Hyperbaric oxygen-stimulated proliferation and growth of osteoblasts may be mediated through the FGF-2/MEK/ERK 1/2/NF-?B and PKC/JNK pathways

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Running title: Signal pathway of Hyperbaric Oxygen stimulated proliferation in Osteoblast

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ABSTRACT

Background: To investigate whether the hyperbaric oxygen (HBO) can promote the growth-arrest osteoblast (OB) cells to proliferate and differentiate, and the probable mechanism.

Methods: OB cells were exposed to O2 with different levels of saturation and pressure for 3 days and 7 days. The OB cells were divided into four groups: (1) The Control Group (Group C): cells were cultured under ambient oxygen (21% O2) and normal pressure (1ata). (2) The Pressure Group (Group P): with high pressure (2.5ata) twice daily. (3) High Oxygen Group (Group O): with high concentration oxygen (50%) twice daily. (4) Pressure and High Oxygen group (Group P+O): with high pressure (2.5ata) and high concentration oxygen (50%) twice daily.

XTT was used to detect the cells proliferation and cell cycle progression was determined by Flow analysis. Expression of growth factors was assayed by RT-PCR. In addition, we determined HBO activated signaling pathway in OB cells by Western Blot analysis.

Results: HBO significantly promote OB cell proliferation and stimulated cell cycle progression after the cells had been treated for 3 days. Afterward, the effect attenuated day by day. HBO also stimulated the OB cells to produce the FGF-2 growth factors. Multiple signaling pathways, including FGF-2/MEK/ERK 1/2/Akt/ $p70^{s6k}$ /NF- κ B and PKC/JNK, are involved in the proliferation of OB cells by HBO stimulation .

Conclusions: Pressure and high saturation of pressure has a positive effect on stimulating

grow-arrested OB cells to proliferate and differentiate through activation of FGF-2/MEK/ERK 1/2/Akt/ p70^{s6k} /NF-κ B and PKC/JNK signaling pathway.

ABBREVIATIONS

Hyperbaric oxygen	HBO
Osteoblast	OB
Reverse transcription-polymerase chain reaction	RT-PCR
Bone morphogenetic protein	BMP
Fibroblast growth factor-2	FGF-2
Vascular endothelial growth factor	VEGF
Mitogen-activated protein kinase	MAPK
MAPK/extracellular signal-regulated kinase	MEK
Extracellular signal-regulated kinase	ERK
c-Jun N-terminal kinase	JNK
Protein kinase A	PKA
Protein kinase C	PKC
Nuclear factor-κB	NF-κB

INTRODUCTION

Nonunion and delayed union can occur in the process of fracture healing and the likelihood of such delay occurring is up to 10%(1). They present great challenge in the clinic treatments for fracture. These conditions not only result in patients' prolonged disability but also lead to tremendous economic loss.

In the past, orthopaedic surgen treated the nonunion and delayed union by surgical decortication and autogenous bone graft. These methods are not always successful and can cause patients to suffer more pain and increase risks of wound infection. The search for better alternatives has been ongoing for a long time.

When an injury occurs, hypoxia is a commonly seen microenvironment in the bone or soft tissue at the injury site (2). The damage to the vascular flow resulted from the fracture or surgical wound sometimes lead to transient hypoxic situation around the injury site, where the oxygen tension can fall to 0-2% in the center (3).

This detrimental situation will impede the subsequent repair,such as inflammatory cell recruitment, matrix processing, angiogenesis and activation of mesenchymal osteoblast (OB) precursors(4).

It has been well demonstrated that hyperbaric oxygen (HBO) therapy promotes osteogenesis on osteoradionecrosis by increasing the OB activity and neoagiogenesis (5,6). Various studies also showed that HBO might improve the healing of fractures in some

conditions. HBO can increase the accumulation of bone–making minerals such as calcium, magnesium, phosphorus, etc (7,8).HBO also help to accelerate bone repair by encouraging vessel in-growth(9),migration of connective tissue from surrounding soft tissue(10),and increasing the bone mineral density(11).In some clinic experience, HBO was also shown to enhance osteogenesis in the fracture area (12,13). Despite the abundance of studies in this area, the actual mechanism that promotes osteoblastic & angiogenic growth is still not completely understood.

