

IN SITU ASSESSMENT OF OSTEOCYTE VIABILITY & CYTOSKELETAL INTEGRITY IN NATIVE BONE TISSUE AND OSMOTIC STRESS EFFECTS ON THE SAME

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INTRODUCTION - Osteocytes are the putative mechanosensors in bone; despite their implicit role in bone physiology, *in situ* study of osteocytes has been limited due to their remote location within the mineralized bone matrix. Nonetheless, native bone remains the ideal environment in which to study the function of osteocytes. Histological methods allow one to study osteocytes in their native milieu, but they do not allow cells to be observed while they remain viable and active. *In vivo* observation techniques are in development but are currently too technically demanding and expensive to be implemented as a standard laboratory technique. Hence, the purpose of this study was twofold, (i) to adapt and implement, for the first time to our knowledge, two cytochemical methods developed for *in vitro* cell culture to study osteocyte activity *in situ* in semi-thin sections of native bone, (ii) to determine what percentage of osteocytes remain viable over time in bone specimens obtained from a recently sacrificed animal, and (iii) to assess the response of osteocytes to osmotic stress while in their native milieu.

METHODS - Studies were carried out on specimens from cortical bovine femur obtained fresh from the slaughterhouse. All specimens were refrigerated for 2 to 3 days prior to processing for this study. Two cytochemical methods, *i.e.* live/dead viability assay and cytoskeletal staining, were adapted and implemented to study *in situ* cell viability and cytoskeletal integrity, respectively. The cytochemical studies were carried out in semi-thin sections that were stored in media or buffer solution over the course of the study. Osmotic stress studies were carried out on semi-thin longitudinal sections 24 hours following immersion in hypo- or hyper-tonic solutions. *Specimens and Specimen Preparation:* Small bone blocks were obtained using a linear precision saw (Isomet 4000, Buehler) and then sectioned with a diamond microtome saw (SP1600, Leica Microsystems). Multiple sets of four, 200 μ m sections were cut in cross section from the tissue block. Sections were stored in Minimum Essential Medium alpha formulation (a-MEM) supplemented with 5% fetal calf serum (FCS) and 5% fetal bovine serum (FBS) for maintenance of cell metabolism, or phosphate buffered saline (PBS), a CO₂-controlled, humidified incubator at 37°C (standard culture conditions). Media was replenished regularly. *Live/Dead Viability Assay:* Cell viability was evaluated in sections at 0, 48, 96, and 144 hours with the Live/Dead Cytotoxicity and Viability Kit (L-3224, Molecular Probes). A 2 μ M ethidium homodimer-1, 4 μ M Calcein-AM Live/Dead solution was made in PBS. Media was aspirated off the bone sections, and then replaced with the Live/Dead solution. The sections were incubated for 25 minutes under standard culture conditions. After removal of the solution, the sections were rinsed and then immersed in PBS prior to imaging on the confocal microscope (AOBS SP2, Leica Microsystems, Mannheim). Image stacks were obtained for three random fields of view per section in each section using He laser excitation at 488 nm and 568 nm, whereby live cells fluoresce green and dead cells fluoresce red. *Cytoskeletal Staining:* The actin cytoskeleton was marked in bone sections using Texas Red conjugated phalloidin (T-7471, Molecular Probes). One part stock solution was diluted into 50 parts PBS. Media was aspirated off the bone sections, which were then fixed with formalin for 30 min. The sections were rinsed, extracted with 0.1% Triton X-100 (Sigma) for 5 min and rinsed again. The sections were treated with the stain solution for 40 min, rinsed with PBS, then mounted and cover-slipped with Vectashield (Vector Labs). Confocal image stacks were obtained using He laser excitation at 568 nm. *Data Analysis:* NIH Image was used to count the total number of live and dead cells in each field. Image stacks were reconstructed in three dimensions (Volocity 3.1, Improvion).

