Effects of Hyperbaric Oxygen on Proliferation and Differentiation of Osteoblasts from Human Alveolar Bone

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Abstract

In view of the controversy of the clinical use of hyperbaric oxygen (HBO) treatment to stimulate fracture healing and bone regeneration, we have analysed the effects of daily exposure to HBO on the proliferation and differentiation of human osteoblasts *in vitro*. HBO stimulated proliferation when osteoblasts were cultured in 10% foetal calf serum (FCS), whereas an inhibitory effect of HBO was observed when cultures were supplemented with 2% FCS,. On the other hand, HBO enhanced biomineralization with an increase in bone nodule formation, calcium deposition and alkaline phosphatase activity, while no cytotoxic effect was detected using a lactate dehydrogenase activity assay. The data suggests that the exposure of osteoblasts to HBO enhances differentiation towards the osteogenic phenotype, providing cellular evidence of the potential application of HBO in fracture healing and bone regeneration.

Introduction

The healing of bony fractures is a complex and multifaceted process. However, extensive trauma, bone loss, unstable fixation, premature mobilisation, infection, extensive osteonecrosis and ageing are factors that may delay or even stop the healing [1]. The re-establishment of the structural integrity of the fractures is then a major challenge for surgeons world-wide.

Besides standard methods for treating delayed and non-union bone fractures such as bone grafting, internal and external fixation and electrical stimulation [1], hyperbaric oxygen (HBO) therapy, which typically involves the administration of 100% oxygen at atmospheric pressures greater than one atmosphere absolute (ATA), has been proposed as an adjunctive therapy to improve the outcomes of patients suffering from bone fractures [2-4], osteoradionecrosis [5-7], distraction osteogenesis [8, 9], as well as of patients with bone grafts [2, 10] and dental implants [11]. Even though, animal studies showed that HBO can be used to treat delayed fracture healing [12-14] or an established non-union of a bony fracture [12, 15, 16]. The clinical application of HBO is still subject of debate [12, 17-19] due to the lack of *in vitro* studies and large randomised controlled trials to demonstrate its effect on osteoblast activity [20].

The effects of HBO on different cell types have been reported previously. To evaluate the potential role of HBO in skin wound healing, its effects on human dermal fibroblasts have been investigated and a stimulatory effects of HBO were reported [21, 22]. Rat hepatocytes have been used to study HBO treatment on primary liver nonfunction [23] and benign and malignant mammary epithelial cells have been investigated to elucidate the inhibitory role of HBO in tumor growth. [24] To understand the cellular mechanisms of the observed therapeutic effects of HBO on fracture healing, this study investigated the effects of HBO on the proliferation and differentiation of human osteoblasts *in vitro* using a laboratory-scale hyperbaric unit.

Materials and Methods

Isolation and culture of osteoblast from human alveolar bone

Human osteoblasts were isolated from alveolar bone as described previously [25, 26]. Briefly, normal human alveolar bone specimens, obtained from consenting healthy young orthodontic patients (13-19 years old) with institutional ethics committee approval, were used as explants for establishment of cell cultures. The cells obtained were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen Corporation, Melbourne, Australia) supplemented with 10% foetal calf serum (FCS, HyClone, Logan UT) and 1% penicillin/streptomycin (GIBCO, Invitrogen Corporation, Melbourne, Australia) in a standard humidified incubator at 37°C containing 5% CO_2/95% atmospheric air. The cells were subcultured and characterized by morphological and functional criteria of osteogenic differentiation potential. Fourth to sixth generation cultures were used in this study.

Hyperbaric oxygen treatments

Osteoblast cultures were treated daily for up to 10 days in a temperature and humidity controlled custom-made 7-litre hyperbaric unit (Fink Engineering, Cheltenham, VIC, Australia). The metal chamber was sealed and flushed for 2 minutes with oxygen and the pressure was subsequently increased to 1.5 or 2.4 atmosphere absolute (ATA). The pressure was maintained for either 30 or 90 minutes, after which the chamber was slowly de-pressurized over 5 minutes.

Proliferation assay

Osteoblasts, prepared from human alveolar bone as described above, were seeded in 24 well plates (2500 cells/well) in 1 ml of culture medium supplemented with either 2% or 10% FCS. After the cells were allowed to adhere for 24 hours, plates were treated daily in the hyperbaric chamber with 100% oxygen at 2.4 ATA for 90 (2.4A90M) or 30 minutes (2.4A30M) or at 1.5 ATA for 90 minutes (1.5A90M) or 30 minutes (1.5A30M) for up to 10 days. Control samples were incubated in a standard humidified incubator at 37°C containing 5% CO_2 and 95% atmospheric air. Proliferation over the 10 days of culture was evaluated by WST-1 assay (Roche Applied Science, Penzberg, Germany) [27]. Briefly, 10 hours after HBO treatment, 100 μl of WST-1 reagent was added to each well containing 1 ml of culture media and incubated for 4 hours at 37 ^{0}C and 5% CO_2. The colour reaction was measured at 440 nm using a plate reader. The reference wavelength for the absorbance was set at 600 nm. A standard curve was used to calculate the actual cell numbers.

