Technical Article

In vivo Technique for Cellular Calcium Waves Documentation: A Light Microscopy Method

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Abstract

Background: We are introducing a novel *in vivo* technique to document cellular calcium deposits, which reflect a snapshot of the effect of calcium wave propagation. This technique however is not advocated enough to replace the accuracy and resolution of the confocal laser technique. Light microscopy equipment, calcium chelators and a histological calcium staining kit are essential. **Aims:** The purpose of this study is to introduce the use of standard light microscopy to display *in vivo* ionic cellular calcium deposits. **Materials and Methods:** Oxalic Acid (OA) (100 millimol) was the calcium chelator used in the study. This substance was injected into the dog right atrial tissue *in vivo* in an area of 1 cm^2. Samples were fixed and stained by the calcium specific von Kossa protocol. **Results and Conclusions:** Histological slides demarcated the intracellular calcium as black dots. Heterogeneity of calcium deposits mimicked images of both, the calcium sparks and calcium waves theories. This light microscopy technique could expand the number of experimental studies in the function of cellular calcium physiology.

Keywords: Calcium chelation, Calcium waves, Cell membranes, Cellular gap junction, *In vivo* chelation, Intercellular calcium propagation, Light microscopy method, Von Kossa

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Introduction

In this study, we are describing an *in vivo* technique aimed at the documentation of tissue calcium deposits, resulting from calcium waves' propagation. Light microscopy equipment, calcium chelators and a histological calcium staining kit are essential. Confocal Laser Microscopy has been recognized as the "Gold Standard" technique of choice to accurately visualize the calcium wave propagation phenomena.[1] The intent of the study is not to replace the established Confocal Technique, however instead, establish our technique as a complementary method.

Oxalic acid (OA) is a known calcium chelator, described as a diprotic acid. It has two negative charged areas, which are attracted to the positively charged calcium ion Ca^{++}. When calcium chelators such as OA are introduced selectively *in vivo* into the tissue, an interesting series of events takes place starting with the conversion of the ionic cytosolic Ca^{++} into a salt such as calcium carbonate ($CaCO_3$). The von Kossa staining is used to display the cytosolic calcium stores.

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The published literature on oxalates calcium chelation dates back to the end of the nineteenth century,[2] when it was first observed how oxalates caused a decrease in the conduction of the neuromuscular apparatus. From the 1950's until recently, intravenous (IV) systemic calcium chelation was advocated as a cure for vascular diseases. Due to reasons beyond the scope of this communication, in 2008, clinical trials were halted in the United States.[3] The controversy continues with some researchers advocating for additional clinical trials for illnesses other than xenobiotics metal toxicity, with IV administration of ethylenediaminetetraacetic acid (EDTA) as the drug of choice.[4] The oral systemic chelation therapy is still utilized mainly in the treatment of heavy metals intoxication in children.[5] Targeted injections of chelators were experimentally done *in vitro* as early as 1961,[6] where a calcium chelator (EDTA) was utilized to dissociate the cardiac cells at the intercalated

disc area. Further, in 2012,[7] oxalates were injected *in vivo* in millimolar amounts in the cardiac sympathetic neural system of the dog's heart. The goal was to evaluate if neural calcium chelation would have an effect on heart rhythm disturbances, that is, Atrial Fibrillation. Post publication of the electrophysiological findings, a review of the stained slides of the experiments began to show that the chelators had infiltrated the adjacent and subjacent atrial muscle. A pattern of heterogeneity of calcium chelation in the infiltrated myocytes began to emerge. This manuscript emphasizes the potential applications of these findings in intercellular cell signalling, such as calcium propagation.

Materials and Methods

After the *in vivo* oxalate injections, Haematoxylin and Eosin staining and von Kossa staining were carried on biopsied samples according to the procedure described by Sheehan DC and Hrapchak BB, and as referenced by The American Master Tech Von Kossa stain kit procedure.[8] von Kossa stains were performed on non-buffered formalin fixed paraffin-imbedded tissue sections, cut at 4 μ thicknesses**.** The slides were deparaffinised using xylene, rinsed in distilled water and then placed in silver nitrate 5% solution for 40 minutes, under a 100-watt incandescent lamp. These slides were then rinsed in distilled water, and placed in 5% sodium thiosulfate solution for 2 to 3 minutes. Then they were rinsed in tap water, and placed in nuclear fast red for 5 minutes. Finally, the slides were dehydrated in fresh absolute alcohol, and then cleared in xylene and cover slipped. Additionally, control slides were placed in 1% Hydrogen Chloride (HCL) solution for 5 minutes prior to the above von Kossa staining procedure, in order to confirm the presence of calcium. Calcium dissolves in acid, whereas urates or phosphates salts do not. The calcium presence was confirmed. The equipment used was a Leica DM2000 bright field transmission light microscope with 4 FLUOTAR lenses (5X, 10X, 20X and 40X) and 2 N-Plan lenses (2X and 60X high dry). Using Photographic Camera Moticam 1000 and 16mm lens mounted on TL 160 BS adapter, digital images were taken using corresponding Motic Images Plus software program. Images of the slides presented in this manuscript are originals, without any physical or digital alterations.

