UNIVERSITY OF CALIFORNIA, SAN DIEGO

A Novel Assay For The Crystal Formation Activity in Human Serum

A Thesis submitted in partial satisfaction of the requirements for the Degree Master of Science

in

Biology

by

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The Thesis of Yasaman Pirahanchi is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

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Master of Science in Biology

University of California, San Diego, 2016

Professor Paul A. Price, Chair

Many previous studies have shown that artery calcification is linked to increased bone loss. Other studies have suggested that an agent released from bone causes artery calcification by traveling through blood. Previous studies have also shown that serum has crystal formation activity, and our goal is to develop a quantitative assay for this activity. Our long term goal is to determine if serum crystal forming activity is elevated in patients with increased bone loss.

In previous studies, Erin Hourigan developed a method to monitor serum crystal formation activity by diluting serum 50 fold with 2mM Calcium and Phosphate at 37C and pH 7.4. Hourigan's assay measured Calcium decline at multiple time-points, and was very labor intensive. In contrast, my assay allows the Calcium decline to go to completion and then directly monitors serum's crystal formation activity by counting the number of crystals present in solution in addition to measuring Calcium decline in solution.

The number of crystals counted by our assay were significantly higher ($p<.0005$) in human neonates than in human adults, which may be due to the fact that the rate of bone turnover is significantly higher in newborns than in adults, causing a rise in neonate's serum crystal formation activity. We found that crystals counted by our assay probably originate from the growth of much smaller crystals already present in serum and are not formed by a serum catalyst. This assay is the first demonstration that human serum has crystals that can be directly counted

INTRODUCTION

Artery calcification is the buildup of calcium phosphate mineral within the artery wall and is used an indicator for coronary artery disease [6], which affects 16.8 million in the U.S [1].There is a strong correlation between bone loss and artery calcification, where the patients with the highest rate of bone loss have the highest increase in artery calcification [2]. The mechanism for the link between bone loss and vascular calcification is still being investigated.

Carbonate apatite is the mineral phase of both bone and of atherosclerotic plaques. Therefore, to gain a better understanding of the pathophysiology of mineral deposition in the plaque, examining the normal physiological process of mineral deposition in bone is valuable. Bone is collagen that has been mineralized by the deposition of apatite mineral within the collagen matrix and strengthens it to form bone [7]. The mineral in bone is recycled with Calcium and Phosphate ions in blood, in cycles of bone buildup and breakdown. This process of bone buildup and breakdown is called remodeling or bone turnover. Normally, bone remodeling is balanced between the action of osteoclasts, the bone cells that breakdown bone, and osteoblasts, the bone cells that form bone [8], and no net bone loss occurs. When bone breakdown exceeds bone buildup, apatite is dissolved into ionic Calcium and Phosphate, which are released into blood [10].

Previous studies have shown that serum has crystal formation activity [4, 10-13], and our goal is to develop a quantitative assay for this activity. Our long-term goal is to determine if serum crystal forming activity is elevated in patients with increased bone loss.

In previous studies, Erin Hourigan developed a method to monitor serum crystal formation activity by reducing the activity of crystal growth inhibitors in serum: serum was diluted 50 fold with 2mM Calcium and Phosphate at 37C and pH 7.4 in order to reduce the concentration of the serum inhibitors, and alkaline phosphatase was added in order to inactivate some of the serum inhibitors. Hourigan's assay measured Calcium decline (a measure of apatite mineral formation) at multiple time-points, and was very labor intensive [5]. In contrast, my assay uses the same reagents but allows the Calcium decline to go to completion and then directly monitors serum's crystal formation activity by counting the number of crystals present in solution.

The number of crystals counted by our assay were significantly higher ($p<.0005$) in human neonates than in adults, which may be due to the fact that the rate of bone turnover is significantly higher in newborns than in human adults, causing a rise in neonate's serum crystal formation activity. We found that crystals counted by our assay probably originate from the growth of much smaller crystals already present in serum and are not formed by a serum catalyst. This assay is the first demonstration that human serum has crystals that can be directly counted.

MATERIALS AND METHODS

Materials

MOPS buffer was purchased from Fischer (Fair Lawn, NJ). Intestinal alkaline phosphatase was purchased from Calzyme (San Luis Obispo, CA). Calcium levels were determined colorimetrically using cresolphthalein complexone (JAS Diagnostics, Miami FL). Hemocytometer was purchased from Fischer Scientific. The Zeiss Axioplan 2 with Zeiss Axiocam monochrome camera was courtesy of the Chisolm Lab. A 50mM EDTA pH 7.4 stock solution was used for EDTA experiments.

