

Validation of a Novel Micro-Capillary Array Fluid Collection Technology for Determination of Biomarkers of Bone Metabolism in Human Sweat

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BONE LOSS DURING SPACE flight is a well documented event that is a serious issue with regard to crew health and safety, especially on extended duration missions that involve physical activity in other than microgravity (i.e., Lunar and Mars exploration-class missions). To date, two approaches have been suggested to prevent bone loss during space flight: physical stimulation of the skeletal system (i.e., vibration or resistive loading) or pharmaceutical intervention (i.e., bisphosphonate drugs). Both approaches appear to successfully prevent at least some of the bone loss that occurs in ground-based models of space flight such as bed rest. However, the utility of these approaches has yet to be fully tested and validated during space flight.



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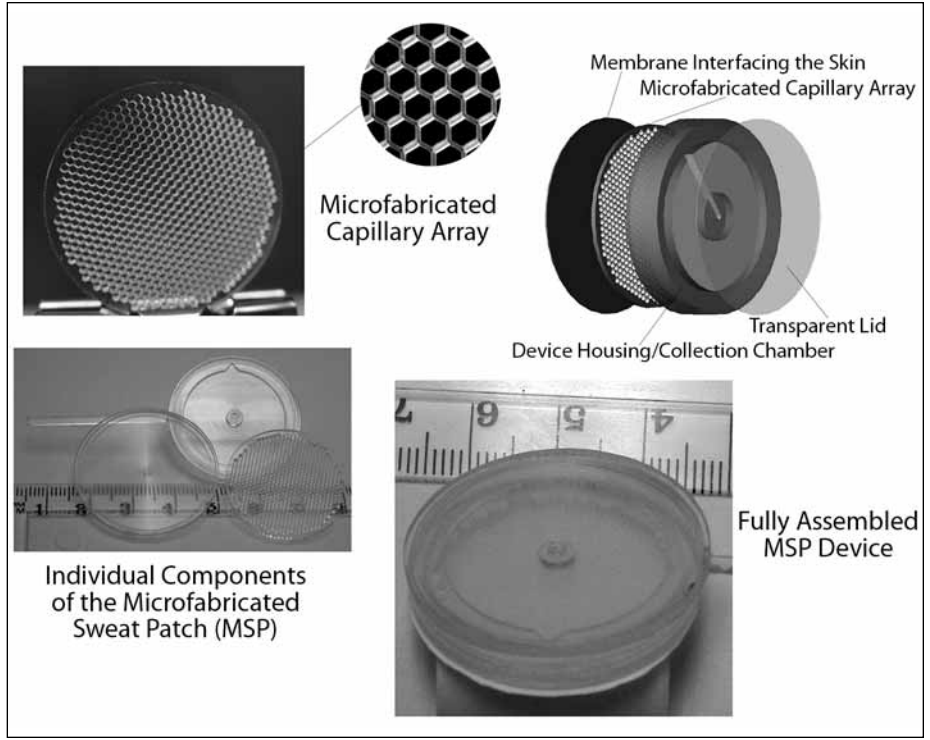
One of the central challenges faced in the manned space program is how to provide real-time biomedical monitoring of crew members in order to assess the efficacy and efficiency of countermeasures designed to combat physical de-conditioning induced by extended space flight. This challenge is especially problematic in the case of bone loss, for the underlying physiology of bone remodeling/bone loss, either during space flight or in diseases such as osteoporosis, considers loss over an extended period of time. The gold standard measurement for assessing bone mineral loss is dual X-ray absorptiometry (DEXA), a method that requires bulky equipment, well-trained personnel, and an assessment period measured in months to years. This approach, although well accepted, does not lend itself to the space flight environment or to the real-time biomedical monitoring goal required to assess the efficacy or efficiency of countermeasures employed to protect astronauts during extended space flight missions.

