**Author's response to reviews**

**Title:** Effects of Hyperbaric Oxygen on the Osteogenic Differentiation of Mesenchymal Stem Cells

**Authors:**

Song-Shu Lin (lss1192001@yahoo.com.tw)
Steve WN Ueng (wenneng@adm.cgmh.org.tw)
Chi-Chien Niu (niuchien@adm.cgmh.org.tw)
Li-Jen Yuan (lry51524@adm.cgmh.org.tw)
Chuen-Yung Yang (ycyfl@yahoo.com.tw)
Wen-Jer Chen (chenwenj@adm.cgmh.org.tw)
Mel S. Lee (mellee@adm.cgmh.org.tw)
Jan-Kan Chen (jkc508@mail.cgu.edu.tw)

**Version:** 5  
**Date:** 5 February 2014

**Author's response to reviews:**

Jan-Kan Chen, Ph.D.
Department of Physiology, College of Medicine,
Chang Gung University
Kweishan, Taoyuan 333, Taiwan
E-mail: jkc508@mail.cgu.edu.tw
MS: 7007722911118033

5th of Feb, 2014
Dear associate editor:

We would like to resubmit our revised manuscript entitled “Effects of Hyperbaric Oxygen on the Osteogenic Differentiation of Mesenchymal Stem Cells” for the consideration of publication in BMC Musculoskeletal Disorders (Section: Orthopedics and biomechanics) (MS: 7007722911118033). The followings are our point-by-point responses to the reviewer’s comments and suggestions.

For the suggestion from reviewer 1, the percentage of cells expressing the MSC markers has been shown in Fig 1. The mean percentages of CD146+, CD105+, Stro-1+, and CD34+cells in the cell preparations has now stated in page 9, lines 2-7 as “The percentage of cells expressing the MSC markers CD146, CD105, and Stro-1 and hematopoietic cell marker CD34 were shown in Fig. 1. The mean percentages of CD146+, CD105+, Stro-1+, and CD34+cells in the cell preparations from 4 patients (n = 4) were calculated to be 28.2% ± 1.66%, 90.0% ± 1.94%, 32.3% ± 0.89%, and 0.10% ± 0.03%, respectively. “

We hope that the revised manuscript is now up to the caliber of BMC Musculoskeletal Disorders. We are looking forward to hearing from you soon.
Thank you very much for your attention.

Very Sincerely
Jan-Kan Chen, ph.D.

Editorial Requirements:
1. We have reformatted the title page at the front of our manuscript file. It contained the names, institutions, countries and email addresses of all authors, and the full postal address of the submitting author.
2. “The experimental protocol was performed in accordance with the Declaration of Helsinki and approved by the human subjects Institutional Review Board of the Chang Gung Memorial Hospital. Written informed consent was obtained from all patients. Demographic and clinical data such as age, gender and surgery reason were collected.” The above description has now stated in page 5, lines 2-5.

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Version: 4 Date: 2 December 2013
Author's response to reviews: see over

Reviewer's report: Referee 1
Title: Effects of Hyperbaric Oxygen on the Osteogenic Differentiation of Mesenchymal Stem Cells
Version: 4 Date: 2 December 2013
Reviewer: Rogeria Serakides
Reviewer's report:
The authors hypothesized that the effect of hyperbaric oxygen (HBO) on bone formation is mediated via increases in the osteogenic differentiation of mesenchymal stem cells (MSCs) which are regulated by Wnt signaling. The effects of HBO on Wnt signaling and osteogenic differentiation of MSCs, mRNA and protein levels of Wnt3a, beta-catenin, GSK-3beta, Runx 2, as well as alkaline phosphatase activity, calcium deposition, and the intensity of von Kossa
staining were analyzed. The manuscript is interesting. But there are some methodological flaws that I consider to be important.