Many recent investigations in this area recently have yielded significant insights into the transcriptional regulation of osteogenesis and differentiation (14,15,16). Multiple signal transduction pathways are shown to be involved in the regulation of the OB-specific transcription (17). These signal pathways mostly correlated with environment stimuli, include osteogenic growth factors like bone morphogenic proteins (BMPs), fibroblast growth factor-2 (FGF-2), extracellular matrix (ECM), mechanical loading, and hormones such as parathyroid hormone (PTH). But it is not clear whether HBO would promote osteogenesis through these environment-stimuli pathways. Many endogenic growth factors (like BMP, FGF-2, VEGF, etc.) produced by OB may play an important role in osteogenesis and differentiation in the early stage of bone injuries. In vivo studies, it was demonstrated that application of these growth factors systemically helps increase bone formation, promote fracture healing and induce bone growth around the fracture site (18). However, it is yet to be established how HBO stimulate OB cells to produce these growth factors.

Four conditions are designed in our attempt to investigate the in vitro effect of hyperbaric oxygen in the OB cells. They are:

1.ambient oxygen (21% O2) under normal pressure

2.ambient oxygen combined with high pressure (2.5 ata)

3.high concentration oxygen (50% O2) under normal pressure (1ata)

4.high concentration oxygen (50% O2) combined with high pressure (2.5 ata)

In consideration of the in vitro culture system, the combination of 50% oxygen concentration and 2.5 ata pressure is closer to the oxygen tension in the human muscle and bone surface tissues (300-400 mmHg) under therapeutic HBO condition. Pure oxygen (100%) combined with 2.5ata was not considered because it will elevate the oxygen tension to a very high level (about 600-700 mmHg) in the culture medium and may result in the cell damage (19).

In this study, the mechanism by which HBO stimulated the proliferation of the OB cells was investigated. It was found that under certain concentration of oxygen and pressure, HBO wound induce proliferative change of OB cells and the process was regulated through the FGF2/MEK ERK1/2/ Akt/ $p70^{s6k}$ /NF-κB and PKC/JNK pathways.

MATERIALS AND METHODS

Cell Culture and biological regimens

The mouse OB-like immortalized cells, MC3T3-E1, (American Type Culture Collection, Manassas, VA) were maintained in α-minimal essential medium supplemented with 10% fetal bovine serum, penicillin/streptomycin (0.0002g/L) (Sigma). Early passage (<3) cells were utilized for experimentation. The OB cells were then seeded at 10^4 cell/well in a 6-well plate. The final volume of all wells was 100 μL. All cells were maintained at 21% O2 and 5%CO2 in humidified incubators at 37°C prior to hyperbaric oxygen experiments. The fresh medium was changed every 2 days.

Exposure to various O2 tension and pressure condition

OB cells were cultured in the 6-well plates then transferred to an HBO chamber (small animal chamber, 50×50×57 cm) made of transparent acrylic plastic and steel. The HBO chamber temperature was maintained at 37°C±1°C. The cells were flushed with O2 with different levels of oxygen concentration and pressure.

The different combination of O2 concentration and levels of pressure were designed to investigate how O2 affect the OB cells .The four groups are as followed: **1.**The Control group(Group C):cells were cultured under ambient oxygen (21% O2) and normal pressure(1ata). **2.**The Pressure group (Group P): cells were treated with high pressure (2.5ata) only twice daily. **3.**The High oxygen group (Group O): cells were treated with high

concentration oxygen (50%) only twice daily. **4.**Pressure and high oxygen group (Group P+O): cells were treated with high pressure (2.5ata) and high concentration oxygen (50%) twice daily.

Evaluation of cell proliferation and cell cycle progression

XTT labeling mixture reagents were used (cell proliferation kit II, Roche Molecular Biochemicals, Indianapolis, IN). The cells were treated in various conditions for a period of 3 days and 7 days. The XTT mixture reagent was added to each well and incubated for four hours; the absorbance at 490 nm was measured for cell proliferation.