Methods

Preparation of calcification buffer

The calcification buffer used to investigate the calcification activity in serum was prepared at 0C in wet ice, utilizing a procedure that was designed to achieve the near instantaneous mixing of calcium and phosphate. Depending on the number of samples for the given experiment, a certain volume of 0.14M MOPS, pH 7.4 with 4mM $CaCl_2$ was placed into one glass tube and an equal volume of 0.14M MOPS pH 7.4 with 4mM Na_2HPO_4 was placed into a 300ml beaker. A stir bar was placed in the $CaCl_2$-MOPS solution and stirring was initiated. Next, the Na_2HPO_4–MOPS solution was quickly poured into the $CaCl_2$-MOPS solution. While stirring continued, 1% of the total volume's worth of alkaline phosphatase was added to the final solution to facilitate crystal formation in our assay.

This would achieve a final calcification buffer containing 0.14M MOPS, pH 7.4, 2mM calcium and phosphate. This final calcification buffer was equilibrated at room temperature for an hour by aliquotting the calcification solution into 1mL amounts in individual tubes and parafilming each tube. The temperature of the calcification solution was equilibrated to room temperature before serum was added to ensure dispersal of serum into the buffer upon mixing.

Preparation of alkaline phosphatase

5mg of 2000U/mg intestinal alkaline phosphatase was added to 5ml of Milli-Q water in a 15ml conical. The conical was rotated end-over-end for 10 minutes until the powder was fully dissolved. The final 2000U/ml alkaline phosphatase solution was stored in 0.5ml aliquots at -70°C until use.

Source of Human Adult Serum

Human adult blood was obtained from volunteer individuals, spanning from age 22-69, blood was drawn into a tube, and plasma was obtained by centrifugation to remove erythrocytes and white blood cells. This was allowed to clot for 30 minutes at room temperature. Serum was collected by centrifugation at 1400g for 10 min and was stored in 0.5ml aliquots and stored at -80°C. Samples stored in the -80C freezer were thawed, sub-aliquotted into 50uL volumes, and put back into the -80C freezer. For each experiment, sub-aliquoted serum samples were thawed, used for the experiment, and discarded. Each aliquot was discarded after one use so that each aliquot of sera was thawed only once for

each experiment, so no sample was repeatedly frozen and thawed. This allowed us to eliminate any possible effects of repeated freezing and thawing on the serum integrity.

Source of Human Cord Serum

Before the placenta was discarded after birth, blood was removed from the placenta and drawn into a tube. Plasma was obtained by centrifugation to remove erythrocytes and white blood cells. This was allowed to clot for 30 minutes at room temperature. Serum was collected by centrifugation at 1400g for 10 min and was stored in 0.5ml aliquots at -80°C until use.

Mineral formation by 2% normal human serum in a 96-well plate system

After the calcification solution was prepared and equilibrated at room temperature and was aliquoted in 1mL amounts into individual 10x75 Fischer glass tubes. Then, 2% sera from various individuals were into calcification solution in each of their corresponding tubes with continuous mixing by a stir bar. The solutions in each tube containing its corresponding serum were then immediately transferred to our 96 well plate system in quadruplicates, with 4 wells being tested for each sample. Each well contained 200uL from the respective tube in which the sample was originally mixed, and the remainder of the 1mL of solution originally in the tube was used for taking aliquots for testing calcium levels before incubation. The negative control, which only contained serum-free calcification buffer, was also added to the 96 well plate system in quadruplicates to verify that the calcification buffer did not form mineral spontaneously. The 96 well plate system was then capped and wrapped with plastic seran wrap to minimize any side effects of evaporation, and incubated in a 37C incubator for 24 hours. Each of the 4 wells was analyzed for Calcium and crystal count (using a Hemocytometer, see below).

Hemocytometer Analysis

Each 200uL of solution in each well of the 96 well plate was re-suspended by pipetting the solutions each five times to make crystals in solution homogenously spread in each well. Of this re-suspended solution, 10uL were taken and put into the hemocytometer for analysis.

The Hemocytometer was counted under a 40x magnification and later pictured under a 20x magnification. The hemocytometer has a total of 25 large squares, and each individual large squares contain 16 smaller squares within, the top 4 of which were counted for each of the 4 wells. The average and standard deviation of crystals/mL was obtained from counting crystals in each of these 4 wells. This raw count of crystal objects was then converted to crystals/mL of calcification buffer by multiplying the averaged number by 2.5 x 10^5 for conversion into crystals/mL. This formula effectively converts the raw number of crystals to the number of crystals/mL.

