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Influence of high-concentration hyperbaric oxygen therapy on bone metabolism

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Summary

Objectives: To learn how high concentration in hyperbaric oxygen therapy (HBO) acts on the expression of genes related to bone metabolism in osteoblast cell lines and human trabecular bone.

Material and methods: The differential expression of several genes related to bone metabolism (SOST, RUNX2, MMP14, OPG, HIF-1 α and SIRT1) in two human osteoblastic cell lines (Saos and Super-Saos) and in human trabecular bone fragments subjected to one, three or five HBO sessions (90 minutes, 100% oxygen; 2.3 atmospheres). In each experiment, a control that did not receive HBO was used.

Results: We did not find significant differences after HBO in the expression of the genes studied, neither in the cells nor in trabecular bone. Only in the Super-Saos cell line the expression of OPG after 5 sessions of HBO decreased 6 times with respect to that of the control group ($2^{-\Delta\Delta Ct}$ of 72; $p=0.01$).

Conclusions: High concentration oxygen in the hyperbaric chamber (HC) does not seem to influence the expression of genes related to bone metabolism.

Key words: oxygen, hyperbaric chamber, bone, genes.

INTRODUCTION

Oxygen is required to produce cellular energy and is involved in numerous processes, such as enzymatic activation, molecular signaling and regulation of gene expression¹. Also in angiogenesis, the maintenance of hematopoietic stem cells and bone formation². In fact, changes in the partial pressure of oxygen can influence the function of osteoblasts and osteoclasts³. In hypoxia, bone formation and mineralization decreases, while resorption increases⁴⁻⁶. In the opposite direction, hyperoxia could have a beneficial effect on the bone. Treatment with high concentration of oxygen in the hyperbaric chamber has proven useful in osteomyelitis and osteonecrosis of the jaw caused by radiotherapy or by the use of bisphosphonates⁷⁻⁹. HC accelerates osteogenic differentiation of mesenchymal cells and decreases the activation of osteoclasts¹⁰⁻¹².

In this work we wanted to analyze the actions of oxygen at high concentration in HBO on the expression of genes related to bone metabolism in osteoblastic cell lines and human bone^{5,6,13,14}.

MATERIAL AND METHODS

Cell lines

Two osteoblastic cell lines, Saos-2 and Super-Saos, were used. Saos-2, derived from a human osteosarcoma. Super-Saos is a line generated in our laboratory, derived from the previous one and with a high capacity to express the sclerostin gene (SOST)¹⁵. Both lines were grown in T25 bottles with 5 ml of DMEM culture medium (Dulbecco's modified Eagle culture medium) plus 1% P/S (penicillin-streptomycin) and 1% amphotericin B, and stored in an incubator at 37°C for one week, changing the culture medium every 4 days to cover between 60-80% of the sur-



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face of the bottle. The plates were introduced into the HC (Galeazzi, Italy; 100% oxygen; 2.4 atmospheres) for 90 minutes per session receiving one, three or five consecutive sessions (Figure 1). The same cell line was used as control group subjected to identical culture, transport and handling conditions, but without undergoing HBO.

Bone fragments

Trabecular bone fragments extracted from the femoral head of patients with osteoporotic fracture hip replacement surgery were used. After extraction, the bone fragments received a single session of HC (Galeazzi, Italy; 100% oxygen; 2.4 atmospheres) for 90 minutes and subsequently frozen at -70°C . Bone fragments subjected to the same conditions of conservation, culture, transport and handling were used as controls but without receiving HC. This experiment was approved by the Clinical Research Ethics Committee (CEIC) of Cantabria. All patients gave informed consent.

RNA extraction and quantification

24 hours after the last HBO session, RNA was extracted, both in the cell lines and in the bone. In the homogenization process in cell lines, the samples were washed with phosphate buffered saline (PBS) prior to the use of TRIzol[®]. In the case of bone fragments, TRIzol[®] was also used, as well as homogenization for 20-30 seconds until the sample was pulverized, and subsequently centrifuged. In both cases the manufacturer's recommendations were followed and the RNA separation, precipitation and resuspension process continued.

Quantitative RT-PCR (polymerase chain reaction with reverse transcriptase) was carried out to detect gene expression: SOST (sclerostin gene), RUNX2 (protein related to transcription factor 2), MMP14 (metalloproteinase 14), HIF-1 α (hypoxia-inducible factor), SIRT1 (sirtuin1), OPG (osteoprotegerin) and RANKL (kappa-nuclear nuclear factor receptor activator ligand) using Taqman assays and following the manufacturer's instructions. The threshold cycle (Ct) values were obtained and the data normalized to the expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and TBP (TATA box binding protein) using the ΔCt method. To calculate the relative level of mRNA, the formula $2^{-\Delta\text{Ct}}$ was used, where ΔCt is the difference between the Ct average of the normalizing genes and the Ct of the gene of interest.