The cells treated for three and seven days were also elucidated for cell cycle progression. The harvested cell pellet was added to 3ml of cold 70% ethanol and maintained at -20°C for 30 min. The cell pellet was resuspended with 1% Triton X-100,0.1 mg/ml Rnase A and 4 μ g/ml propidium iodide after centrifuging. The flow cytometry (FC 500,Beckman Coulter,

Inc., Fullerton, CA) was used to elucidate cell cycle progression

RNA Extraction and RT-PCR

Total RNA was isolated using TRI reagent (Molecular Research Center). The cDNA was primed with oligo (dT) $_{12-18}$ and extended with reverse transcriptase (Clontech, BD biosciences, USA). cDNA was amplified by polymerase chain reaction using following primer pairs: BMP-2,forward, 5'-AAGAAGCCATCGAGG AACTTTCAG -3';reverse, 5'-CCTGAGACCAGCTGTGTTCATCTT-3';   VEGF,   forward,

5'-ACCCTGGCTTTACTGCTGTACCTC-3';reverse, 5'-TCACCGCCT
TGGCTTGTCACA-3'; FGF-2,forward, 5'-AAGCGGCTCTACTGCAAGAA-3';reverse,
5'-CCGTTTTGGATCCGAGTTTA-3'; cyclinD1,forward, 5'-CTGACAC
CAATCTCCTCAAC-3';reverse, 5'-GTAGATGCACAACTTCTGG-3'.

The PCR reaction production was run on 2% agarose gel in a TAE buffer and identified after staining with ethidium bromide. The intensity of the signal was quantified (Image Station 2000R, Kodak), and normalized against GAPDH messages.

Extraction of Protein and Western Blot Analysis

Total cellular proteins were extracted using a lysis buffer. The protein concentration was determined using Bio-Rad protein assay (Bio-Rad,Hercules, CA).

Antibodies against cyclin D1, Akt, Ser473-phosphorylated Akt,phosphorylated p70^{S6K}(Thr421/Ser424), p70^{S6K}, phosphorylated ERK1/2, ERK1/2,phosphorylated JNK,JNK, phosphorylated P-38,P-38,and β-actin were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against NF-κB and cyclin D1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies were purchased from Pierce Biotechnology (Pierce Biotechnology, U.S.A.). Cell proteins were fractionated by electrophoresis on a 10% SDS-polyacrylamide gel and were transferred onto nitrocellulose membrane, blocked and probed with various primary antibodies. Following incubation with

primary antibodies (1:1000) overnight, the membrane was washed and incubated with

horseradish peroxidase-conjugated secondary antibody (1:10000) for 1 hour. The blot was

washed and visualized by enhanced chemiluminescence (Pierce Biotecnology, U.S.A.). The

bands were quantified and normalized against β-actin messages.

Statistical Analysis

Statistical analyses were carried out using SPSS/Windows (SPSS Science, Chicago, IL)

software. The statistical significance was evaluated by one-way analysis of variance (ANOVA).

RESULTS

HBO promote Osteoblast (OB) proliferation

To investigate the effects of HBO on OB proliferation, the growth-arrested OB cells (cultured in serum-free medium for 48 hrs) were treated in various conditions twice daily. (**1**) Control group(C): cells were cultured under ambient oxygen (21% O2) and normal pressure (1ata). (**2**) Pressure (P) group: cells were treated with high pressure (2.5ata) twice daily. (**3**) High oxygen (O) group: cells were treated with high concentration oxygen (50%) twice daily. (**4**) Pressure and high oxygen (P+O) group: cells were treated with high pressure (2.5ata) and high concentration oxygen (50%) twice daily.

After treated for three days, the morphology of cells was observed (Fig1A). The rate of proliferation of cells was determined by XTT. The net proliferation rate (ΔOD) was determined(OD3 day－OD0 day) (Fig 1B).After treatment for three days, the cells of all treated groups had higher proliferation rate than the rate of the control group as showed in Fig 1A. It revealed that the cells in the P+O group had the most growth. The cells in P group and O group had similar proliferative rate. However, the pressure combined with high saturation of oxygen did not seem to have the synergistic effect.