Major Compulsory Revisions

1) The authors do not sufficiently provide that the cells used in this study are stem cells. The phenotypic characterization of the cells is necessary. This characterization is important because the bone marrow contains mesenchymal stem cells (MSC), hematopoietic cells and fibroblasts. The International Society for Cellular Therapy stipulated that MSC must express markers such as CD105, CD73, and CD90 and others, and must not express hematopoietic cell markers.

Response:
The percentage of cells expressing the MSC markers has been shown in Fig 1. The mean percentages of CD146+, CD105+, Stro-1+, and CD34+ cells in the cell preparations has now stated in page 9, lines 2-7 as “The percentage of cells expressing the MSC markers CD146, CD105, and Stro-1 and hematopoietic cell marker CD34 were shown in Fig. 1. The mean percentages of CD146+, CD105+, Stro-1+, and CD34+ cells in the cell preparations from 4 patients (n = 4) were calculated to be 28.2% ± 1.66%, 90.0% ± 1.94%, 32.3% ± 0.89%, and 0.10% ± 0.03%, respectively.”

2) The sample size (n) was not described in any of the tests. How many patients the cells were extracted? What is the age, gender and health status of each patient? The cells from each patient were separately evaluated? The cells from each patient were subjected to all treatments?

Response:
The sample size (n) has been described in any of the tests. “MSCs were harvested from 12 patients (5 females and 7 males) who underwent iliac bone grafting for spine fusion. The mean age was 58.3 years-old, where the age ranged from 39 to 77 years old. The cells from each patient were separately evaluated. The cells from 3 or 4 patient were subjected to each treatment.” The above description has now stated in page 5, lines 6-9.

3) Why the periods of 1, 4 and 7 days were used for RT-PCR and western blot and 7, 14 and 21 days were used for the other analyzes? Seven days is sufficient for osteogenic differentiation in human cells? Authors should justify the difference between the periods studied for each test.

Response:
Under the influence of osteogeneic induction medium for 1 week, an increased alkaline phosphatase activity and calcium accumulation was evident in human MSCs culture and increased over time (Jaiswal et al., J Cell. Biochem. 1997; Bruder et al., CORR 1998; Pittenger, et al., Science 1999). The osteoblast mRNA levels of alkaline phosphatase and osteopontin were up-regulated after osteogeneic induction for 1 week (Bruder et al., CORR 1998). Seven days is sufficient for osteogenic differentiation in human MSCs.

“Osteoblasts originate from MSC via a stepwise maturation process. During the early stage of osteogenesis, the cell cannot deposit calcium to form mineralized
bone [23]. In order to deposit calcium, the cells must enter the late stage of osteogenesis [24]. Because we cannot find the short-term effects of HBO (7 d) on calcium production, we therefore, investigated the long-term effects of HBO (14 and 21 d) on the osteogenesis of MSCs and found that HBO significantly increased the expression of osteogenic markers (Fig. 5a, b). Enhanced positive matrix von Kossa staining at the surface layer of the HBO group was seen compared to the control group (Fig. 5c).” The above description has stated in page 12, lines 15-21.

4) Why the groups I (induction), II (induction + HBO), III (Induction + HBO + siRNA) and IV (Induction + HBO + scrambled siRNA) were not evaluated in all tests and in all figures?

Response:
We used siRNA to confirm the role of interest mRNAs or proteins in the regulation of Wnt pathway after HBO treatment.

In the Fig.2, group I, II, III, and IV were all evaluated. We found no significant effect of scrambled siRNA (IV) on MSCs after HBO treatment, group IV was not evaluated in Fig 3 to Fig 8.

In the Fig.3, we investigated the effect of HBO on Wnt 3a related signal first and then we used #-catenin siRNA to confirm in the Fig.4.

In the Fig.6-Fig.8, we investigated the effect of HBO on Wnt 3a related signal and then we used GPR177 siRNA (Fig.6), VPS35 siRNA (Fig.7), and ATP6V0 siRNA (Fig.8) to confirm our results.