Evaluation of differentiation

Osteoblasts were cultured for 3 days in 96 well plates (seeding density: $1x10^4$ cells/well), after which the culture medium was changed to osteogenic medium (DMEM supplemented with 10% FCS, 1% penicillin/streptomycin, 50 μg/ml ascorbic acid (Sigma-Aldrich, USA), 10 mM β-glycerophosphate (Sigma-Aldrich, USA), and 10 μM dexamethasone (Sigma-Aldrich, USA). Subsequently, cultures were exposed daily to HBO (2.4A90M or 1.5A90M) for up to 13 days. To evaluate calcium deposition, cultures were washed 3 times with PBS without calcium and magnesium, treated with 0.6 N HCl (200 μl per well) and 10 μl of sample was added into 300 μl calcium reaction buffer (Sigma Diagnostic Calcium Procedure, Sigma, USA) in a 96-well plate. The color reaction was measured using an ELISA plate reader at a wavelength of 575 nm after 5 minutes of incubation.

Alkaline phosphatase activity (ALP) was measured at days 5, 7 and 13 following the manufacturer's instructions (Sigma Diagnostic ALP Procedure, Sigma, USA). Briefly, 200 μl of pre-warmed (30 °C) ALP reagent was added to a 20 μl sample, mixed and incubated for 30 seconds. The color reaction was measured for the initial absorbance at 405 nm. After continuing incubation at 30 ^{0}C for another 2 minutes following the initial reading, the sample was measured for the final reading. ALP activity was determined by measuring the changes of the absorbance at 405 nm over 2 minutes.

Mineralization was also monitored using von Kossa staining. Briefly, cell culture plates were washed with distilled water and flooded with 5% silver nitrate solution. The plates were placed in bright light for 60 min. Subsequently, the plates were rinsed 3 times with distilled water and 5% sodium thiosulphate was added. After an incubation of 5 minutes at room temperature, the plates were washed with distilled water. The cultures were finally photographed using a digital camera (Nikon Coolpix 4500; Maxwell Optical, Lidcombe, NSW, Australia) mounted on a microscope.

Cell membrane integrity assay

To evaluate the cytotoxicity of the exposure to HBO (2.4A90M or 1.5A90M), cells were cultured in 24 well plates at a cell density of 2500 cells/well in medium supplemented with either 10% FCS or 2% FCS. Membrane integrity of cells after HBO treatment was measured by the amount of lactate dehydrogenase (LDH) leakage into the medium using LDH based *in vitro* toxicology assay kit (Sigma, Missouri). Briefly, 200 μl of cell supernatant was transferred to a clean flat-bottom plate and 100 μl of LDH assay mix was added. The plate was covered with aluminium foil and incubated for 20-30 min. The reaction was stopped by adding 30 μl of 1N HCl and the absorbance was measured at a wavelength of 490 nm.

Statistics

Data are shown as mean ± standard deviation. To compare the differences between HBO-treated and control samples multi-way ANOVA and a Student-Newman-Keuls posthoc test was performed using the statistical package SPSS v14 (Chicago, IL). The level of significance was set at $p \leq 0.05$.

Results

HBO treatment promotes proliferation of osteoblasts in the presence of 10% FCS

To assess the effect of HBO on cell proliferation, osteoblasts were cultured in 10% FCS for 24 hours, and then treated with HBO using 4 treatment conditions (2.4 ATA for 90 minutes (2.4A90M), 2.4 ATA for 30 minutes (2.4A30M), 1.5 ATA for 90 minutes (1.5A90M), or 1.5 ATA for 30 minutes (1.5A30M)). Cell proliferation was evaluated daily by a WST-1 assay for 10 consecutive days (Figure 1a). Cell number was significantly higher (p=0.031) for all HBO treated cultures compared to the untreated controls at day 3. Similarly, at day 4, cell number was significantly higher for all HBO treated cultures (except the 1.5A30M group) compared to the control (p=0.002). However, at day 6, only the cell number for the 2.4A30M and 1.5A90M HBO treated groups were significantly higher than the controls (p=0.024). Finally, at days 8 and 10, cultures reached confluence and no difference in cell number was noted between the treated and untreated groups. Without oxygen, air pressure at either 1.5A90M or 2.4A90M did not induce any significant change in cell number compared with control cultures (Figure 1b). To assess whether the HBO treatments applied in our experiments induced any cytotoxic effect, cell membrane integrity was studied using LDH leakage assay before and after HBO treatment. No significant increase of extracellular LDH activities was detected after HBO treatments, which indicated no change in cell membrane integrity before or after HBO treatments in all treatment groups supplemented with 10% FCS (Figure 1c).

