (ROS) and reactive nitrogen species (RNS) that affect signaling processes critical to wound healing.

In recent years, the use of bone augmentation and sinus floor augmentation for dental implant treatment has increased. Therefore, it is important to determine the effect of HBO on bone regeneration. Although animal studies have shown that HBO can be used to treat delayed fracture healing [12]-[14] or an established nonunion of a bony fracture [12] [15] [16], the clinical application of HBO is still subject to debate [12] [17]-[19] due to the lack of *in vitro* studies, for example, to demonstrate its effect on osteoclast activity.

Osteoclasts are a member of the monocyte/macrophage lineage and are formed by multiple cellular fusions from their mononuclear precursors. They play an important role in bone metabolism by regulating bone resorption. Receptor activator of nuclear factor-*κ*B ligand (RANKL) induces the differentiation of osteoclasts from myeloid precursors at various intermediate stages, as well as from well differentiated tissue macrophages, such as alveolar macrophages [20]. Osteoclasts attach to the surface of bone and secrete protons into an extracellular compartment between the osteoclast and bone surface. The osteoclast proton pump is essential for bone mineral solubilization and digestion of organic bone matrix by acid proteases [21]. There are two crucial steps in osteoclast formation: commitment of progenitor cells to osteoclast precursor cells, and fusion of mononuclear cells to form TRAP-positive, multinucleated osteoclasts. The activation of the receptor for RANKL (RANK) is a key step in the differentiation of mature osteoclasts during the late stage of this process [22]-[24]. RANKL is a member of the tumor necrosis factor (TNF) superfamily, and acts as a key regulator of osteoclasts. It induces transcription factors such as NF-*κ*B, c-Fos, and nuclear factor of activated T cells (NFAT) cl, which play an essential role in osteoclast differentiation, fusion, and function [25]-[27]. The regulation of irreversible cell lineage commitment depends on a delicate balance between positive and negative regulators, which comprise a sophisticated network of transcription factors. Robust osteoclast-specific induction of NFATc1 is achieved through an autoamplification mechanism, in which NFATc1 is constantly activated by calcium signaling, while the negative regulators of NFATc1 are suppressed. However, it has been unclear how such negative regulators are repressed during osteoclastogenesis. NF-*κ*B induces the initial induction of NFATc1, the expression of which is autoamplified by NFATc1 binding to its own promoter in cooperation with c-Fos [28]. This autoamplification mechanism enables the strong induction of NFATc1 specific to osteoclasts [26].

RAW264.7 cells were employed as a model system for developing osteoclasts in this study. Upon treatment with RANKL, these cells stain positively for TRAP within 1 day and start to fuse after 4 to 5 days to generate multinucleated cells, a hallmark of fully differentiated osteoclasts [29]. RAW264.7 cells have served as a model for osteoclasts in multiple contemporary studies dealing with osteoclast physiology or the RANK/RANKL/OPG interaction [30]-[32]. Although RAW264.7 cells are of murine origin, which might be considered a drawback of the study design, they represent a well-established and reproducible model, and the cells respond robustly to human RANKL, which legitimates the experimental approach of co-culturing human PDL cells with murine monocytes/macrophages [33]. The characterization of osteoclasts by the TRAP assay is a well-accepted method to characterize these cells, and was also adopted in recent reviews on osteoclast biology [34]. The purpose of this study was to clarify the influence of a hyperbaric oxygen environment on bone regeneration at the cellular level. The information obtained from such studies should therefore advance the overall understanding of osteoclastogenic cytokines and the mechanism of bone regeneration under HBO, thereby potentially leading to novel therapeutic approaches to bone diseases.

2. Materials and Methods

2.1. Reagents

Recombinant human soluble RANKL (sRANKL) was purchased from Chemicon International (Temecula, CA, USA). Mouse monoclonal antibodies against NFATc1, rabbit polyclonal antibodies against c-FOS, RANK, and

goat polyclonal antibodies against β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell Culture

The mouse monocyte-macrophagic cell line, RAW264.7 cells, were obtained from Dainippon Pharmacy Co. (Osaka, Japan). RAW264.7 cells were cultured in α-minimal essential medium (α-MEM, GIBCO) containing 10% heat-inactivated fetal bovine serum (FBS; CSL, Victoria, Australia), 100 U/ml penicillin, 100 µg/ml streptomycin, and 1 mM L-glutamine at 37˚C in a humidified atmosphere of 5% CO_2 in air.