A second approach gaining momentum in the clinical arena is the monitoring of biomarkers known to indicate the loss of bone mineral content during the earlier phases of bone remodeling/bone loss. These include the measurement of excreted calcium and collagen cross-links in urine or blood, both biomarkers of

bone breakdown. This approach has been successfully used to monitor patients undergoing treatment for osteoporosis in a terrestrial setting. This type of measurement has also been shown to reflect bone loss in individuals undergoing bed rest as a ground-based model of space flight.

In order to employ this approach to provide real-time biomedical monitoring of overall bone loss rates and the efficiency of in-flight countermeasures during space flight, it is essential that a miniaturized, rapid, non-invasive, gravity-independent technique be developed for collection and analysis of these biomarkers. This project was designed to address one element of the use of such a technique based on the collection and analysis of sweat for subsequent analysis of calcium content. This technique utilizes a micro-fabricated array of capillary tubes arranged in disc form, manufactured from biocompatible material known as the Micro-fabricated Sweat Patch (MSP). Sweat is collected as an unadulterated liquid by a gravity-independent means, specifically capillary action. Sweat is then recovered by centrifuging the sample out of the capillary array into a collection tube in preparation for analysis.

Ionized calcium levels in normal human sweat have previously been reported to be in the micro-molar levels (i.e., 50-250 micro-molar) as compared to the low milli-molar range for serum.¹ In addition, existing sweat collection technologies that collect sweat and its constituents by way of absorption/evaporation into a filter material (i.e., Osteopatch™ technology) indicate that calcium loss from an area of skin similar to that covered by the MSP device is in the region of 20-30 μg per 24-hr period. Pre-existing biochemical analysis techniques (colorimetric in nature) normally used for clinical sample analysis of calcium are inappropriate because of their lack of sensitivity at this detection level. To overcome this limitation, we investigated the use of a stoichiometric fluorescent dye, Calcium Green-1, for the determination of Ca^{2+} in sweat. This dye has been utilized in cell biology to measure free calcium levels in the 0-50 μM range.



MUSCLE CELLS—Stuart Lee with a B.S. and M.S. from Virginia Tech is involved in the study of muscle cells for the purpose of maintaining muscle integrity in astronauts while in space. Lee is enrolled in the doctoral program in Health and Human Performance at UH.

Project Goal

The goal of this project was to determine (1) the volume of sweat that could be reproducibly recovered from the MSP device and (2) the appropriate analysis method capable of providing the level of sensitivity required for detection of excreted calcium in human sweat, with the ultimate goal being a means of monitoring bone loss.

Results

Gravimetric Determination of Fluid Collection Rates

Micro-fabricated Sweat Patch (MSP) devices (Fig. 1) were weighed using a Mettler Analytical Balance prior to saturation with sweat stimulant after saturation and, again, after the sample was collected by centrifugation.

These gravimetric values (grams) were used to determine the volume of total liquid collected, the volume of liquid collected by centrifugation from the MSP device (i.e., sample volume), and the amount of liquid that remained in the MSP device after centrifugation (i.e., carry-over volume) (Table 1). Significant amounts of liquid remained in the MSP device after centrifuga-

Figure 1. Micro-Fabricated Sweat Patch. Diagrammatic representation shows individual components and the fully assembled device.

Table 1. Volumetric Testing of the MSP

N	Volume Collected (g) Mean +/- SD	Volume Carry-Over (g) Mean +/- SD	Sample Volume (g) Mean +/- SD
12	0.668 +/- 0.086	0.118 +/- 0.054	0.551 +/- 0.079

Table 2. Calcium Recovery from MSP Devices Using the Fluorescent Calcium Dye, Calcium Green-2

N	Ca Concentration (μM)	FU (Pre) Mean +/- SD	FU (post) Mean +/- SD	% Ca Recovery
3	13 μM	6.19 +/- 0.47	6.82 +/- 0.98	101
3	25 μM	7.24 +/- 0.72	7.42 +/- 1.47	102
3	50 μM	10.78 +/- 0.97	8.16 +/- 1.61	76

FU - Fluorescent Units

tion (approximately 17% of the total volume collected). This may reflect incomplete removal of the liquid from the capillary array but is more likely associated with liquid being trapped at the edge of the secondary collection chamber edge. However, the volume collected for analysis was reproducible (550 microliters) with a variance of approximately 14% among MSP devices.

