Ischemic Preconditioning to Enhance Osteogenic-Angiogenic Coupling

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Statement of Purpose: Formation of vascularized bone is required to repair critical-size bone defects. When this process occurs at a defect site, the oxygen levels are far below those commonly used for *ex vivo* culture. Osteogenic differentiation of stem cells is impaired in hypoxic environments when compared to atmospheric conditions. Ischemic preconditioning (IPC) should be able to mitigate this effect as well as stimulate secretion of angiogenic factors, yet IPC protocols have not been closely studied or optimized. In addition to using the same oxygen level as will be expected *in vivo* (modeled as 2% O_2), we will use a higher oxygen level (4%, "step-down IPC") or a lower oxygen level (1%, "step-up IPC"). Better understanding of the role of hypoxic signaling in vascular and bone development will help guide regenerative medicine efforts and enhance strategies based on implantation of cell-seeded scaffolds.

Methods: Human adipose-derived stem cells (ADSCs) were seeded at $3x10^4$ cells/cm^2 in 6-well tissue culture treated plates. All plates were placed in atmospheric oxygen levels for 12 hours to allow attachment. After this period, plates were moved to 1%, 2%, 4% environments for ischemic exposure or left in atmospheric conditions. After 72 hours of exposure the cells were transferred to 2% O_2 in osteogenic media for 4 weeks. Osteogenic differentition was tracked by measuring: alkaline phosphatase (ALP) activity, Alizarin Red staining and western blotting to quantify the Osteonectin (ON) and Osteopontin (OPN) expression. Secretion of vascular endothelial growth factor (VEGF) was tracked by ELISA in varied oxygen environments.

Results:
We observed significantly enhanced ALP activity with both the conventional and step-down IPC. We did not see any difference between the step-up IPC and the cells directly transferred from 20%. For mineralization, we did not observe a significant difference for any of the 3 IPC protocols that we used. ON expression was 1.5-2 fold higher with our varied IPC methods, all of them showing a significant increase vs no IPC. These results are shown in Figure 1. Similar trends were observed for OPN. Culture of ADSCs in low oxygen conditions also increased VEGF secretion. These results are shown in Figure 2.

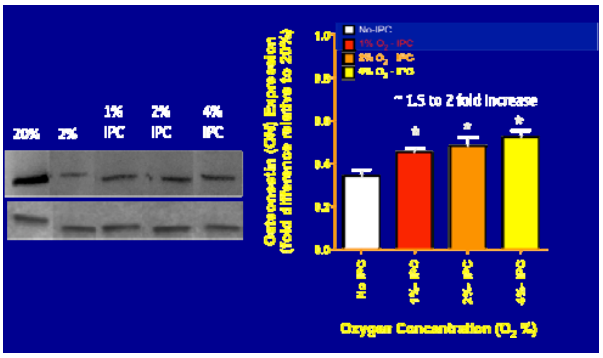

Figure 1. ON expression of ADSCs following 28 days of culture in 2% O_2 for cells preconditioned at 1%, 2%, 4% O_2 or control cells transferred from 20% O_2 (No IPC).

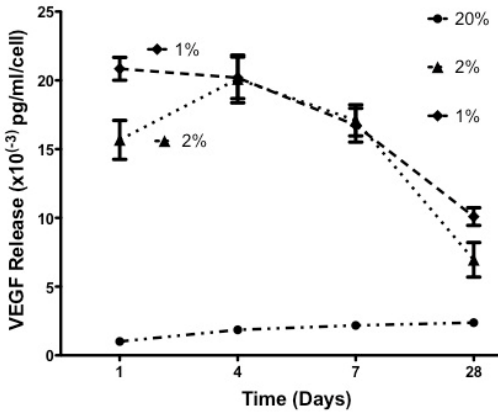

Figure 2. VEGF release by ADSC over 28 days in varied culture conditions.

Current studies are investigating the molecular mechanisms underlying these effects. Efforts are focused on the hypoxia-inducible factor (HIF) family. Initial findings indicate that the elevated secretion of VEGF in 1 and 2% O_2 requires activity of HIF-1.

Conclusions: IPC of ADSCs show promising results on osteogenic differentiation and on the secretion of angiogenic factors. Much of the reduction in osteogenic differentiation in 2% O_2 relative to 20% O_2 can be recovered. Also, 1-4 days of low oxygen culture can trigger high levels of VEGF secretion. Optimization of IPC methods can accelerate formation of vascularized bone at defect sites and coupled with appropriate biochemical and mechanical stimuli.