2.3. Stimulation of Raw264.7 Cells

RAW264.7 cells were treated daily for up to 7 days in a custom-made hyperbaric oxygen chamber (**Figure 1**). The chamber was sealed and flushed for 2 minutes with 95% O_2 and 5% CO_2, and the pressure was subsequently increased to 1.4 atmosphere absolute (ATA). The hyperbaric oxygen (HBO) treatment consisted of 95% O_2 and 5% CO_2/1.4 ATA, hyperbaric with room air (HBA) was room air/1.4 ATA, and high concentration oxygen (HCO) was 95% O_2 and 5% CO_2/1.0 ATA. Following various conditions were set up to search for a more effective conditions. The pressure was maintained for 20 or 60 minutes, after which the chamber was slowly depressurized over 5 minutes. RAW264.7 cells were stimulated under the various conditions for 1) 20 minutes, 2) 20 minutes × 3, 3) 20 minutes × 7, 4) 60 minutes, 5) 60 minutes × 3, 6) 60 minutes × 7. After 8 days, both cell proliferation and differentiation were analyzed.

2.4. Effect on Proliferation

We analyzed the proliferation of the cells by the MTS assay using a CellTiter 96®AQueous One Solution Cell Proliferation Assay kit (Promega KK, USA) according to the manufacturer's instructions.

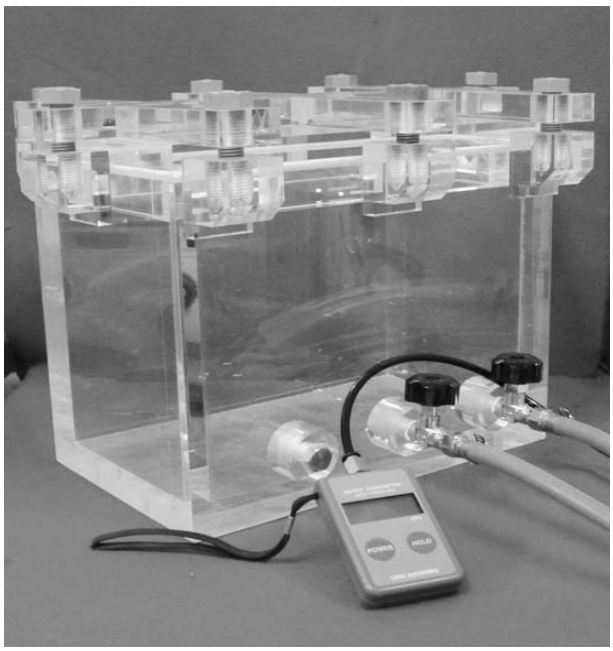

Figure 1. The custom-made hyperbaric oxygen chamber. The chamber was sealed and flushed for 2 minutes with 95% O_2 and 5% CO_2, then the pressure was subsequently increased to 1.4 atmosphere absolute (ATA). HBO treatment consisted of 95% O_2 and 5% CO_2/1.4 ATA, HBA of room air/1.4 ATA, and HCO treatment consisted of 95% O_2 and 5% CO_2/1.0 ATA. The pressure was maintained for 20 or 60 minutes, after which the chamber was slowly depressurized over 5 minutes.

RAW264.7 cells were seeded at 3.0×10^3 cells in 96-well plates and cultured in α-MEM at 37˚C in a humidified atmosphere with 5% CO_2. After one day, the RAW264.7 cells were stimulated according to our protocol.

2.5. Effect on Differentiation

We analyzed the ability of the cells to differentiate by the Tartrate-resistant acid phosphatase (TRAP) activity assay. RAW264.7 cells were seeded at 3.0×10^3 cells in 96-well plates and cultured in α-MEM supplemented with RANKL (100 ng/ml) at 37˚C in a humidified atmosphere with 5% CO_2. TRAP activity was examined with a TRAP staining kit (Hokudo, Hokkaido, Japan) according to the manufacturer's instructions. After stimulation, 30 µl of cell culture media were incubated with 170 µl of staining solution in the kit for 3 hours. Thereafter, the absorption of the reaction mixture was measured at 550 nm with a microplate reader (Colona Electric, Ibaragi, Japan).

2.6. Real-Time PCR

RAW264.7 cells were seeded at 4.5×10^5 cells in 10 cm plates and cultured in α-MEM supplemented with RANKL (100 ng/ml) at 37˚C in a humidified atmosphere with 5% CO_2 and stimulated according to our protocol.