HBO treatment inhibits proliferation of osteoblasts in the presence of 2% FCS

In order to evaluate the influence of serum factors on the HBO-mediated proliferation, proliferation experiments were carried out using culture medium supplemented with 2% FCS. A decrease in cell number was observed after HBO treatment (Figure 2a). At days 3 and 4, HBO groups 2.4A90M and 1.5A90M had significantly lower cell numbers compared to the untreated controls (p=0.0089), whilst at days 6, 8 and 10, all HBO treated groups showed a significantly lower cell number compared to the control group (p=0.017) (Figure 2a). Interestingly, hyperbaric air also induced a significant decrease in cell number from day 5 onward in 2% FCS culture condition (p=0.032) (Figure 2b). The cell membrane integrity study indicated that no significant increase in LDH activity was noted after HBO treatment in 2% FCS cell culture condition, which indicated the inhibitory effect of HBO on proliferation in 2% FCS-supplemented culture medium could not directly be related to cytotoxicity of the HBO treatment (Figure 2c).

To further demonstrate that the upregulation of osteoblast proliferation after HBO treatment in 10% FCS and downregulation of cell proliferation after HBO treatment in 2% FCS, the results of the HBO treatment (1.5A90M) in 10% and 2% FCS are shown in Figure 3. Compared the control groups cell proliferation was significantly higher in 10% FCS than 2% FCS after 3 days (p=0.015). Interestingly, cell proliferation observed in 10% FCS cultures after HBO treatment was highest, while the lowest cell growth rate was observed in 2% FCS cultures after exposure to HBO.

HBO treatment stimulates early osteogenic differentiation

Under osteogenic conditions, the level of calcium deposition was significantly higher for HBO-treated cultures after 3 days compared to the untreated controls (p<0.001) (Figure 4a). Calcium deposition was not detectable within the control cultures until 6 days of differentiation culture. In contrast, significant calcium deposition was observed after 3 days of HBO treatments and the calcium deposited was consistently higher in HBO treatments as was evaluated for up to 19 days. No difference was detected in the amount of calcium deposition between the treatments of hyperbaric air and the untreated control group (Figure 4b), which indicated that pressure alone (hyperbaric air) did not enhance osteogenetic mineralization (Figure 4b). In addition, ALP activity, a biomarker for osteogenic differentiation, was also significantly increased within the HBO-treated cultures (Figure 5).

The effect of HBO on osteogenetic differentiation of osteoblasts was also assessed by bone nodule formation using von Kossa staining. Bone nodules were observed after 7 days of HBO treatment, whereas in the non-treated cultures no clear bone nodule could be detected until day 12. Overall, increased numbers of nodules as well as increased nodule size were found present within the HBO-treated cultures compared to the untreated controls (Figure 6).

Discussion

In the present study, osteoblasts derived from alveolar bone were used to study the effects of the exposure to HBO on cell proliferation and osteogenic differentiation *in vitro*. The results showed that HBO treatment stimulated cellular proliferation when osteoblasts were cultured in the presence of 10% foetal calf serum (FCS),.However, when the concentration of FCS was lowered to 2% an inhibitory effect of HBO on proliferation was observed. Nevertheless, no direct cytotoxic effects were detected by means of a LDH activity assay. Furthermore, the results demonstrated that HBO enhanced differentiation, which was associated with increased bone nodule formation, calcium deposition and alkaline phosphatase activity.

Previously, variable effects of HBO on cell proliferation have been reported. For example, whilst a stimulatory effect of HBO has been reported for the growth of human skin fibroblasts [21, 22] hepatocytes [23] and endothelial cells [28, 29], an inhibitory effect has been described for lymphocytes [30], promyelocytic leukemic HL60 cells [31] and benign and malignant mammary epithelial cells [24]. Our results demonstrated an initial significant stimulatory effect of daily HBO treatments on osteoblast proliferation after 3 days of culture. Interestingly, an inhibitory effect of HBO on cell proliferation was noted, which appeared to be dose-dependent, when the FCS concentration was lowered to 2%. Although the exact role of FCS in the cellular response to HBO is not clear, its protective role from the oxidative environment has been previously described [32]. In addition, the fact that cells are more sensitive to environmental changes in low serum conditions may have contributed to these responses. Importantly. this underlines the need of careful selection of culture system and conditions when evaluating the effects of HBO *in vitro*, since it will impact on the final observations [33]

LDH assay has been used to assess cytotoxic effect by measuring the cell membrane integrity [34]. LDH activity of the culture media did not reveal cytotoxic effects of HBO regardless of the culture condition. However, it has been demonstrated that a prolonged exposure to HBO at 2.5 ATA decreases cell proliferation as a result of increased apoptosis [22, 35]. It remains unclear, however, whether in the 2% FCS culture condition, the inhibitory effect of HBO on osteoblasts proliferation is due to an increased cell damage or a decrease in DNA and protein synthesis.

Early studies demonstrated the relationship between the oxygen concentration and bone formation in the 1960s [36]. It was also observed that low oxygen culture conditions (5% oxygen) or hypoxia conditions resulted in cartilaginous matrix synthesis, and high oxygen conditions (35% oxygen) induced mesenchymal tissue differentiation towards bone. Additional studies have also demonstrated the regulating role of oxygen in bone remodelling by directly affecting collagen synthesis, ALP activity, and the production of transforming growth factor-beta (TGF-beta) in fracture sites [37, 38]. Our study demonstrated daily exposure to HBO promoted osteogenic differentiation in cellular level, which was associated with an increase in bone nodule formation, calcium deposition and ALP activity.