RESULTS - The live/dead viability assay and cytoskeletal staining methods were adapted and implemented in native bone sections. Total cell numbers remained constant throughout the time course of the study, indicating that the red/green assay was reproducible and accurate to account for changes in viability of the whole cell population over time. Although distinct actin stress fibers were not readily apparent following phalloidin staining, the osteocyte body and processes stained in great

detail, and the integrity of the osteocyte syncytium could be observed (Fig. 1B). More than 40% of osteocytes remained viable (Fig. 1A,2) in bone sections following slaughter of the animal and cold storage. From this initial time point and throughout the rest of the experiment, a layer of dead cells persisted 10.38 \pm 1.32 μ m from the surface of the sample. Over the time course of the experiment (144 hours or six days), no statistically significant difference was observed in percentage of viable cells observed throughout the volume in bone stored in Media or PBS (Fig. 2). However, after 98 hours, viable cells remained distributed throughout the bone in media, but had migrated to the surface, and begun to detach in PBS. Bone sections exposed to hypotonic PBS showed 60.38% viability (st.dv. \pm 8.63) and hypertonic showed 60.96% viability (st. dv. \pm 4.81), both of which were significantly higher ($p < 0.05$) than per cent viable cells in isotonic PBS (st. dv. $\% \pm 15.70$).

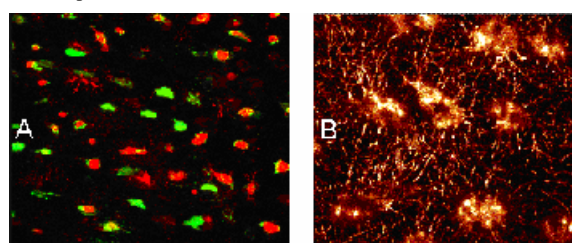


Fig. 1 *In situ* study of osteocytes in their native environment. **A:** Live cells fluoresce green, dead cells fluoresce red. **B:** Actin staining delineates the cell body and processes of osteocytes.

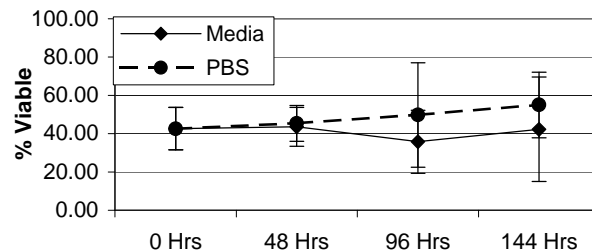


Fig. 2 Cell viability in Media and PBS over time.

DISCUSSION - This study proves the feasibility of adapting and implementing the live/dead viability assay and cytoskeletal staining *in situ* in native bone sections, which has not been reported in the literature previously. The viability assay functioned well and total cell counts were constant over time, indicating that the method is appropriate for assessing viability in a population of cells over periods of time up to one week. It was surprising to note the resiliency of osteocytes in these bone sections – more than 40% of osteocytes remained viable well after animal slaughter and tissue refrigeration. A layer of dead cells did persist on the surface of the section throughout the experiment; these cells were likely killed during the sectioning process. It is expected that a greater proportion of cells would be viable at early (and later) time points if bone samples were obtained immediately after slaughter. Exposure to non-isotonic media resulted in volumetric strain and enhanced viability in osteocytes, similar to previous studies in cultured osteoblasts [1]. Actin cytoskeletal staining revealed a surprisingly clear picture of cellular integrity. Although numerous imaging techniques have been reported to examine the structure and morphology of the pericellular network, this technique allows for direct observation of the osteocytes and their processes. Hence, it can be applied to detect changes in cellular integrity before gross structural changes become apparent. These methods begin bridge the gap between the petri dish and *in situ*, *in vivo* study of bone physiology and may provide a methodological basis for new insights into the cellular basis of bone pathophysiology.

REFERENCES – [1] Rashewsky, SE, Sorkin, AM, Knothe Tate, ML. BMES Annual meeting, 2003 **ACKNOWLEDGEMENTS** – Whitaker grant RG-02-0527.