Optimized oligonucleotide primers were purchased from NIHON GENE RESEARCH LABORATORIES (Sendai, Japan). Real-time PCR was performed on a light-cycler (Roche, Tokyo, Japan). The PCR cycling conditions were 10 min at 95˚C for 1 cycle followed by 45 cycles each of 95˚C for 30 s, 65˚C for 30s, and 72˚C for 60 s. The following oligonucleotides [35] [36] were used: β-actin sense 5′-AGCACCATGAAGATCAAG-3′, anti-sense 5′-GTAAAACGCAGCTCAGTAA-3′; NFATc1 sense 5′-GGGAGATGGAAGCAAAGACT-3′, anti-sense 5′-AAGGTACGTGAAACGCTGGT-3′; c-Fos sense 5′-CTGTCCGTCTCTAGTGCCAACTT-3′, anti-sense 5′-ATCTGTC TCCGCTTGGAGCGTAT-3′; TRAP sense 5′-ACGTGCCCAACTGCTACA-3′, anti-sense 5′-GGGGTCATCAACGAGTCCT-3′; RANK sense 5'-CATCATCTTCGGCGTTTAC-3′, anti-sense 5′-GATACCTT-ACACACTTCTTGC-3′; TNF-α sense 5′-CTCTTCTCATTCCTGCTTG-3′, anti-sense 5′-GTCTGGGCCATAGAACTGAT-3′; C-FMS sense 5'-ACAAGGCAGGCTGGAATA-3′, anti-sense 5′-CCAGAGACGTCACAGAACAG-3′.

2.7. Western Blot Analysis

RAW264.7 cells were seeded at 4.5×10^5 cells in 10 cm plates and cultured in α-MEM supplemented with RANKL (100 ng/ml) at 37˚C in a humidified atmosphere with 5% CO_2 and stimulated according to our protocol.

Samples were lysed in lysis buffer, and the aliquots of the samples were run on 10% SDS-polyacrylamide gels, and transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) at 50 mV for 10 hours. The nitrocellulose membrane was incubated with 5% bovine serum albumin in TBST for 1 hour to eliminate non-specific binding, and incubated with a 1:200 dilution of polyclonal antibody against NFATc1, c-Fos or RANK. Protein bands were visualized using HRP-conjugated secondary antibodies and the Enhanced Chemiluminescence Reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The bands were scanned by computer-assisted densitometry (ChemiDoc XRS-J, Bio-Rad) and acquired using the Quantity One software package (Bio-Rad).

2.8. Statistical Analysis

All quantitative results are expressed as the means ± SD. Statistical differences between experimental groups were analyzed by Student's *t*-test. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Proliferation of Raw264.7 Cells

We examined the effects of HBO on RAW264.7 cell proliferation using the MTS assay. As shown in **Figure 2(a)**, at hyperbaric oxygen (HBO), the proliferation was significantly higher in the cells incubated for 20 minutes × 7, 60 minutes, 60 minutes × 3, and 60 minutes × 7 compared with the untreated controls. In the cells in-

cubated for 20 minutes or for 20 minutes × 3, no significant difference was noted between the treated and the untreated controls. Under hyperbaric with room air (HBA) conditions, the proliferation was significantly lower for the cells treated for 20 minutes × 3, but under the other conditions, no differences in proliferation were noted between the treated cells and untreated controls (**Figure 2(b)**). After incubation under a high concentration of oxygen (HCO), the proliferation was significantly higher for the cells incubated for 20 minutes × 3, and 20 minutes × 7. The other conditions did not induce any significant change in proliferation compared with control cultures (**Figure 2(c)**).

3.2. Differentiation of Raw264.7 Cells

We next examined the effects of stimulation on RAW264.7 cell differentiation by the TRAP assay. As shown in **Figure 3(a)**, under HBO, there was significantly more differentiation in the cells incubated for 20 minutes, while it was lower for those incubated for 60 minutes, 60 minutes × 3, and 60 minutes × 7 compared with the untreated controls. For the cells incubated for 20 minutes × 3, and 20 minutes × 7, no significant difference in differentiation was noted between the treated cells and the untreated controls. In the cells cultured under HBA, the differentiation was significantly higher for cells incubated for 20 minutes compared with the untreated controls. No difference in the differentiation was noted between the untreated cells and those incubated under the