Although the responses of osteoblasts to hypoxia have been well documented [37-40], the direct effects of HBO on human osteoblasts have, to the best of our knowledge, not been investigated previously, despite the indications that HBO can improve fracture healing [3, 7, 41, 42]. A considerable number of studies have, on the other hand shown enhanced osteogenic activity as a result of HBO treatment. For example accelerated levels of bone morphogenetic proteins [43], earlier union of autologous bone grafts [10], and improved bone formation in titanium implants [44] were observed *in vivo* after exposure to HBO. This requirement for oxygen during healing is the rationale underlying HBO therapy and generally, it is assumed that HBO stimulates the ingrowth of blood vessels, resulting in increased blood supply and consequently in enhanced bone formation [45]. Our study indicates that HBO also stimulates initial proliferation and directly enhances osteogenic differentiation of osteoblasts, as was assessed by calcium deposition, bone nodule formation and ALP activity.

The pathophysiological mechanisms underlying the mitogenic and differentiative effects of HBO remain to be elucidated, hampering the full exploitation of its therapeutic potential of HBO therapy. Enhanced autocrine production of growth factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and TGF-beta1, as a result of exposure to HBO [21] has been proposed as a reason for mitogenic effects observed. The mechanism of oxygen tension on cell differentiation has been proposed to be related with Smad and p38 MAPK pathway and through the regulation of transcriptional activities of Sox9 and Runx2 [46]. Furthermore, the nitric oxide (NO) levels in tissues and the bone marrow has also been increased in the HBO treatment due to the stimulation of nitric oxide synthesis [29], which has been demonstrated to, in turn, mobilize stem/progenitor cells and endothelial progenitors [29, 47].

Despite the limitations of 2-dimentional cell culture models, including the unnatural geometric and mechanical constraints imposed on cells [48], they have demonstrated to be a valuable research tool for investigating the responses of osteoblasts to the exposure to HBO. However, future studies will employ 3-dimensional models to further elucidate the effects in a more physiological environment and to identify potential beneficial effects for the development of tissue-engineered grafts.

In summary, the results from our current study provide direct cellular evidence of the effects of HBO on osteogenesis, further supporting its use as an adjunctive clinical treatment to promote bone fracture healing and bone regeneration. However, the underlying mechanism of the stimulatory responses to HBO and the potential benefit for the development of tissue-engineered bone grafts requires additional investigation.

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Legends

Figure 1: Effect of elevated oxygen levels and pressure on proliferation of osteoblasts cultured in 10% FCS. Human osteoblasts derived from alveolar bone were cultured in 10% FCS for 24 hrs and subsequently treated with (A) HBO (2.4A90M, 2.4A30M, 1.5A90M, or 1.5A30M) or with (B) pressure (2.4A90M or 1.5A90M). Compared with the untreated control group, cell number was significantly increased under HBO treatment. When cultures were treated with pressure alone, there was no significant effect on cell proliferation. (C) Extracellular LDH activity before and after HBO treatment was not significantly affected. *= significantly different from control ($p<0.05$).

Figure 2: Effect of elevated oxygen levels and pressure on proliferation of osteoblasts cultured in 2% FCS. Human osteoblasts derived from alveolar bone were cultured in 2% FCS for 24h and subsequently treated with (A) HBO (2.4A90M, 2.4A30M, 1.5A90M or 1.5A30M) or with (B) pressure (2.4A90M 0r 1.5A90M). Compared with the untreated control group, cell number was significantly decreased under HBO treatment When cultures were treated with pressure alone, an inhibition of cellular proliferation was observed. (C) Extracellular LDH activity before and after HBO treatment was not significantly affected. *= significantly different from control ($p<0.05$).

Figure 3: Comparison of the effect of elevated oxygen level and pressure on proliferation of osteoblasts cultured in 2% and 10% FCS. Human osteoblasts derived from alveolar bone were cultured for 24h and subsequently treated with HBO (1.5A90M).

Figure 4: Effect of elevated oxygen levels and pressure on calcium deposition by osteoblast cultured under osteogenic conditions. Osteoblasts were treated with (A) HBO (2.4A90M or

1.5A90M) or with (B) pressure (2.4A90M or 1.5A90M). Significant increase in calcium deposition was detected under HBO treatment compared with the untreated control group, whereas pressure alone did not have any significant effects on calcium deposition.. *= significantly different from control (*p*<0.05).

Figure 5: Effect of the exposure to HBO on the expression of ALP in osteoblasts. Osteoblasts cultured under osteogenic conditions were treated with HBO and the extracellular ALP activity was measured at days 5, 9 and 12. Significant increase in ALP activity was detected as a result of HBO treatment compared with the untreated control group. *= significantly different from control (*p*<0.05).

Figure 6: Effect of the exposure to HBO on bone nodule formation. Osteoblasts cultured under osteogenic conditions were treated with HBO and bone nodule formation was assessed by von Kossa staining at days 7 and 12. At day 7 bone nodules were only detectable in HBO treatmented cultures. At day 12 bone nodules were obvious in all cultures, however they appeared larger in the HBO treated cultures..