Hemocytometry data was analyzed as follows: For each data point, the crystal counts in the four replicates of each serum sample diluted 2% in calcification buffer were averaged (Tables 1-6). The hemocytometer has a total of 25 large squares, and each individual large squares contain 16 smaller squares within, the top 4 of which were counted for each sample. Each sample was counted in duplicates, averaged, and this raw count of crystal objects was then converted to crystals/mL of calcification buffer by multiplying the averaged number by 25 and this then by 10^4 for conversion into crystals/mL. Each data point in Tables 1-6 is the average crystal counts/mL ± standard deviation.

Calcium analysis

After filling the 96 well plate with our test solutions, the 25ul of the remainder of each solution remaining in its original tube was mixed with 25ul of 150mM HCl and stored for assaying. Also, at the 24 hour endpoint of our assay, 50ul aliquots from each well containing cloudy solutions were removed and spun down in an Eppendorf Centrifuge 5415C for 5 minutes at 7,000 rpm. 25ul of the supernatant solution was added to 25ul of 150mM HCl and then quantitatively assayed for calcium by a colorimetric assay purchased from JAS Diagnostics Inc. Calcium levels were determined at a wavelength of 575nm using a Infinite® 200 PRO 96-well plate reader from TECAN Group Ltd.

The difference in calcium concentrations at the end compared to the beginning of incubation in our assay was used to calculate the moles of calcium incorporated into the crystals counted by our assay. The calcium data was analyzed as follows: For each data point, the calcium levels in the four replicates of the serum-free calcification buffer were averaged before and after 24 hours of incubation. The difference between the average calcium level in the quadruplicates before and after 24 hours of incubation was obtained by subtraction. This calculated the change in mM of Calcium for each solution. To convert this to Moles of Calcium incorporated in mineral per mL, we simply multiplied this number by the volume. The calcium levels from the four dilutions containing serum from normal adult humans were also averaged before and after 24 hours of incubation, and the difference between these numbers was taken. This was then again converted to moles of Calcium incorporated into mineral/mL by multiplying by the volume (Table 1-6). This calculation of converting changes in average calcium levels to moles of calcium

incorporated into mineral/mL was repeated for each human serum sample that was diluted (Table 1-6). Each value in Tables 1-6 is the average Moles Calcium Incorporated/mL ± standard deviation.

EDTA testing to confirm that crystals counted by our assay originate from nascent crystal nuclei in the serum

The same general protocol used in the previous experiments with the 96 well plate assay set-up, crystal counting, and calcium analysis was followed to assess the impact of EDTA on crystal growth. The following modifications were made: 5mM EDTA was added to serum from a normal adult human and was incubated at 37C for 15 minutes, and 5mM EDTA was also added to serum from the same normal adult human was also incubated at 37C for 60 minutes. An incubation control was included by adding the same amount of water to the serum of the same normal adult human and incubating this for 60 minutes as well to compare. An additional control was added by diluting 2% serum into Calcification growth solution with 0.1 mM EDTA, such that 1.9mM of Ca would remain in the calcification growth solution.

Each of these samples were separately diluted in individual 10x75mm glass tubes. Each dilution in each tube was aliquoted into four wells of the 96 well plate. Aliquots of each well were taken at 24 hours and analyzed for calcium (See *Calcium Analysis)*. Calcium levels were averaged based on experimental group and data points were shown as the mean calcium level ± standard deviation (Tables 5 and 6).

RESULTS

Development of a 96 well plate system to study the crystal formation activity in serum

In previous studies, Erin Hourigan developed a method to monitor serum crystal formation activity by diluting serum 50 fold with 2mM Calcium and Phosphate at 37C and pH 7.4. Hourigan's assay was kinetic and measured Calcium decline at multiple time-points, and was very labor intensive. In contrast, my assay (Figure 1) directly monitors serum's crystal formation activity by counting the number of crystals present in solution in addition to measuring Calcium decline in solution. Typically, the decline in Calcium is complete at about 6 hours. In my assay, I have allowed crystal growth to reach completion at a 24 hour endpoint, at which time I counted crystals with a hemocytometer. Calcium decline has fallen to the solubility product of mineral at 6 hours but I halt my assay at 24 hours to make sure Calcium levels have fallen to equilibrium and that the reaction has gone to completion.

Our main goal was to test our 96 well plate model system and calcification growth solution with different adult human serum. Sera from different healthy adult humans were tested with each serum diluted 2% by volume in calcification growth solution at room-temperature. These dilutions were mixed in individual tubes, then aliquoted into the 96 well plate system and incubated at 37C for 24 hours. We then counted crystals in each well by using a hemocytometer and measured the level of calcium decline in the solution (see Figure 1).