Calcium Determination Using Calcium Green-2 Fluorescent Indicator Dye

Sweat patches were saturated with sweat stimulant containing defined amounts of ionized calcium. Calcium recovery was assessed by determining fluorescent signal (FU) associated with the fluorescent calcium indicator Calcium Green-2 (Molecular Probes, Eugene, OR) (Fig. 2) in the samples before and after being collected in the MSP device and collected by centrifugation (Table 2). At higher concentrations of calcium, significant loss of calcium occurred, presumably associated with contact of the sample with the micro-fabricated device.

However, it must be noted that the pre- and post-values are not significantly different and, as such, the lower calculated Ca^{2+} recovery rate at the highest calcium concentration (50 μ M) may be misleading. In addition, the fluorescent calcium dye, CG-2, becomes saturated at higher levels of Ca^{2+} (Fig. 2) and this saturation may have also led to the apparent reduction in calcium recovery at 50 μ M levels.

Alternate Ca^{2+} Analysis Approach in Unadulterated Sweat Collected Using the MSP Device

Based on the results described above using CG-2 to determine calcium concentration in sweat stimulant, the next stage of the project was to measure calcium contained in real sweat collected from an individual. The MSP device had previously been tested for the ability to collect sweat from the skin of an individual undergoing physical activity. After collection of several samples of unadulterated sweat from exercising individuals, researchers assessed calcium concentration using the CG-2 protocol described above. However, all samples tested induced a saturation of the CG-2 dye indicating a much higher concentration of calcium in sweat than had been anticipated.

Utilizing a newly available commercial colorimetric calcium assay, we performed a series of experiments in order to determine the actual values of calcium present in unadulterated sweat. This assay is available from BioAssay Systems (Hayward, CA) and is sensitive to calcium in the 25-2000 μ g/ml (62 μ M-50 mM range – Calcium atomic weight = 40). It utilizes a binary reagent system and produces a colored product measured at 612 nm using a standard 96-well plate reader.

Testing was carried out using sweat collected from the forearm of five separate subjects participating in physical activity. An MSP device was attached to the skin of the forearm with an adhesive patch after it had been washed with distilled water and then dried with a clean cotton towel. After 20 minutes of active sweat collection, the MSP device was removed and unadulterated liquid sweat was collected by centrifugation into a sample tube attached to the MSP device. Samples were then assayed for calcium content using the commercially available calcium assay based on a colorimetric end product described above.

As seen in Table 3, a wide range of calcium concentrations were detected in the sweat of several different individuals collected using the MSP device, ranging from approximately 230 μ g/ml Ca^{2+} to 1350 μ g/ml Ca^{2+} (5 mM to 34 mM). This is in contrast to the amount of calcium in sweat previously reported¹ using a collection device known as the Osteopatch™, which reported a daily loss of around 35 mg/day Ca^{2+} in sweat or in an