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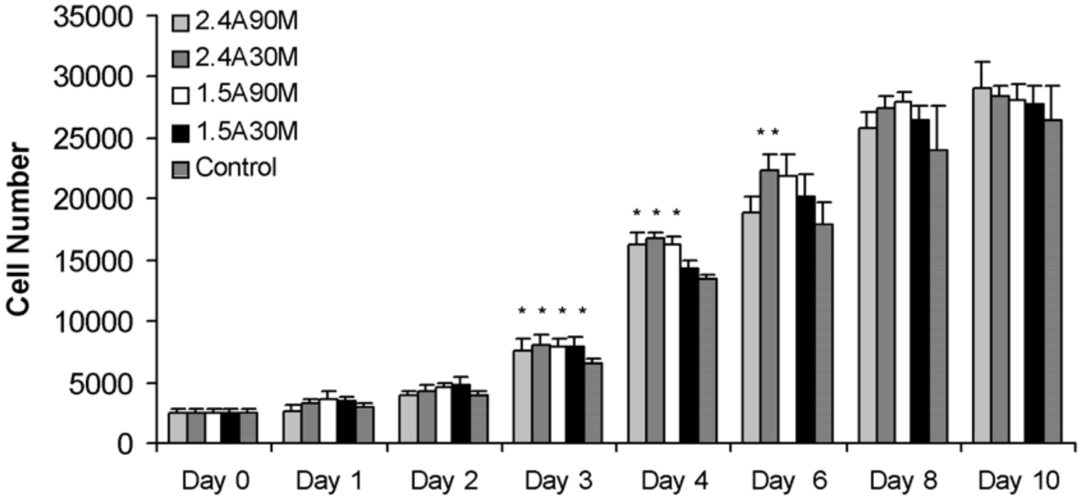
Figure 1
(a)


2.4A90M
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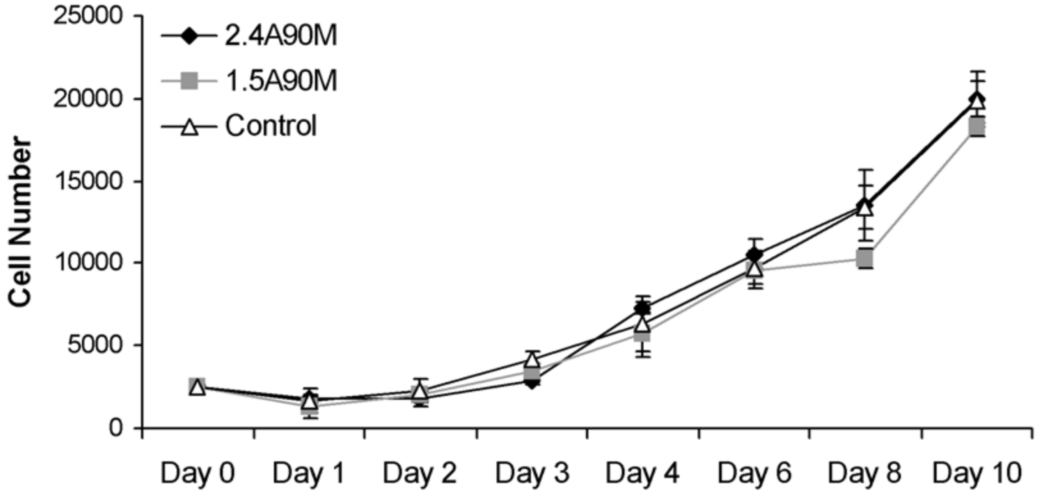
Day 8
Day 10

(b)


2.4A90M
1.5A90M
Control
Cell Number
25000
20000
15000
10000
5000
0
Day 0
Day 1
Day 2
Day 3
Day 4
Day 6
Day 8
Day 10

(c)