After treatment for 7 days, the OB cells of all treated groups still maintained the elevated proliferative rate compared to that of control group. The proliferative rates then gradually decreased in individual treated groups. These results indicated that a profound mitogenic effect of HBO on OB cells in the first 3 days of the treatment then the effect

attenuated gradually.

HBO stimulates cell cycle progression to S and G2/M phase

The effects of HBO on the cell cycle progression were elucidated. Growth-arrested OB cells were stimulated by different conditions for 3 days and cell cycle profiles were obtained by flow cytometric analysis (Fig 2). In control group, the most of the cells (67.5%) remained in G1/G0 phase (Fig 2A). In the treated groups, especially the O group and P+O group, more cells entered S and G2/M phase than the control group(Fig.2B).Current data indicated that HBO and high oxygen saturation could prompt OB cells to entry cell cycle.

Cyclin D1 is an important regulator protein in the early G_1 phase of the cell cycle (20). D-type cyclins including cyclin D1, cyclin D2, and cyclin D3 are cell cycle regulators that promote progression through the early-to-mid G1 phase of the cell cycle (21). Our investigation was to try to determine whether hyperbaric oxygen influenced the level of cyclin D1 after growth-arrested OB cells were stimulated with hyperbaric oxygen for 3 days and 7 days. The expression of cyclin D1 protein increased most significantly in the P group and P+O group after hyperbaric oxygen stimulation for 3 days. (3days mean: control group v.s pressure group=0.13 v.s 0.51, P<0.05; control group v.s hyperbaric oxygen group =0.13 v.s 0.54, P<0.05). After treatment for 7 days, the expression of cyclin D1 of treated groups was still higher than that of the control group but the strength abated gradually. (7days mean: control group v.s hyperbaric oxygen group= 0.10 v.s 0.33, P<0.05) (Fig. 3). Overall, we concluded that HBO could promote OB cells to entry the cell cycle, especially in the first 3

days.

HBO promote expression of FGF-2

Many growth factors, including FGF-2, BMP-2, BMP-4, and VEGF, are involved in the regulation of osteoblast proliferation. These growth factors are produced by OB and other bone cells and have effects on OB proliferation and differentiation (22). To investigate whether HBO can stimulate the gene expression of these growth factors, we examined the mRNA expression of growth factors by RT-PCR.

There was no difference in the transcriptional expression of the BMP-2 m, BMP-4, and VEGF between control group and treated groups (data not shown). Using semi-quantities RT-PCR analysis, the expression levels of FGF-2 in O group and P+O group were significantly higher than in that of the control group (Fig 4)(3day mean: control group v.s oxygen group=0.14 v.s 1.01,$P<0.05$;control group v.s hyperbaric group=0.14 v.s 0.92,$P<0.05$). On the 7th day, the expression of FGF-2 in the P group caught up with the other two treated groups and continue to show significant predominance over the control group. These data indicated that FGF-2 might be an important growth factor in this system. To investigate which downstream of signal pathways the FGF-2 activated, we need to check multiple signaling pathways, including PKA, Akt, and MAP kinase pathway, to elucidate the effect of HBO in the OB cells.

Akt/p70^{s6k} *signaling pathway in hyperbaric oxygen-induced proliferation of osteoblasts*

We determined the expression of Akt and p70^{s6k} at the end of 3 days and 7 days treatment of different levels of pressure and oxygen saturation. In this study, on the 3rd day, the pressure group and the hyperbaric oxygen group induced the expression of Akt (3days mean, the control group v.s pressure group=0.73 v.s 1.21,P<0.05;control group v.s hyperbaric oxygen group =0.73 v.s 1.10, P<0.05). After 7 days of treatment, these three treated groups expressed Akt more significantly than the 3rd day, but the difference between these three groups was not obvious (7 days mean, control group v.s pressure group=0.67 v.s 1.02,P<0.01;control group v.s O group=0.67 v.s 0.96,P<0.01;control group v.s hyperbaric oxygen group =0.67 v.s 0.97, P<0.01)(Fig 5A).