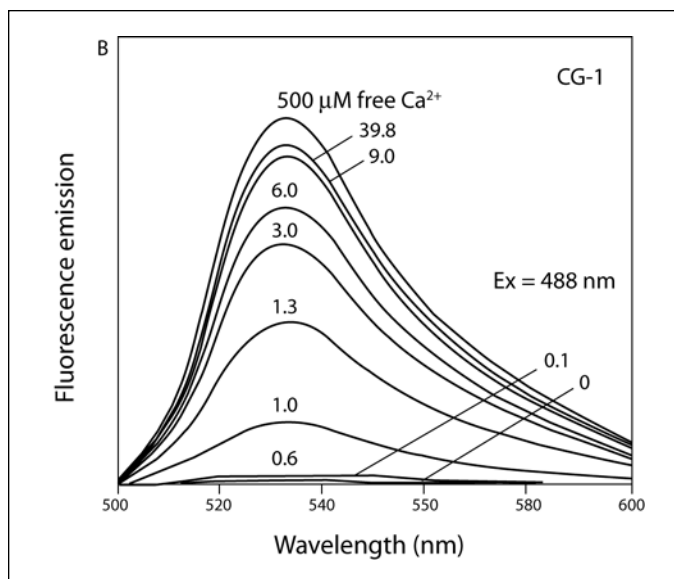


Figure 2. Calcium Green-2 (CG-2) Fluorescent Emission Spectra at Different Ca^{2+} Concentrations

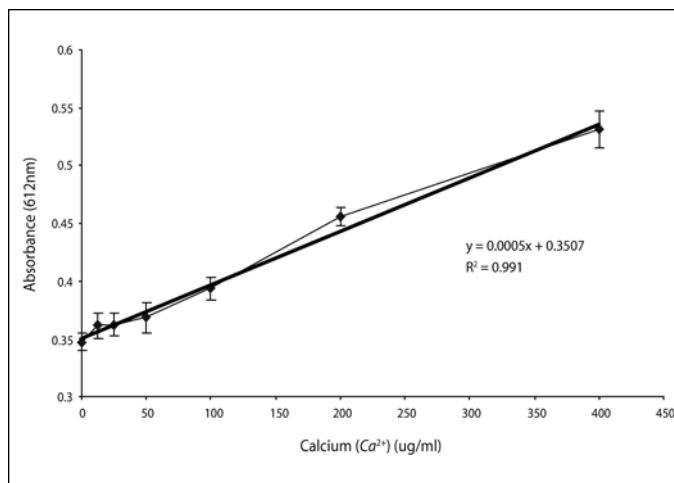


Figure 3. Representative Standard Curve for Colorimetric Determination of Calcium (Ca^{2+}) in Sweat Samples

Table 3. Calcium (Ca^{2+}) Concentration in Sweat Collected from Exercising Subjects Using MSP Devices

SUBJECT ID	MEAN Ca^{2+} (μ g/ml)	Standard Deviation
A	432	52
B	751	31
D	378	33
E	235	17
G	1345	198

approximately 23 μ g Ca^{2+} /24 hr collection period recovered from a single Osteopatch™.

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Discussion

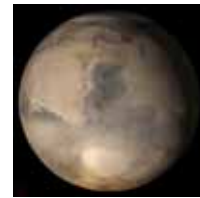
Data presented above indicate that unadulterated liquid sweat contains a significantly higher amount of Ca^{2+} than had been previously reported. In order to ensure adequate sweat sample production, all samples were collected from individuals participating in physical activity. This is recognized as a limitation of our study in that active sweating may produce more calcium than passive sweating in sedentary individuals, a phenomenon previously reported.² However, notwithstanding this limitation, the use of unadulterated sweat for determining the absolute amount of calcium excreted in sweat appears to be a more accurate method than collection of the non-aqueous components of sweat by evaporation as in the case of other collection technologies such as the Osteopatch™.

As such, the micro-fabricated sweat patch utilized in this study allows the rapid and accurate collection of approximately 650 micro-liters of unadulterated liquid sweat using a micro-fabricated array of capillary tubes located in the form of a plastic disc. Further studies are warranted that compare the relative sweat calcium loss rate as detected in sweat samples collected using the Micro-Fabricated Sweat Patch (MSP) device with the Osteopatch™ in the same individual.

References

¹N. Rianon, D. Feeback, R. Wood, T. Driscoll, L. Shackelford, and A. LeBlanc, "Monitoring Sweat Calcium Using Skin Patches," *Calcif. Tissue Int.* 72 (2003): 694-97.

²J. Y. Chu, S. Margen, D. H. Calloway, and F. M. Costa, "Integumentary Loss of Calcium," *Am. J. Clin. Nutr.* 32 (1979): 1699-1702.



Courtesy NASA-Kennedy Space Center