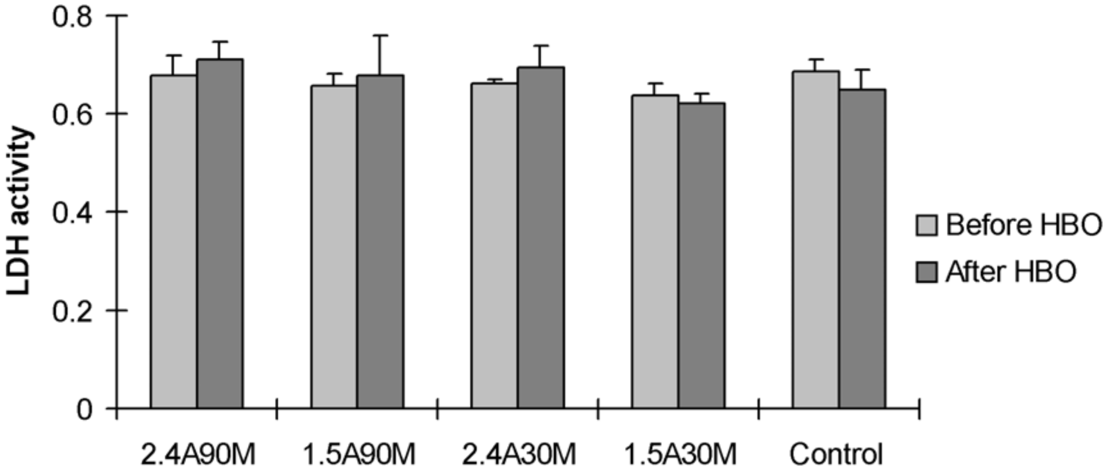
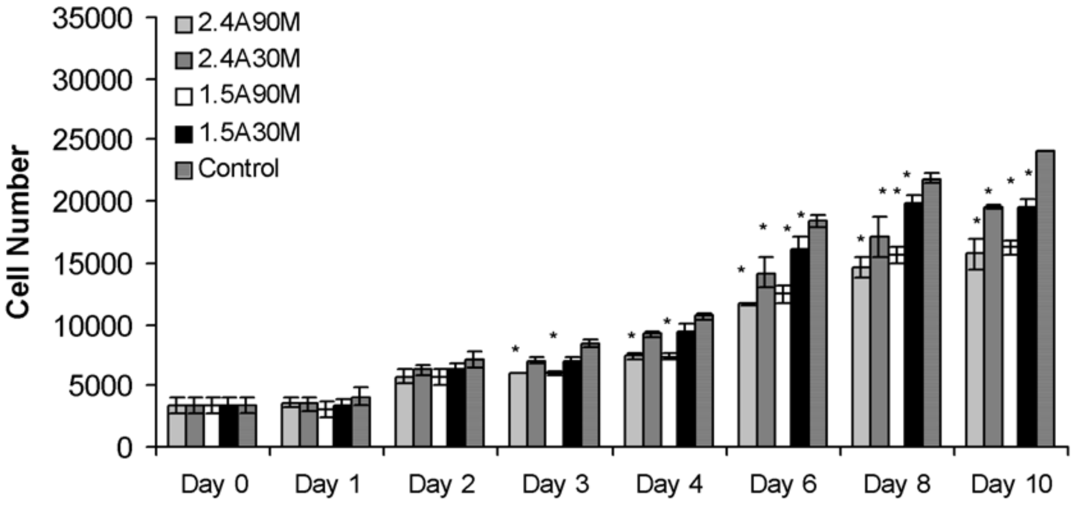

LDH activity
0.8
0.6
0.4
0.2
0
Before HBO
After HBO
2.4A90M
1.5A90M
2.4A30M
1.5A30M
Control

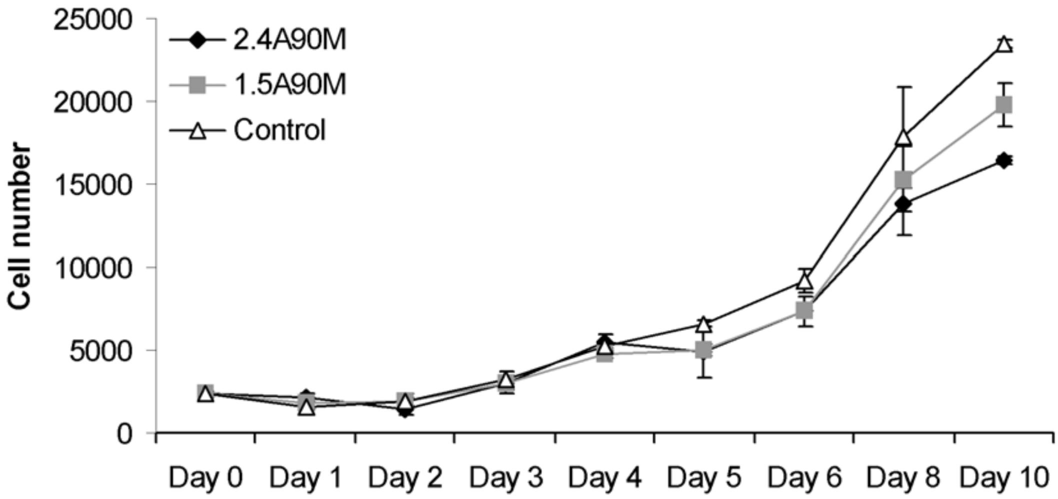
Figure 2

(a)