The expression of p70^{S6K} in the P+O group was the most significant after 3 days of treatment. (3days mean, control group v.s hyperbaric oxygen group =0.37 v.s 0.82, P<0.01)(Fig. 5B). After 7 days of treatment, only the O group and the P+O group expressed more significantly than the control group (7 days mean: control group v.s O group = 0.48 v.s 0.79, P<0.05;control group v.s P+O group=0.48 v.s 0.70,P<0.05). These results indicated that hyperbaric oxygen might be able to increase the expression of cyclin D1 via the Akt/p70^{s6k} signaling pathway.

Phosphorylated ERK1/2 and NF-κB was significantly expressed in HBO induced OB proliferation

The MAPK pathway is also a major pathway in cell proliferation. On the 3rd day, the expression of phosphorylated ERK1/2 in the P+O group was even weaker than the control

group. However, on the 7th day, in all treated groups, the phosphorylat -ed ERK1/2 significantly increased (Fig 6A). Nevertheless, when compared with control groups, there was no significant difference among each treated groups. NF-κB is a target protein of Akt and MAPK, which was also activated in the treated groups, especially in the P+O group, either at the end of the 3rd day or on the 7th day(3 days mean, control group v.s hyperbaric oxygen group= 0.12 v.s 0.33,$P<0.05$;7 days mean, control group v.s hyperbaric oxygen group=0.15 v.s 0.61,$P<0.01$)(Fig. 6B).

According to these data, HBO might activate the FGF-2/MEK/ERK1/2/ NF-κB pathway to increase cell proliferation.

The phosphorylation of JNK and PKCα in hyperbaric oxygen-induced proliferation of osteoblasts

We also checked other downstream proteins of signaling pathways through which HBO might activate OB cells proliferation. Protein kinase A (PKA) and Protein kinase C (PKC) are two well-defined signal transduction pathways in osteoblast proliferation. They activate formation of 1,4,5-inositol trisphosphate, which stimulates a rise in intracellular free Ca^{2+} and related signaling events. PKA and PKC pathways can regulate transcription factors such cAMP response element binding proteins (CREBs), AP-1 family members and Runx2 (23). We also determined phosphorylation of PKCα by Western blotting. We found HBO could stimulate expression of PKCα (3 days mean, control group v.s. hyperbaric oxygen group =0.19 v.s 0.91, $P<0.01$)(Fig. 7A). In this study, the involvement of JNK in the proliferation

of signal transduction initiated by hyperbaric oxygen in OB cells was studied. The PKC may

be the intermediates in the activation of JNK during hypoxia-reoxygenation (24).

Thereafter, we determined the downstream kinase of PKC signal. JNK was found to be

a major MAPK whose activation upon hyperbaric oxygen stimulation is clearly (3 days

mean, control group v.s hyperbaric oxygen group= 011v.s 0.49,P<0.01). On the 7th day, the

expression of JNK in the treated groups was still stronger than that of the control group but

the difference between the treated groups attenuated.(7days mean, control group v.s

hyperbaric oxygen group =023 v.s 0.38,P<0.05)(Fig. 7B). Therefore, we found that JNK

might be the major downstream kinase of PKC signal in hyperbaric oxygen-induced

proliferation of OB cells.

DISCUSSION

The treatment of fractures is the most common orthopedic surgery. Different modalities have been used for fracture reconstruction with excellent results in most case, but delayed union and nonunion of fracture still remained a major complication.

The reason of delayed union and nonunion can be determined in most cases; it can be a certain type of open fracture, comminution of the fracture, and wound for satisfactory reduction or extension the original, etc (25). However, some factors of delayed union and nonunion in fracture remained unclear. It is believed that the proliferation and differentiation of osteoblast play a key role in the healing of fracture (26) while some unknown factors leaded to delay even stop the healing process totally.