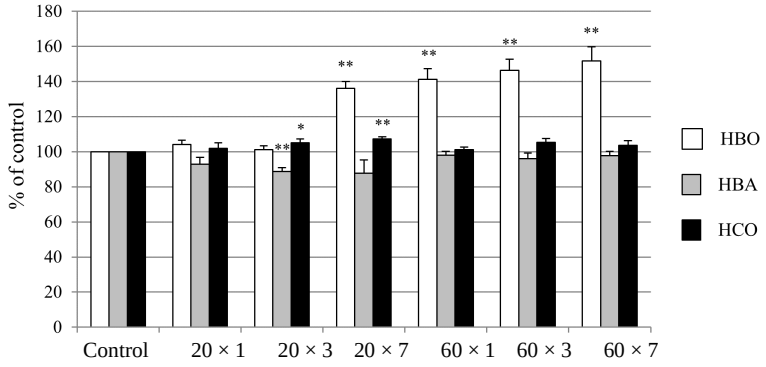

Figure 2. The effects of stimulation on the proliferation, as determined by the MTS assay. RAW264.7 cells were cultured in 10% FCS for 24 hr and subsequently treated with HBO, HBA or HCO for the indicated times (20 min or 60 min, once, three times or seven times). Proliferation was analyzed by the MTS assay. **Significantly different from the control ($p < 0.01$). *Significantly different from the control ($p < 0.05$).

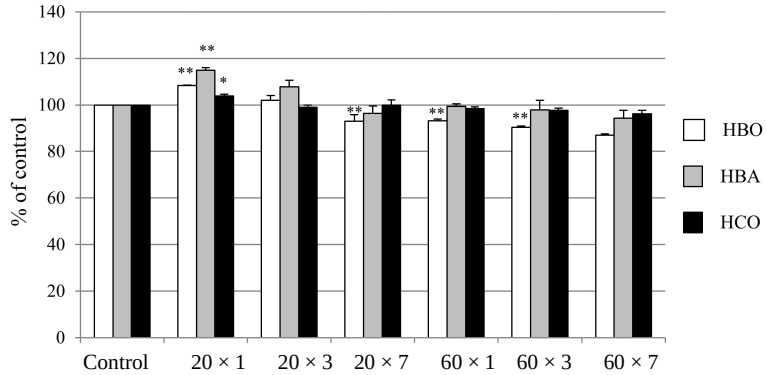

Figure 3. The effects of stimulation on differentiation determined by the TRAP assay. RAW264.7 cells were cultured in 10% FCS and treated with 100 ng/ml RANKL for 8 days, then were treated with HBO, HBA or HCO. Differentiation was analyzed by the TRAP assay. **Significantly different from the control ($p < 0.01$). *Significantly different from the control ($p < 0.05$).

other conditions (**Figure 3(b)**). The differentiation was significantly higher for cells incubated under HCO for 20 minutes compared with the untreated controls. However, no significant difference in the differentiation was noted between the cells treated for other periods of time and the untreated controls (**Figure 3(c)**).

3.3. Expression of mRNA

To investigate whether the stimulation of RAW264.7 cell differentiation alters the expression of osteoclastogenic cytokines, we examined the expression of NFATcl, c-Fos, TRAP, RANK, TNF-α, and C-FMS. As shown in **Figure 4**, NFATc1 increased after all forms of stimulation. While c-Fos was observed to decrease in cells incubated for 20 minutes, 20 minutes × 3, and 60 minutes under HBO, HBO increased cellular differentiation in a time-dependent manner in the cells incubated for 60 minutes. Under HBA and HCO increased too. RANK and C-FMS were increased by HBO, HCO, and decreased by HBA. On the other hand, TNF-α increased by HBO and HBA, and decreased by HCO. TRAP was increased by HBA, and decreased by HCO. TRAP also generally decreased by HBO.