Oxalic Acid: Molecular Weight = 126- pH 1.6

For concentration of 100 mmol/L, dilute 126 mg in 10 ml of solvent.

Results

The infiltration of *in vivo* targeted delivery of calcium chelators, viz. OA into the atrial myocardium combined with the von Kossa staining technique resulted in images which demonstrate the process of chelation which changes the ionic Ca^{++} into a larger molecule, calcium carbonate ($CaCO_3$) It has been reported that stress in the endoplasmic reticulum causes excessive release of calcium into the cytosolic space.[9] This sudden increase in cytosolic calcium, in turn, induces down regulation of the intracellular gap junction communication (IGJC), thereby trapping the chelated calcium at the gap junction. The technique hereby presented, could thus enable and expand research for the study of cell physiology, as it relates to intra- and intercellular calcium propagation. The display of the focal phenomenon in post chelated atrial myocardium [Figure 1], correlates with the prevalent calcium sparks and calcium wave theories.

Discussion

In cardiac cell-to-cell communication, it can be said that, "permeability of the nexus falls with increasing molecular weight of the compound".[10] Our findings clearly suggest that intracellular calcium (now converted to $CaCO_3$) is not allowed to permeate through the gap junctions. An interesting aspect of the study is the localization of calcium in the tissue samples, from the same longitudinal plane, showing a random or heterogeneous distribution. Others have addressed the question of an extracellular communication of calcium signaling as, "extracellularly mediated $Ca2^+$ signaling also exists and this could be employed to supplement or replace gap junction communication." The function of intercellular $Ca2^+$ waves may be the coordination of cooperative cellular responses to local stimuli".[11,12] Further, the tissue chelation process could be the stimuli needed to start this cooperative cellular response.

The unexplained randomness of chelated calcium in some cells, and not others in the same longitudinal

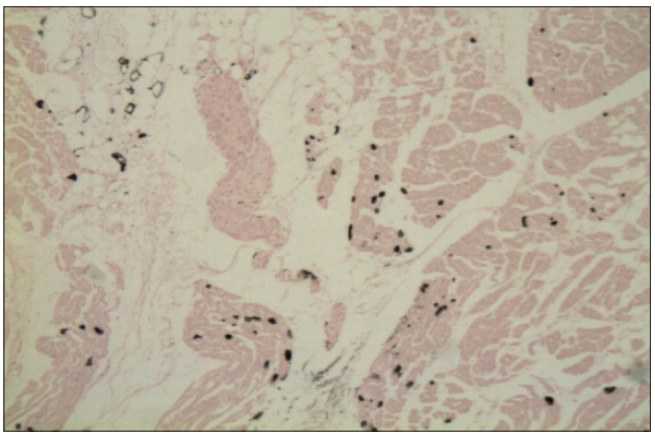

Figure 1: Example of atrial myocardium showing focal random chelated calcium after injection of 100 mol/L of oxalic acid (stained black) ×5 magnification

plane, could also be explained by the prevalent calcium sparks or calcium wave phenomenon. It has been stated that "these $Ca2^{+}$ sparks appeared to recruit other sparks along the wave front so that the wave progressed in a saltatory manner."[13] Furthermore, other researchers have found that nonlinear gap junctions enable long distance propagation of calcium waves.[14] It should be noted that in our nine individual studies, that all chelated samples exhibited a focal or heterogeneous pattern of calcium presence, whereas all control samples did not show evidence of calcium deposits. Further research is warranted to substantiate the findings biochemically, molecularly and functionally. Our studies focused on calcium chelation in the context of myocardial cells that resulted in the introduction in the medical literature of a technique that falls in a branch of cytology, known as Cell Biology. This *in vivo* targeted cellular calcium chelation method was introduced in the medical literature for its effects on the calcium cardiac neural tissue, and subsequently for its utility as a cancer-fighting hypothesis.[15]

In Jeon's 2008 book "International Review of Cytology: A Survey of Cell Biology 'Intercellular Ca^{++} Waves: From Cultures to Living Tissues',[16] discussing the state of the art of cellular Ca^{++} waves, the author states: "The question is whether Ca^{++} waves can also be observed in preparations somewhat closer to the *in vivo* situation or at least under conditions that allow some conclusions on the existence mechanisms, and role of Ca^{++} waves for the *in vivo* situation". We have partially answered that question by documenting a snapshot of the *in vivo* saltatory phenomenon of the propagation of ionic calcium waves in the atrial myocardial cells.

This technique also introduces the field of cell physiology to the emerging complex field of cellular ionic calcium propagation, which is a reflection of cell function and calcium architecture. In the emerging field of bioelectric cells signals, a very recent publication correlates bioelectric cell membrane signal that can identify cells likely to develop into cancer.[17] *In vivo* targeted chelation (by interrupting the cell membrane signals) could become a useful provocative diagnostic tool, or perhaps a therapeutic mode in the cancer war. Do cancerous cells also exhibit a saltatory intercellular ionic calcium wave phenomenon? Is that a factor in the already documented unreliability of some chemotoxic treatments? The technique presented in this manuscript could further facilitate the correlation of therapy outcome with the saltatory calcium propagation phenomenon. However, further research is warranted for conclusive evidence.

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