Previous studies showed that the expression of growth factors mRNA returned to baseline prior to healing of fracture might contribute to the delayed union or nonunion (27). Therefore, it is important to activate and maintain the expression of growth factors during the healing period.

It has been well documented in past decades that HBO can increase the activity of OB cells *in vivo* in necrotic bone after irradiation, but not to the level in the normal bone. Other studies showed that angiogenic response can markedly increased by HBO (28), the activities of OB were prolonged at a high level in HBO treatment (29) and HBO can relieve the ischemia change of avascular necrosis of femoral head by enhancing the fibroblastic,

angioblastic, osteoblastic, and osteoclastic activities (30) .In all, HBO therapy is beneficial in the process of fracture healing.

We do note that some researchers in the past had concluded that HBO had no effect on OB proliferation in vitro and sometimes even resulted in apoptosis (31).

Similar results were also found in our earlier studies. However in this study, after modifying the tension of oxygen from 100% to 50%, the effect of HBO proved to be positive rather than cell-killing. The difference may be resulted from the excessive free radicals in the 100% oxygen environment. In fact, the oxygen tension in culture medium treated with 2.5ata and 50% O2 is more similar to the oxygen tension in human muscle (about 300 to 400 mm-Hg) under clinical HBO therapy in vivo.

Accord this cell cycle profile, it meant that HBO and high oxygen saturation may activate the arrested OB cells. HBO stimulated OB cells to entry cell cycle progression and increase cell proliferation.

Many growth factors control the process of osteogenesis .In the present study, we found that only FGF-2 was demonstrated in the HBO-induced proliferation of OB cells .It is well known that HBO will induced the FGF-2 expression in soft tissue healing but there is no similar study done in bone healing. FGF-2 is an important regulator of bone differentiation and growth in vivo. In vitro,FGF-2 could be produced by OB cells and is stored in the extracellular matrix(32,33),stimulating OB proliferation (34) and TGF-β production(35).The expression of FGF-2 will not only contribute to the soft tissue healing but also to the

angiogenesis (36) through further inducing the expression of VEGF by OB (37).The

cooperative interaction between vascular endothelial cells and osteoblasts is suggested in

recent studies in which it was demonstrated that several factors produced by endothelial cells

can affect osteoblast function and differentiation(38,39). It is advantageous to the generally

ischemic situation at the fracture sites.

The process of OB cell proliferation can be regulated via multiple different signal

transduction pathways. Results from our study show that HBO promote the proliferation of

osteoblast through at least two signaling pathways. The FGF-2/MEK/ERK (MAPK) pathway

may be the principal mechanism. Evidences suggested that signaling through MAPK pathway

is essential for the early stage of osteoblast differentiation (40,41). The following gene

expression of cascades required in the pathway was examined by a series of studies. The

significantly increased phosphorylated ERK ,P-38, Akt/p70^{s6k},and NF-κB confirmed the

priority of this pathway. The less prominent enhancement of phosphorylated PKC/JNK may

suggest a more accessory role of this pathway.

In our study, the partial pressure of oxygen in the P group and O group is the same(P

group:760mm-Hg $\times$ 20% $\times$ 2.5 ata=380 mm-Hg; O group:760mm-Hg $\times$50% $\times$ 1 ata=380

mm-Hg).Under similar partial pressure of oxygen, we can compare the influence between

pressure and oxygen. According to our results, we are unable to conclude which one of these

two factors play a more important role. On the other hand, the pressure and oxygen

combination did not seem to be synergistic in some expression of signal proteins. The optimal

treatment duration still remained uncertain. Some expression of signal protein decreased on the 7th day despite it reached a higher level on the 3rd day. It may indicate that over-treatment may suppress the expression of signal pathway.

In conclusion, we have tested and confirmed a novel concept: a combination of 50% oxygen exposure and 2.5 ata HBO could elicit the proliferation of OB *in vitro* mainly through the enhancing FGF-2 expression. Through activating FGF-2/MEK/ERK /Akt/P70^{S6K}/NF-κ B ,the process promotes HBO induced bone healing .