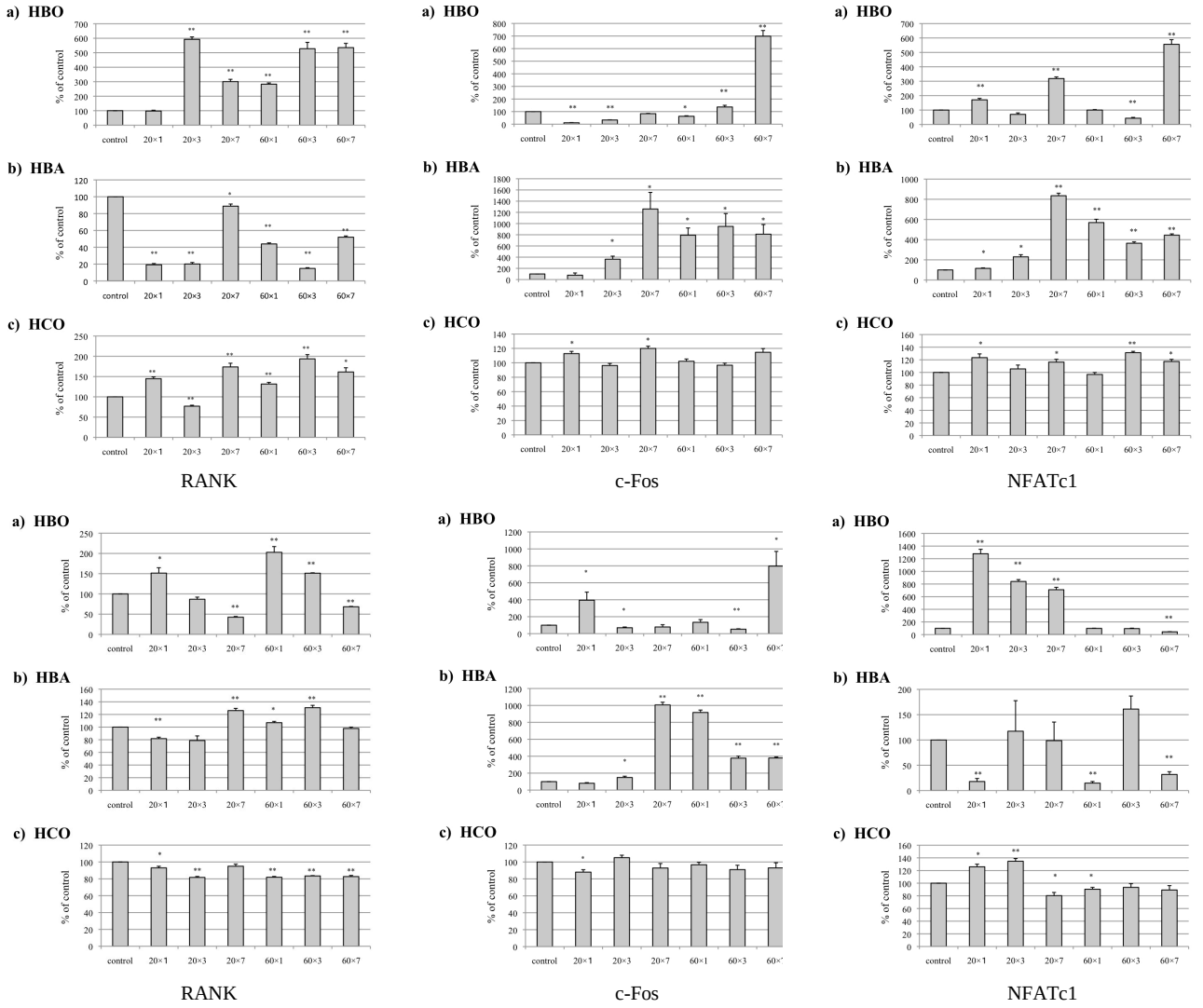

Figure 4. The effects of stimulation on the expression of osteoclastogenic cytokines in RANKL-treated RAW264.7 cells. RAW264.7 cells were treated with 100 ng/ml RANKL for 8 days and subsequently treated with (a) HBO, (b) HBA or (c) HCO. The expression of NFATcl, c-Fos, TRAP, RANK, TNF-a, and C-FMS mRNA was measured by real-time PCR. The b-actin mRNA level was used as an internal quantity control. **Significantly different from the control ($p < 0.01$). *Significantly different from the control ($p < 0.05$).

3.4. Western Blot Analysis

The Western blotting analyses were performed to confirm whether stimulation affects c-Fos and NFATcl protein expression. We observed that the bands of c-Fos were detected in the range of 42- to 50-kDa and the bands of NFATcl were detected in the range of 83- to 119-kDa after each stimulation (**Figure 5**). Consistent with the marked increase in c-Fos mRNA, the c-Fos protein level was substantially increased in RAW264.7 cell-derived osteoclasts. However for NFATcl, despite the marked increase in the NFATcl mRNA level, the NFATcl protein level was decreased in RAW264.7 cell-derived osteoclasts.