5) I did not understand why the authors in Statistical Analysis write “the results from three or four independent experiments”. Was not performed a single experiment? I suggest that the statistical analysis and statistical tests are described in detail.

Response:
The revised statistical analysis and statistical tests have now stated in page 8, lines 17-20 as “Data are given as mean ± standard deviation (SD) of the results from three or four different samples in each item of the experiment. The cells from each sample were separately evaluated. Differences between two groups were measured by the Student’s t-test. A p value less than 0.05 was defined statistically significant difference.”

Minor Essential Revisions
1) In the background, authors should explain the role of wnt proteins specifically on bone.

Response:
The revised description has now stated in page 3, lines 15-18 as “Runx2, a member of the runt homology domain transcription factor family, is essential for osteoblast differentiation [23]. Canonical Wnt signaling promotes osteogenesis by directly stimulating Runx2 gene expression and this regulation can be antagonized by secreted frizzled-related protein-1 (SFRP1) [11].”
2) The Graph A of Figure 1 is not clear. The data are being compared to that group?

Response:

The revised description has now stated in page 20, lines 7-10 as "The mRNA ratios of Wnt3a (a, II / I, **p< 0.01, n = 3, Student’s t-test), #-catenin (a, II / I, *p< 0.05, n = 3, Student’s t-test), and Runx2 (a, II / I, *p< 0.05, n = 3, Student’s t-test) were up-regulated, while GSK-3# (a, II / I, *p< 0.05, n = 3, Student’s t-test) was down-regulated after HBO treatment." I, induction; II, induction + HBO

3) The asterisks in figures 5, 6 and 7 have been displaced.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published

Statistical review: Yes, and I have assessed the statistics in my report.

Declaration of competing interests: No to all questions.

Thank you very much for your attention.

It will be our great honor if this paper can be accepted for publication.

Sincerely,

Jan-Kan Chen, ph.D.

Chang Gung University

Reviewer’s report: Referee 2

Title: Effects of Hyperbaric Oxygen on the Osteogenic Differentiation of Mesenchymal Stem Cells

Version: 4 Date: 7 January 2014

Reviewer: kuo-an lai

Reviewer’s report:

Minor Essential Revisions

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: none

Additional material:

The results of calcified matrix ((4c Long-term effects of HBO on MSCs)) should described the detail.

Response:
The revised description has now stated in page 10, lines 8-12 as “The deposition of a calcified matrix on the surface of the culture dish was evident by von Kossa staining. The matrix intensity for the induction + HBO group and induction group were 557505.3 ± 55457.4 and 382909.7 ± 55873.8 which were quantified by image-analysis system. Greater positive staining through the matrix at the surface layer of the induction + HBO group was observed compared to the induction group (1.47 ± 0.23-fold, p< 0.05, Fig 5c). “

Thank you very much for your attention.

It will be our great honor if this paper can be accepted for publication.

Sincerely,
Jan-Kan Chen, ph.D.
Chang Gung University

Reviewer's report: Referee 3
Title: Effects of Hyperbaric Oxygen on the Osteogenic Differentiation of Mesenchymal Stem Cells
Version: 4 Date :13 January 2014
Reviewer: Hwa-Chang Liu

Reviewer's report:
Major Compulsory Revisions: none
Minor Essential Revisions: Please explain the method used for statistical analysis.
Response:
The revised statistical analysis and statistical tests have now stated in page 8, lines 17-20 as “Data are given as mean ± standard deviation (SD) of the results from three or four different samples in each item of the experiment. The cells from each sample were separately evaluated. Differences between two groups were measured by the Student’s t-test. A p value less than 0.05 was defined statistically significant difference.”
Discretionary Revisions: none
Level of interest: An article of outstanding merit and interest in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: I declare that I have no competing interests

Thank you very much for your attention.
It will be our great honor if this paper can be accepted for publication.

Sincerely,
Jan-Kan Chen, ph.D.
Chang Gung University