Statistic analysis

We used a non-parametric test, the Wilcoxon test for the comparison of means of two matched groups. Values of $p < 0.05$ were considered statistically significant.

RESULTS

HBO effect on RNA expression in the Saos-2 cell line

There were no differences in the expression of genes in the

Figure 1. Hyperbaric chamber



cell line after one, three or five HBO sessions. The differences with respect to the control in $2^{-\Delta\text{Ct}}$ after 5 sessions were 0.71 for SOST ($p=0.50$), 0.89 for SIRT1 ($p=0.34$), 0.47 for MMP14 ($p=0.18$), 0.43 for HIF1 α ($p=0.18$), 0.79 for RUNX2 ($p=0.65$) and 7.91 for OPG ($p=0.40$) (Figure 2). No RANKL expression was detected.

Effect of HBO on RNA expression in the Super-SaOS cell line

Compared to the control, we found OPG expression decreases 6 times after 5 HBO sessions ($2^{-\Delta\text{Ct}}$ Ct, 72 $p=0.01$). In the rest of the genes there were no differences: $2^{-\Delta\text{Ct}}$ Ct, from 1.03 for SOST ($p=0.34$), 1.46 for SIRT1 ($p=0.34$), 1.77 for MMP14 ($p=0.18$), 1.08 for HIF1 α ($p=0.18$), 1.14 for RUNX2 ($p=0.18$) and 1.24 for RANKL ($p=0.31$) (Figure 3).

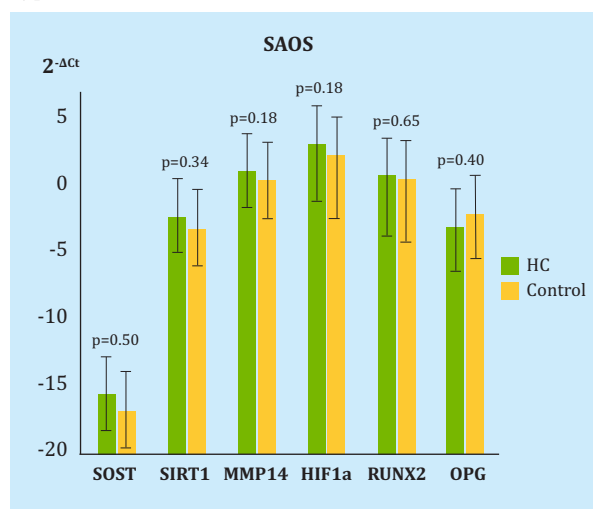
HBO effect on RNA expression in trabecular bone

Nor were there differences in the expression of genes after HBO in bone, only a modest, non-significant increase in the expression of SOST with a $2^{-\Delta\text{Ct}}$ Ct change of 5.39 ($p=0.48$). In the rest of the genes the differences were 0.92 for MMP14 ($p=0.58$), 1.28 for HIF1 α ($p=0.81$), 0.72 for RUNX2 ($p=0.24$), 1.18 for SIRT1 ($p=0.42$), 1.97 for RANKL ($p=0.91$) and 3.9 for OPG ($p=0.55$) (Figure 4).

CONCLUSIONS

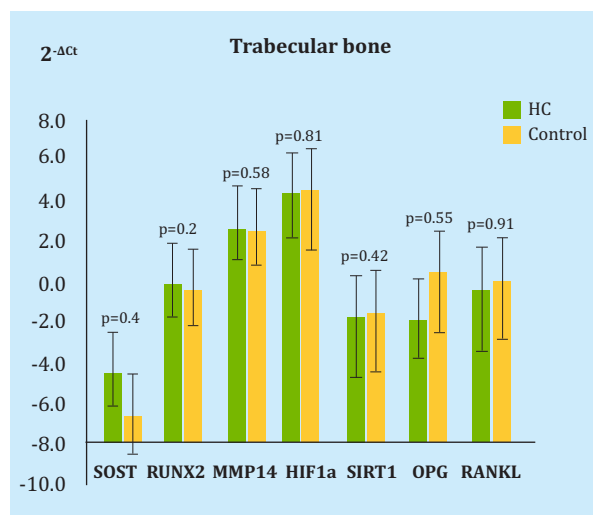
Hyperoxia is considered beneficial for bone by increasing the proliferation and differentiation of osteoblasts¹⁶. Al Hadi et al.⁶ described increased expression of type I collagen and Runx-2 mRNA in osteoblast cell lines (Saos-2) subjected to HBO for 14 days (2.4 ATA, 97% O₂, 90 min/day). HBO also increased the proliferation and differentiation of osteoblasts in human alveolar bone¹⁷. Hyperoxia also seems to decrease bone resorption. Treatment in HC (100% O₂, 2.4 ATA) reduced the expression of RANK, NFATc1 and Dc-STAMP in the serum of patients and also regulated the expression of the hypoxia inducible factor (HIF-1 α)¹⁸. Other described actions of oxygen at high concentration (100% O₂, 2.4 ATA) are the improve-