4. Discussion

The effects of HBO on a variety of cell types have been reported previously. For example, to evaluate the potential role of HBO in skin wound healing, its effects on human dermal fibroblasts were investigated, and a stimulatory effect of HBO was reported [37] [38]. Rat hepatocytes have also been used to study the effects of HBO treatment on primary liver nonfunction [39], and benign and malignant mammary epithelial cells have been used to elucidate the inhibitory role of HBO on tumor growth [40].

A large number of studies have shown enhanced osteogenic activity as a result of HBO treatment. For example, accelerated levels of bone morphogenic proteins [41], earlier union of autologous bone grafts [9], and improved bone formation in titanium implants [42] were observed *in vivo* after exposure to HBO. This requirement for oxygen during healing is the rationale underlying HBO therapy, and it is generally assumed that HBO stimulates the growth of blood vessels, resulting in an increased blood supply, and as a result, enhanced bone formation [43]. It was demonstrated that after bone grafting, the formation of new bone is closely associated with the rate of vascularization [44]. In the posterolateral fusion area, the major blood supply for vascularization of the bone graft was from the upper and lower transverse processes [11]. After dissection, the healing environment between the transverse processes is relatively hypovascular. This hypovascular environment results in cell apoptosis, which ultimately affects the bone-forming ability of the cells. HBO is known to accelerate the

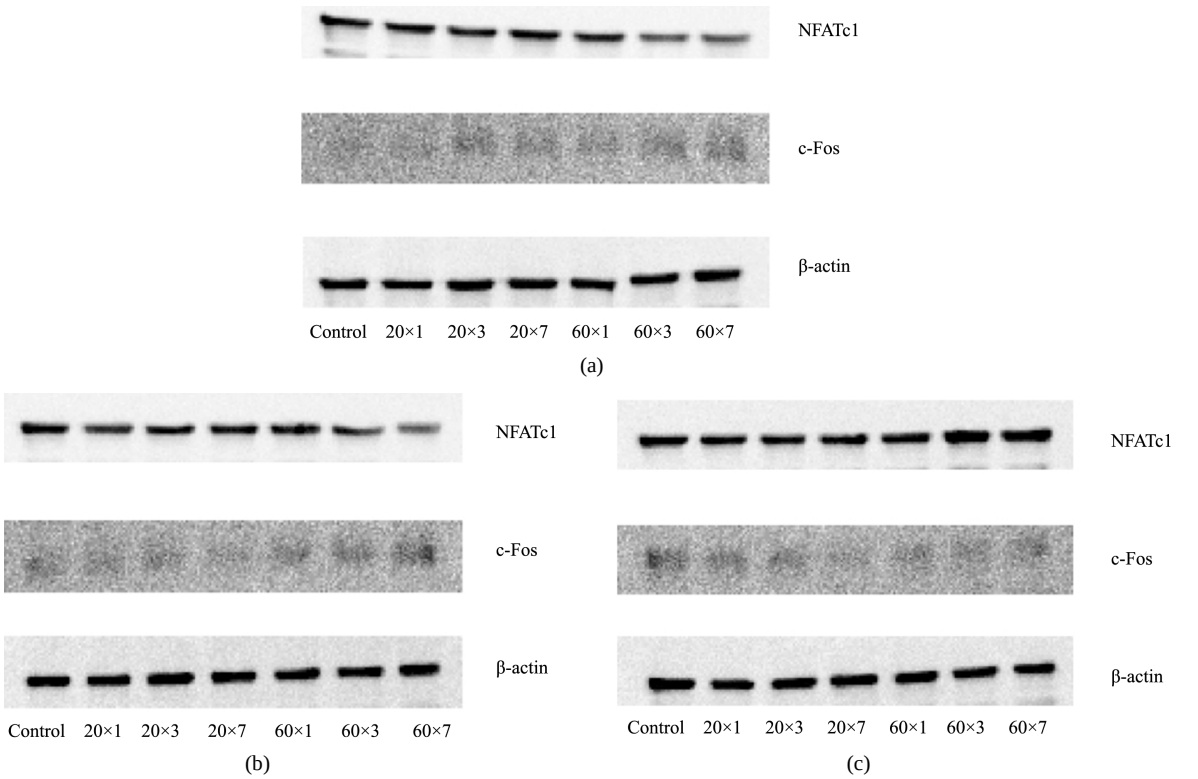

Figure 5. The effects of stimulation on the expression of c-Fos and NFATc1 protein in RANKL-treated RAW264.7 cells. RAW264.7 cells were treated with 100 ng/ml RANKL for 8 days and subsequently treated with (a) HBO, (b) HBA or (c) HCO. The expression of c-Fos and NFATc1 proteins were measured by an immunoblot analysis.