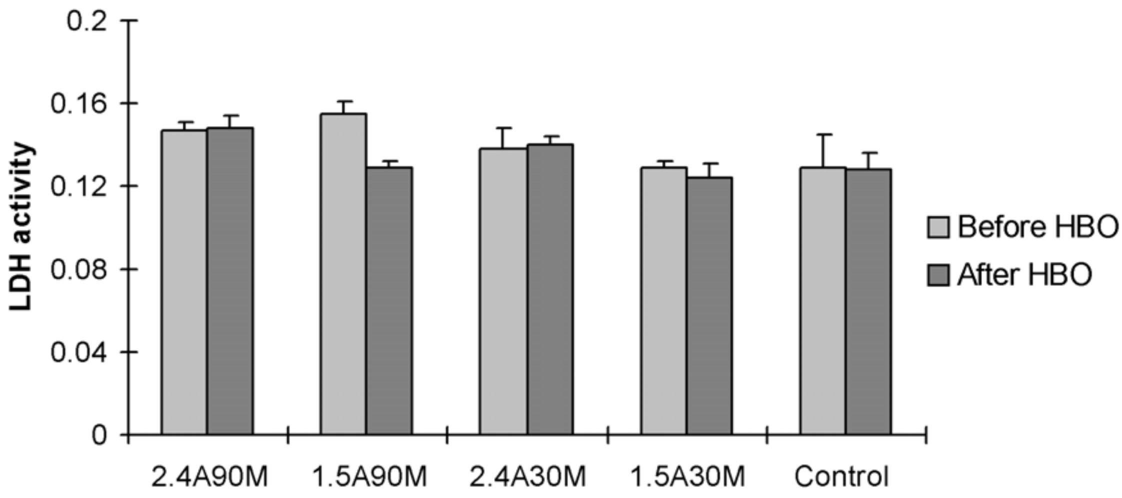
35000
30000
25000
20000
15000
10000
5000
0
Cell Number
2.4A90M
2.4A30M
1.5A90M
1.5A30M
Control
Day 0
Day 1
Day 2
Day 3
Day 4
Day 6
Day 8
Day 10

(b)



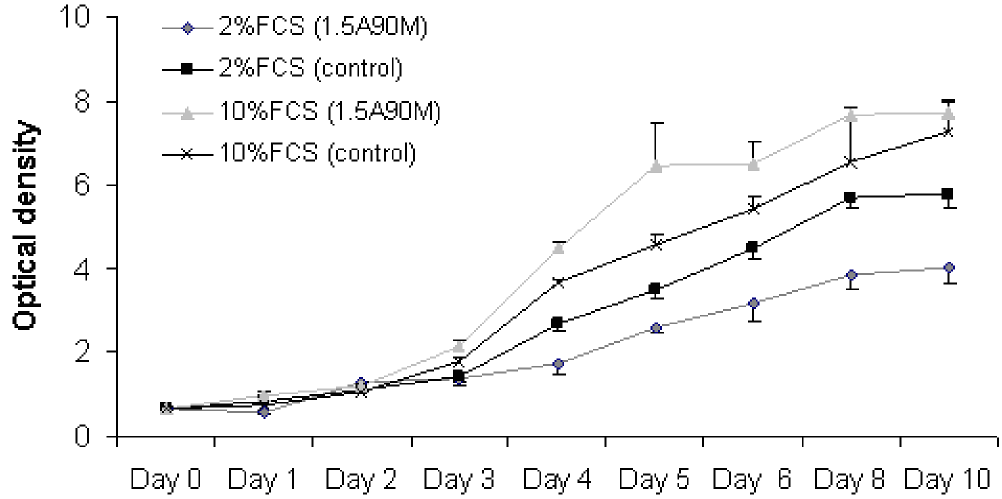
25000
20000
15000
10000
5000
0
Cell number
2.4A90M
1.5A90M
Control
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 8
Day 10

(c)



0.2
0.16
0.12
0.08
0.04
0
LDH activity
Before HBO
After HBO
2.4A90M
1.5A90M
2.4A30M
1.5A30M
Control

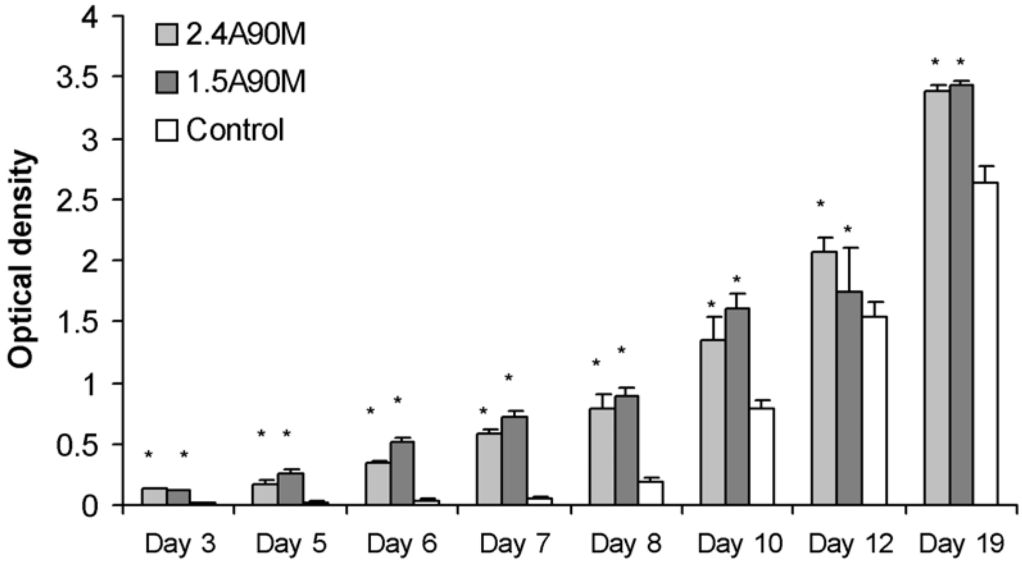
Figure 3



10
8
6
4
2
0
Optical density
2%FCS (1.5A90M)
2%FCS (control)
10%FCS (1.5A90M)
10%FCS (control)
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 8
Day 10