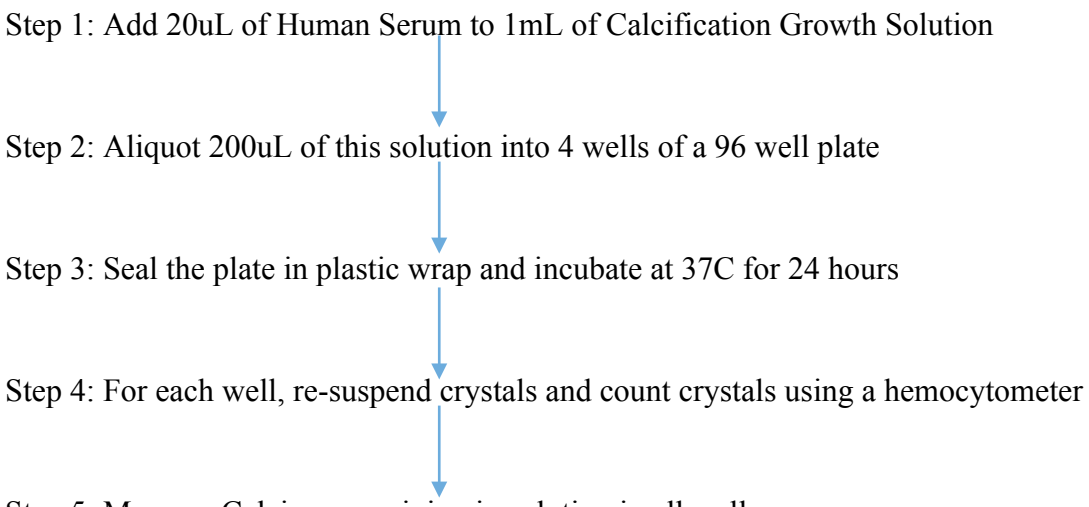

Figure 1. Flow chart of experimental method.

Human sera were diluted to 2% into 1ml of calcification buffer (0.14 M MOPS buffer at pH 7.4 containing 2mM calcium and 2mM phosphate; See *Materials and Methods*) and incubated at 37°C for 24 hours. Serum-free calcification buffer was also incubated at 37°C for 24 hours to verify that our calcification buffer did not form mineral spontaneously. Each of the four solutions with the serum from the same individual diluted in the same calcification buffer was performed in quadruplicate, with each solution distributed into 4 wells in the 96 well system. Aliquots of each solution were taken at 24 hours and analyzed for, crystal counting, and calcium levels (See *Materials and Methods* and Figure 1*)*.

At the start of the experiment, the solutions with and without serum were clear. By hour 24, while the serum-free calcification buffer remained clear, the 2% normal human serum solutions were cloudy. At this time, the mineral was in the form of many small crystals that remained completely suspended in solution. The serum-free

calcification buffer, however, remained clear and free of any detectable mineral throughout the entire 24-hour experiment.

Reproducibility of Assay

We wanted to confirm that variation in average crystals/mL among healthy human individuals was not due to variation in mixing during dilution or well-to-well variation in the 96 well plate system. In order to do this, we tested if serum from a single normal human could reliably and reproducibly form mineral in our calcification system. The degree of reproducibility of this reaction determines the validity of our assay and results. To test this, serum from a single healthy 22 year old adult female was diluted to 2% into 1ml of calcification buffer (0.14 M MOPS buffer at pH 7.4 containing 2mM calcium and 2mM phosphate; See *Materials and Methods*) and incubated at 37°C for 24 hours. At 24 hours, the solutions were re-suspended and crystals were counted using a hemocytometer. Calcium decline in each solution was also measured at 24h, from which we calculated moles of Calcium incorporated into crystals. No significant differences were found in these values (Table 1), validating the reproducibility of our measurements for each individual's serum in our experiments. The well-to-well standard deviation was 2.00% or less or all samples, demonstrating that our method of counting crystals in different wells for each sample has excellent reproducibility. The standard deviation of the average crystal counts for each tube was 2.58 crystals. This suggests that mixing is not a factor in obtaining values of crystal numbers for each sample. Based on this reproducibility in the crystal counting method

and in mixing, all subsequent experiments were performed by using 1 tube for a given sample tested and 4 wells in the 96-well plate.

Table 1. Reproducibility of the Crystal Formation Activity Assay.

An experiment was carried out to determine the reproducibility of the crystal counting assay. The same adult human serum was diluted into 4 different tubes, and each of these tubes were aliquoted into 4 wells of a 96 well plate. After 24 hours of incubation at 37C, the number of crystals were counted using a hemocytometer. This shows that crystal counts obtained for this assay are reproducible for a given individual, and allows us to eliminate mixing as a factor that would contribute to the number of crystals obtained for each sample.