development of new blood vessels in hypovascular tissue, which increases the vascularization in the affected area and enhances bone healing. To understand the cellular mechanisms of the observed therapeutic effects of HBO on fracture healing, our study investigated the effects of HBO on the proliferation and differentiation of RAW264.7 cells *in vitro* using a custom-made hyperbaric oxygen chamber.

Historically, the beneficial effects of HBO have been attributed to the establishment of a favorable oxygen gradients [45] [46]. Recent research has revealed that HBO also generates reactive oxygen species (ROS) and reactive nitrogen species (RNS) that affect signaling processes critical to wound healing [47]-[54] and have shown that HBO-derived ROS and RNS induce stem cell mobilization [50] [55] [56], vasculogenesis [51] [52] [57] [58], mitochondrial biogenesis [53], and preconditioning [59]-[62]. Because ROS and RNS are the basis for HBO's effects on signaling, its clinical consequences in any therapeutic situation will be dose-, tissue-, and time-specific. HBO increases the local concentrations of ROS and RNS [63] by providing a substrate for nitric oxide synthase, as well as by the generation of superoxide. ROS and RNS influence osetoclast differentiation and activity, and regulate other critical aspects of bone metabolism. Endothelial nitric oxide synthase is an enzyme expressed extensively in osteoclasts and osteoblasts, their marrow precursors, and in their descendents [64]. Nitric oxide is constitutively synthesized by both osteoclasts and osteoblasts, and it has contrasting biphasic effects on bone resorption, stimulating turnover at lower signaling levels and inhibiting turnover at higher, proinflammatory, concentrations [57]. ROS stimulate the expression of RANKL [65], changing the RANKL/osteoprotegerin ratio and favoring osteoclast differentiation in animal models [66].

In the present study, we drew a comparison between a hyperbaric environment (HBA) and a high concentration oxygen environment (HCO). Our results showed that the HCO environment increased cellular proliferation in a time-dependent manner. However, when an HBA environment was used to stimulate the RAW264.7 cells, their proliferation was decreased compared with control cultures. In addition, while the effect was dependent on the duration of stimulation, both the HBA and HCO environment enhanced the differentiation of RAW264.7 cells.

These data suggest that the HCO environment enhances proliferation. However, both an HBA and HCO environment are needed to enhance cell differentiation. In addition, the TRAP activity showed that the effect of each type of stimulation was time-dependent. It was presumed that when stimulation was performed many times, both differentiation and bone resorption were inhibited. We found intriguing evidence to support the idea that the HBA and HCO environment act independently of each other on the proliferation and differentiation of RAW264.7 cells, and that they act in concert under HBO. Finally, we observed that the expression of osteoclastogenic cytokines, NFATc1 and c-Fos, increased due to both an HBA and HCO environment. This suggests that both a HBA and HCO environment enhanced the differentiation of RAW264.7 cells via increased cytokine expression. However, the effects of HBA and HCO environments were in opposition to each other in terms of RANK, TNF-α, C-FMS and TRAP.

RAW264.7 cells were employed as a model system for developing osteoclasts in this study. Numerous researches have employed RAW264.7 cells as a single- or mono-culture. However, it is also true that this monoculture system cannot evaluate interactions of bone regeneration under HBO. Co-culture compositions of two different cell type of bone like osteoclast and osteoblast, must be established in order to evaluate cell-cell interactions of a hyperbaric oxygen environment on bone regeneration. Additionally, in this study, the pressure was subsequently increased to 1.4 ATA and was maintained for 20 or 60 minutes. Extermely important agenda is to evaluate the effect of HBO, if RAW264.7 cells are stimulated under 2.0 ATA or the various amount of time.

5. Conclusion

We hypothesized that HBO can stimulate bone turnover through its effects on the chemical mediators of osteoclast activation. Our results were in agreement with this hypothesis, but the more intriguing fact is that the HBA and HCO environments acted independently of each other with regard to cell proliferation and differentiation, as well as the production of osteoclastogenic cytokines, while acting together under a HBO environment.

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