Δ Novel Profiling Method to Detect Hypoxia Biomarker After Cold Therapy at Bone Injury Site

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Purpose: Applying a cold stimulus onto bone has been shown to clearly increase the healing response. However, the mechanisms upon which acute cold stimulates bone-forming cells are paradoxical and not well understood. It has been previously established that when the vascular network in and around bone is compromised, hypoxia occurs near the fracture site, leading to the activation of a key mechanism in fracture healing to restore the blood flow, ie, angiogenesis. Here we hypothesize that localized application of cold temperature positively stimulates fracture healing and bone formation by inducing physiological changes near the injury site indirectly by modifying the vasomotor tone and reducing the bone blood flow. The objective of this study is to elucidate the mechanism by which cold therapy affects bone formation in vivo at the injury site through a new methodology to detect hypoxia markers.

Methods: Nine C3H strain mice aged 2 to 3 months played the role of the control and experimental group by using both hind legs. Cortical rectangular window defects within the ventrolateral aspect of the femoral diaphysis were created. Hypoxyprobe consisting of pimonidazole, a hypoxia marker, was intraperitoneally injected 7 days post operation into the mice. 15 minutes after the time of injection, the experimental hindlimb of the mouse was exposed to a cold-water bath for 15 minutes. Immediately after, the mice were euthanized and their femurs were harvested. Each femur was fixated, decalcified, processed, and embedded for staining. Adducts formed between pimonidazole and hypoxic cells was detected by incorporating anti-pimonidazole fluorescein isothiocyanate (FITC)-conjugated IgG1 mouse monoclonal antibody and horse radish peroxidase conjugated rabbit anti-FITC. 3,3′-diaminobenzidine (DAB) staining was then utilized to visualize the areas of interest through immunoperoxidase staining. ImageJ analysis was applied to assess the area indicative of DAB staining.

Results: ImageJ analysis revealed a noticeable increase in area marked by DAB staining between experimental and control groups. Furthermore, results indicate a strong increase in the number of hypoxic cells within and around the cortical bone defect in the hindlimbs of mice exposed to a cold stimulus in comparison to the control hindlimbs. This demonstrates detectable localized hypoxia induction through the application of a cold stimulus.

Conclusion: A new analytical approach has been developed to measure hypoxic levels at the bone injury site. By detecting adduct formation created through pimonidazole reduction with hypoxic cells, hypoxia levels were assessed at the bone defect site following exposure to a cold stimulus. The results illustrate the development of a detectable hypoxic environment that provides a conjunctive avenue to explore the facilitation or fluctuation of certain regenerative pathways dependent upon hypoxic conditions within the stages of bone repair.

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