Figure 2. Difference in the expression of genes under study and housekeeping in the SAOS cell line after 5 sessions of hyperbaric chamber



HC: cell group undergoing hyperbaric oxygen therapy; SOST: sclerostin; SIRT1: sirtuin1; MMP14: metalloproteinase 14; HIF-1α: hypoxia inducible factor 1α; RUNX2: protein related to transcription factor 2; OPG: osteoprotegerin.

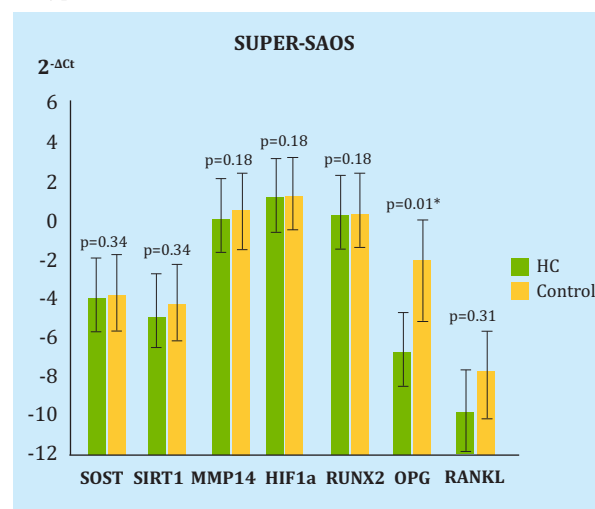
Figure 4. Difference in the expression of genes under study and housekeeping in bone after a single hyperbaric oxygen therapy session



HC: cell group undergoing hyperbaric oxygen therapy; SOST: sclerostin; SIRT1: sirtuin1; MMP14: metalloproteinase 14; HIF-1α: hypoxia inducible factor 1α; RUNX2: protein related to transcription factor 2; OPG: osteoprotegerin.

ment in angiogenesis, increased vascularization of the aspirated iliac crest of mice¹⁹, greater cell proliferation²⁰ or acceleration in the healing of open femoral fractures in experimental animals²¹. However, most of these works have been carried out in animal models and human studies are scarce. In patients with avascular necrosis of the femoral head, serum OPG levels increased after HBO (5.61 ± 1.99 pmol/L at baseline, 7.90 ± 1.9 pmol/L after 15

Figure 3. Difference in the expression of genes under study and housekeeping in the Super-SAOS cell line after 5 sessions of hyperbaric chamber



HC: cell group undergoing hyperbaric oxygen therapy; SOST: sclerostin; SIRT1: sirtuin1; MMP14: metalloproteinase 14; HIF-1α: hypoxia inducible factor 1α; RUNX2: protein related to transcription factor 2; OPG: osteoprotegerin.

sessions, 8.97 ± 2.07 pmol/L after 30 sessions; $p < 0.05$), without changes in RANKL²² levels. After HBO (2.5 ATA, 100% O₂ for 90 min/day), osteogenic differentiation of bone marrow mesenchymal cells was also improved in treated patients, with an up-regulation in Wnt3a, b-catenin and Runx2 and descending GSK-3b, compared to those who did not receive it¹². These same authors also described an increase in bone morphogenetic protein (BMP2) and Osterix in treated patients¹².

In our study, we did not find that oxygen at high concentration in HC influences the expression of different genes related to bone metabolism (SOST, SIRT1, MMP14, HIF1α, RUNX2, OPG and RANKL). However, we would highlight that we found a slight tendency, not significant, to the increase in the expression of SOST in the bone undergoing treatment. We know that oxygen tension influences the regulation of SOST and that in hypoxia (1% oxygen tension) osteoblasts and osteocytes express low levels of SOST and sclerostin²³, perhaps this is due to a lower expression of prolyl hydroxylase (PHD2) since it has been seen that deletion of PHD2 in osteocytes causes a lower production of sclerostin dependent on SIRT1¹⁴. This pathway could elucidate our understanding of the pathophysiological mechanism through which, and in the opposite direction, an oxygen-rich environment could increase the expression of SOST and sclerostin. In fact, our group has found a 25% increase in serum sclerostin levels in 12 patients undergoing HBO treatment. However, other works are contradictory¹³.

In conclusion, it does not appear that hyperoxia in HC influences the expression of genes related to bone metabolism, although we believe that more studies are needed to broaden our knowledge of the actions of oxygen in bone.



Conflict of interests: Authors declare no conflict of interests.

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