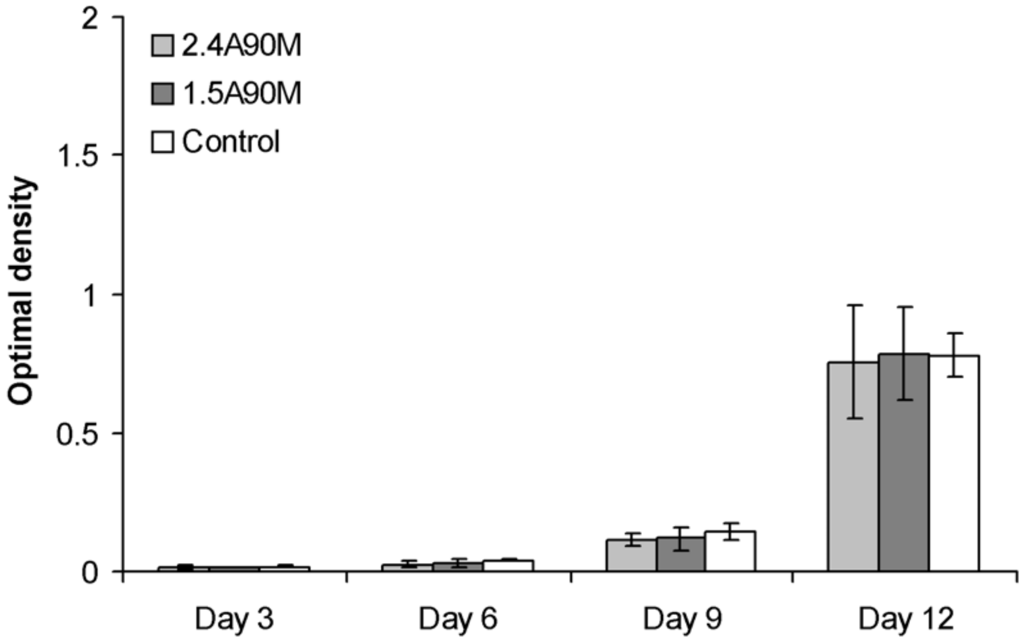
Figure 4

(a)



2.4A90M
1.5A90M
Control
Optical density
4
3.5
3
2.5
2
1.5
1
0.5
0
Day 3
Day 5
Day 6
Day 7
Day 8
Day 10
Day 12
Day 19

(b)



2.4A90M
1.5A90M
Control
Optimal density
2
1.5
1
0.5
0
Day 3
Day 6
Day 9
Day 12

Figure 5

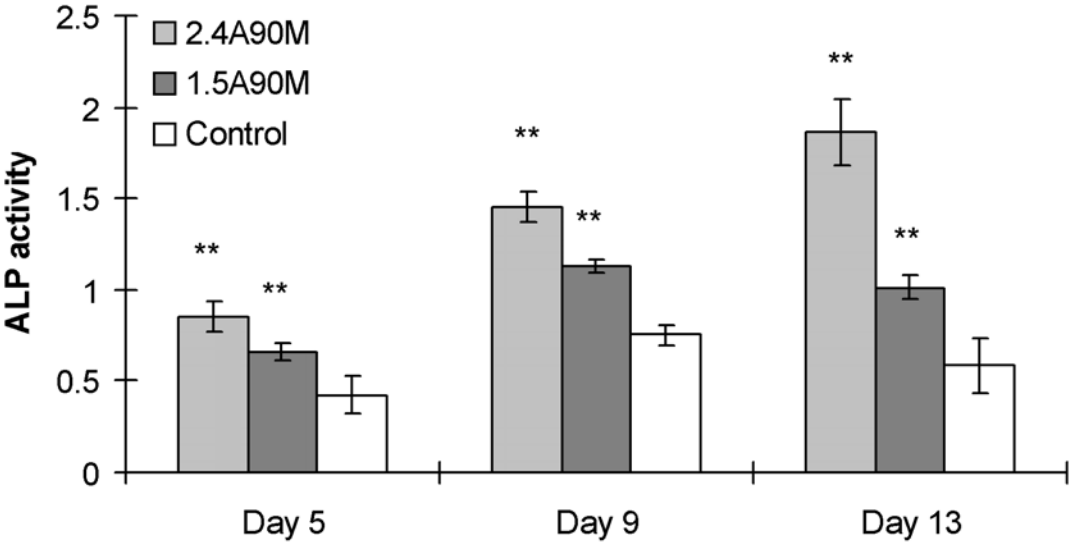

2.4A90M
1.5A90M
Control
ALP activity
2.5
2
1.5
1
0.5
0
**
**
**
**
**
**
Day 5
Day 9
Day 13

Figure 6