Mixing	Number of crystals counted in 4 units of the hemocytometer grid				Average of Wells 1-4 ($\bar{x}\pm$ SD)	Calculated Crystals/mL ($\bar{x}\pm$ SD) x 10^8
	Well 1	Well 2	Well 3	Well 4		
Tube 1	167	165	162	168	167 ± 2.60	1.67 ± 0.04
Tube 2	165	166	167	163	165 ± 1.71	1.65 ± 0.05
Tube 3	166	159	163	165	163 ± 3.09	1.63 ± 0.07
Tube 4	170	169	172	169	169 ± 1.41	1.69 ± 0.03

Figure 2a. Negative Control

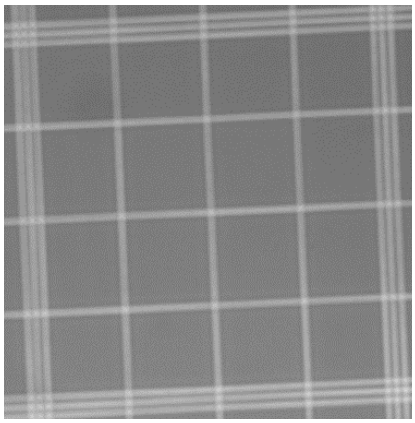

Figure 2b. Example

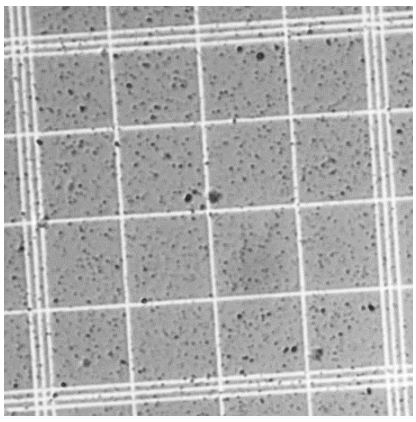

Figure 2. Hemocytometer grid used for Counting Crystals.

Figure 2a is an example of the hemocytometer grid for the negative control, and as expected, no crystals are visible because no serum was added. Figure 2b is an example of the hemocytometer grid when serum is added, and these crystals were counted.

Testing Healthy Adult Human Serum Samples

Serum samples from 5 healthy adult individuals were tested at 2% by volume in my assay. The number of crystals/mL counted by our assay range from 1.42 x 10^8 to 2.46 x 10^8 , with a serum average of 1.99 x 10^8. There are significant differences between these numbers, and this may be attributed to the fact that diet, exercise, age, and gender may be contributing factors to accounting for the variations in the number of crystals/mL among healthy adult humans. The general trend is that the number of crystals/mL decreases with age. Two of the individuals were tested at 2 different times, male 3 was tested at 28 years of age and 32 years of age, respectively, while male 4 was tested at 62 years of age and 69 years of age respectively. For male 3, no significant difference was measured in the number of crystals/mL from the age of 28 to 32 (2.07 x 10^8 to 1.97 x 10^8). For male 4, there was a significant difference ($p<.005$) in the number of crystals/mL from the age of 62 years to 69 years (1.91 x 10^8 to 1.42 x 10^8). While future work may needed to be done to investigate why these differences exist, this may be attributed to the significant decline in bone turnover with age. The number of moles of Calcium incorporated into mineral/mL was not significantly different among the healthy adult individuals, and this measurement was included as a control to ensure that the solubility product of the crystals formed in our assay had been reached by the 24 -hour endpoint.

Table 2. Crystal Formation Activity in Adult Human Serum.

Human Adult serum was tested using 20uL of serum in 1mL of in growth solution containing 2mM Calcium and Phosphate at pH 7.4 and 37C. Results are after 24 hours of incubation. Crystals were counted by using a hemocytometer.

Source of Human Serum	Crystals/mL ($\bar{x}$± SD) x 10^8	Moles of Calcium Incorporated into Mineral/mL ($\bar{x}$± SD) x 10^6
No Serum	0.00 ± 0.00	0.00 ± 0.00
22 y Female 1	1.84 ± 0.09	1.75 ± 0.03
22 y Male 1	2.30 ± 0.07	1.76 ± 0.04
22 y Male 2	2.46 ± 0.09	1.72 ± 0.03
28 y Male 3	2.07 ± 0.06	1.77 ± 0.02
32 y Male 3	1.97 ± 0.04	1.69 ± 0.05
62 y Male 4	1.91 ± 0.06	1.70 ± 0.03
69 y Male 4	1.42 ± 0.10	1.75 ± 0.01

Testing Neonate Human Serum Samples

Serum samples from 4 neonates were tested at 2% by volume in my assay. The number of crystals/mL counted by our assay range from 3.04 x 10^8 to 3.44 x 10^8 , with a serum average of 3.27 x 10^8. The number of moles of Calcium incorporated into mineral/mL was not significantly different among the healthy neonate individuals, and this measurement was included as a control to ensure that the solubility product of the crystals formed in our assay had been reached by the 24 -hour endpoint. There was no significant difference in the number of crystals/mL between different healthy neonate individuals.