HBO also regulated the osteoblast by the PKA/PKC /JNK pathway. We postulated that osteoblast could possess the ability to respond to hyperoxia directly, which causes changes in cell signaling pathway involved in cell proliferation and growth factor production. By increasing the production of FGF-2 in osteoblast, the HBO also promote the angiogenesis, which is a prerequisite for bone formation and fracture healing (42). Though some systematic reviews failed to locate any relevant clinical evidence to support or refute the effectiveness of HBO for the management of delayed union or established non-union of bone fractures (43), these results suggest that it might be a **optimal** combination of oxygen concentration and pressure that can stimulate OB cells proliferation and osteogenesis. Further works are needed to define adequate conditions for clinical application in the future.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1A. HBO promote OB cells growth. The growth-arrested OB cells (cultured in serum-free medium for 48 hrs) were treated in various conditions twice daily. (**1**) Control group (C): cells were cultured under ambient oxygen (21% O2) and normal pressure (1ata). (**2**) Pressure (P) group: cells were treated with high pressure (2.5ata) twice daily. (**3**) High oxygen (O) group: cells were treated with high concentration oxygen (50%) twice daily. (**4**) Pressure and high oxygen (P+O) group: cells were treated with high pressure (2.5ata) and high concentration oxygen (50%) twice daily. The number of OB cells in treated groups significantly increased compared with the control group, esp. in the P+O group.

Figure 1B. Left: The rate of proliferation of cells was determined by XTT. The net proliferation rate (ΔOD) was determined(OD3 day$-$OD0 day). After treated in various O2 concentration and air pressure twice daily for three days, the cells of all treated groups had higher proliferation rate than the rate of the control group. Those figures showed that the cells in the P+O group, P group and O group had similar proliferative rate.

Figure 1B. Right: After 7 days of treatment, the OB cells of all treated groups still maintained the elevated proliferative rate compared to that of control group, but the effect attenuated gradually.

Figure 2. Growth-arrested OB cells were stimulated by different conditions for 3 days and cell cycle profiles were obtained by flow cytometric analysis. The treated groups, especially the O group and P+O group, had more cells to entry S and G2/M phase than in the control

group. Current data indicated that HBO could prompt OB cells to entry cell cycle.

Figure 3. The expression of cyclin D1 mRNA in growth-arrested OB cells increased after hyperbaric oxygen stimulation for 3 days, especially in the P group and P+O group. On the 7th day, the expression of cyclin D1 in the treated groups still maintained a higher level than that of the control group but their strength abated.

Figure 4. The effect of HBO on the production of growth factor by OB cells. Using semi-quantitive RT-PCR analysis, only the expression levels of FGF-2 in the treated groups were significantly higher than in the control group. These data indicated that FGF-2 might be an important growth factor in this system. The expression in the 7th day was more significant than those in the 3rd day.

Figure 5A. & 5B. The effect of HBO on expression of Akt and p70^{S6K} .HBO induced the expression of Akt and enhancing expression of p70^{S6K} . These results indicated that hyperbaric oxygen might be able to increase the expression of cyclin D1 via the Akt/p70^{s6k} signaling pathway.

Figure 6A .On the 3rd day, the expression of ERK1/2 in the control group was more obvious than that of the P+O group contrarily. However, on the 7th day, the phosphorylated ERK1/2 in the treated groups significantly increased when compared with control group.

Figure 6B. NF-κB is a target protein of Akt and MAPK, which was also activated in the treated groups, especially in the P+O group.

Figure 7A. In our study, HBO could stimulate expression of PKCα after the sample had

been treated for 3 days (3 days mean, control group v.s. hyperbaric oxygen group =0.19 v.s

0.91, P<0.01)

Figure 7B. JNK was found to be activated upon hyperbaric oxygen stimulation clearly on

the 3rd day (3 days mean, control group v.s hyperbaric oxygen group= 011v.s 0.49,P<0.01).

On the 7th day, the expression of JNK in the treated groups remained stronger than that of

the control group but the difference among the treated groups attenuated. (7days mean,

control group v.s hyperbaric oxygen group =023 v.s 0.38,P<0.05)