Table 3. Crystal Formation Activity in Neonate Serum.

Human Neonate serum was tested using 20uL of serum in 1mL of in growth solution containing 2mM Calcium and Phosphate at pH 7.4 and 37C. Results are after 24 hours of incubation. Crystals were counted by using a hemocytometer.

Source of Human Serum	Crystals/mL ($\bar{x}$± SD) x 10^8	Moles of Calcium Incorporated into Mineral/mL ($\bar{x}$± SD) x 10^6
No Serum	0.00 ± 0.00	0.00 ± 0.00
Neonate 1	3.04 ± 0.08	1.70 ± 0.07
Neonate 2	3.16 ± 0.08	1.69 ± 0.06
Neonate 3	3.44 ± 0.05	1.71 ± 0.07
Neonate 4	3.44 ± 0.10	1.72 ± 0.06

Comparison of Human Adults and Human Neonates

The number of crystals counted by our assay were significantly higher ($p<.0005$, t-test) in Neonates than in adults, and no significant difference in the moles of calcium incorporated into mineral were found between neonates and adults (Table 4). This could be explained due to the fact that the rate of bone turnover is significantly higher in newborns than in human adults, causing a rise in neonate's serum crystal formation activity.

The standard deviation for the serum average of the healthy neonate individuals is smaller than the serum average of the healthy adult individuals, and may be attributed to the fact that diet, exercise, and age would not be contributing factors to accounting for the variations in the number of crystals/mL, as the serum samples were taken at birth.

Table 4. Crystal Formation Activity in Adult Human Serum vs. Neonate Serum.

The number of crystals counted by our assay were significantly higher ($p<.0005$, t-test) in Neonates than in adults, and there was no significant difference in moles of calcium incorporated into mineral/mL between neonates and adults.

	Crystals/mL ($\bar{x}\pm$ SD) x 10^8	Moles of Calcium Incorporated into Mineral/mL ($\bar{x}\pm$ SD) x 10^6
Human Neonates (n=4)	3.27 ± 0.19	1.71 ± 0.01
Human Adults (n=7)	1.99 ± 0.33	1.73 ± 0.03
Significance of Difference	$p<.0005$	Not Significant

Evidence that Crystals counted by Assay originate from much smaller crystals already present in Serum

We also investigated whether crystals counted by our assay originate from the growth of much smaller crystals that are already present in serum or from crystals formed by a serum catalyst during the 24h incubation. If crystals are already present in serum, pre-treatment of serum with excess EDTA should chelate Calcium and cause the crystals to dissolve. We found that pre-treatment of serum with 5mM EDTA dramatically decreased the number of crystals formed in our assay (Table 5). This result supports the hypothesis that crystals counted by our assay originate from the growth of much smaller crystals already present in serum and are not formed by a serum catalyst.

We also found that pre-treatment of serum with EDTA for 60 minutes has significantly lower numbers of crystals counted by our assay than when serum is pre-incubated with EDTA for 15 minutes (Table 2). This may indicate that EDTA takes time to dissolve all the crystals in serum. The number of moles of calcium incorporated into crystals was not significantly different between serum not treated with EDTA versus serum treated with EDTA. The size of each given crystal, qualitatively observed though imaging, decreased as crystal number increased. This suggests that the less number of crystals in solution, the more moles of Calcium are incorporated into each given crystal, contributing to larger crystals.

Table 5. The Effect of Serum pre-incubation with EDTA on the Assay.

Serum was pre-treated with 5mM EDTA and then tested at 2% by volume in growth solution containing 2mM Calcium and Phosphate. This compares the number of crystals counted by our assay, relative size of crystals, and moles of calcium incorporated into mineral/mL for adult human serum that was pre-incubated for 15 minutes versus for 60 minutes.

Source of Human Serum	Crystals/mL ($\bar{x}$± SD) x 10^8	Relative Size of crystals	Moles of Calcium Incorporated into Mineral/mL ($\bar{x}$± SD) x 10^6
No Serum	0± 0	0	0.00± 0.00
Adult Human Serum Without EDTA	1.91 ± 0.02	+	1.76 ± 0.04
Adult Human Serum pre-incubated with EDTA for 15 minutes	0.21 ± 0.01	++	1.76 ± 0.04
Adult Human Serum pre-incubated with EDTA for 60 minutes	0.05 ± 0.00	+++	1.75 ± 0.02

Figure 3a.	Figure 3b.	Figure 3c.
Adult Human Serum (AHS)	AHS with EDTA	AHS with EDTA
Without EDTA	pre-incubated for 15 min	pre-incubated for 60 min

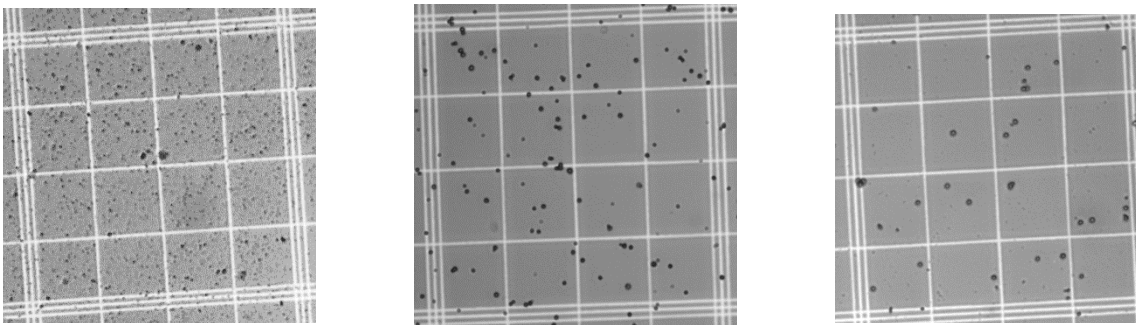

Figure 3. Visual representation of the effect of EDTA pre-incubation on the number of crystals in serum.

After 15 minutes of pre-incubation of EDTA in serum (Figure 3b.), the decrease in the number of crystals (as described in Table 5) can visually be observed using the hemocytometer, as with an increase in the size of individual crystals, as compared to serum without EDTA. After 60 minutes of pre-incubation of EDTA in serum, there is an even greater decrease in the number of crystals, and greater increase in relative size of each crystal, as compared to pre-incubation of EDTA in serum for 15 minutes (Table 5), which can also be visually observed using the hemocytometer.

After seeing that the addition of 5mM EDTA to serum followed by incubation at 37C for 15 minutes and 60 minutes respectively significantly decreased the number of crystals counted by our assay, we wanted to confirm this decrease is a direct cause of the EDTA chelator and not by some other factor such as incubation time. To do this, we added water to 50uL of serum and incubate it for an hour to compare with the EDTA added to 50uL of serum and incubated for an hour. We saw no significant difference in number of crystals counted by our assay, size, or moles of calcium incorporated into

mineral/mL in the serum sample pre-incubated with water for 60 minutes as compared to Adult Human Serum without EDTA (Table 6). This supports the hypothesis that the effect of decline in crystals number counted by our assay is directly due to EDTA chelation and not by the act of incubation.

We also wanted to make sure the EDTA effect of decreasing the number of crystals counted by our assay was primarily due to pre-treatment and was not due to the effect of the small amount of EDTA that accompanied serum when diluted in growth solution. Though the number of crystals/mL counted by our assay decreased when EDTA was added to the calcification growth solution, the decrease in number of crystals was far more drastic when EDTA was pre-incubated with serum (Tables 5 and 6). When EDTA was diluted in the calcification growth solution before serum was added, the number of crystals/mL counted by our assay was 1.21 x 10^8, as compared to the control without EDTA which had 1.91x 10^8(Table 6). In contrast, when EDTA was pre-incubated in serum for 60 minutes the number of crystals/mL counted by our assay was 0.05 x 10^8, as compared to the control without EDTA which had 1.91x 10^8(Table 5). Therefore, almost all of the EDTA effect is due to the pre-incubation of EDTA in serum.

Table 6. Controls for the Effect of EDTA.

Control experiments show that the impact of pre-incubation on serum crystal formation (Table 5) is not due to the 1 hour pre-incubation or the small amount of EDTA in the calcification growth solution.

Source of Human Serum	Crystals/mL ($\bar{x}$± SD) x 10^8	Relative Size of crystals	Moles of Calcium Incorporated into Mineral/mL ($\bar{x}$± SD) x 10^6
No Serum	0± 0	0	0.00± 0.00
Adult Human Serum Without EDTA	1.91 ± 0.02	+	1.76 ± 0.04
Adult Human Serum added to Calcification Growth Solution with 0.1 mM EDTA	1.21± 0.06	+	1.74 ± 0.01
Adult Human Serum pre-incubated with Water for 60 minutes	1.97± 0.04	+	1.77 ± 0.01

DISCUSSSION

Where do the crystals come from?

In my assay, I counted approximately 0.2 billion crystals/mL when adult human serum samples were tested at 2% by volume. This means that in 1mL of 100% serum, there are approximately 10 billion crystals**.** I have shown that these crystals probably originate from the growth of smaller crystals already present in circulation. There are several possibilities for the origin of these crystals, though the exact origin of these crystals is not known.

One possibility is that the crystals originate from bone break down and/or bone buildup in the Bone Multicellular Unit (BMU). The BMU (see Figure 4 [9]) is a vascular compartment with one end having a high level of osteoclasts, which break down bone and release calcium from the bone into the blood when blood calcium levels are low, and osteoblasts, which form bone and store calcium in bone when blood calcium levels are high. These crystals may be formed during the process of bone formation when the mineralization of collagen occurs, at which time some of this mineral may escape into the blood. These crystals also may be formed as a result of bone breakdown by osteoclasts, which releases large amounts of Calcium and Phosphate into the blood within the BMU compartment. These crystals may also be formed in the gut as a mechanism to transport Calcium and phosphate from the gut into the blood.

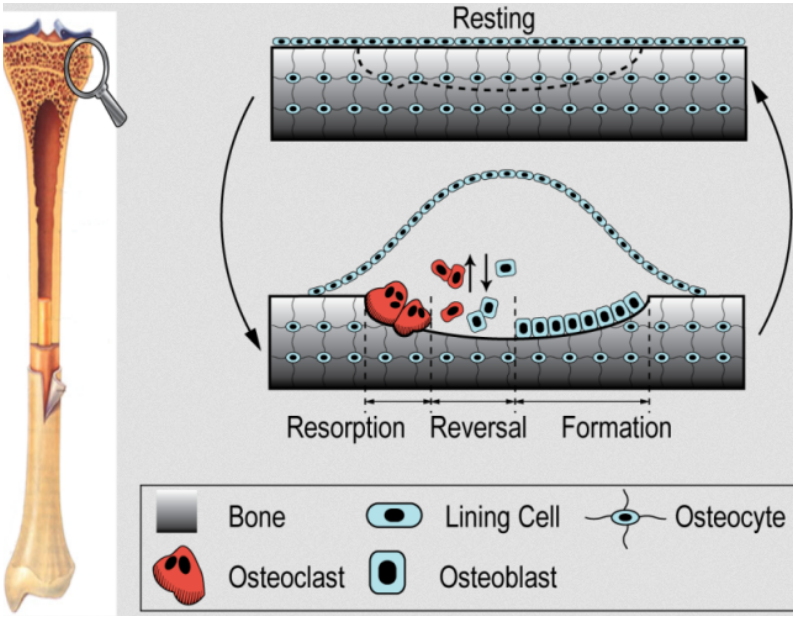

Figure 4. [9] The Bone Multicellular Unit (BMU).

What is the pathophysiological significance of these crystals?

My hypothesis is that the pathophysiological significance of these crystals is that these crystals pass the endothelial layer and cause artery and soft issue calcification. Crystals in blood are extremely small, too small to detect by any method until after they have grown. These Crystals are normally bound to proteins that inhibit their growth. Some crystals in blood may escape the inhibitors that prevent their growth, and become free in blood to penetrate the endothelial layer of the vascular wall into the intima and media and grow to calcify the artery and soft tissue. This process is likely to continue through life, and may account for the fact that hardening of the arteries continue with age.

How could Bone loss and artery calcification be linked?

Patients with the highest rate of bone loss have the highest amount of artery calcification. Our hypothesis is that crystals in blood are the link between bone loss and artery calcification. Elevated bone loss generates crystals in the BMU, which elevates crystals in blood. The increase in the number of crystals in blood increases the number of crystals that pass across the endothelial layer, grow in the intima and media, and calcify the artery.

While much work remains, it is possible that this crystal counting method can be developed into a clinical assay to see if the there is a correlation between the number of crystals in serum and the rate of bone loss. If this correlation is observed, it would support the hypothesis that crystals in blood are the link between bone loss and artery calcification.

How could bone loss increase crystals in blood?

Net bone loss occurs in the BMU whenever bone resorption by osteoclasts exceeds bone formation by osteoblasts. In human diseases with net bone loss, such as osteoporosis and uremia, it is presently unclear whether the defect that causes net bone loss is due to too little bone formation by osteoblasts, too much bone resorption by osteoclasts, or to a combination of the two. Either defect could potentially generate crystals in the BMU, and elevate crystals in blood. If the osteoclast is too active, it will elevate Calcium and Phosphate in the blood within the BMU and thereby form crystals. If the osteoblast is impaired, the defect in collagen calcification could release crystals formed in the BMU into blood.

Future Goals

Our long-term goal is to see if patients with diseases that cause higher levels of bone loss have a higher number of crystals in serum. In order to do such experiments, analysis of many patients would be needed, and would require automation of our crystal counting method. If pictures could be captured for use in programs that could measure the number of crystals, we could compare samples with much less time and potentially automate the assay.

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