Physical factors that trigger cholesterol crystallization leading to plaque rupture

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Abstract

Background: Triggers of plaque rupture have been elusive. Recently it was demonstrated that cholesterol expands when transforming from a liquid to a crystal state, disrupting overlying plaque. This study examined the effect of physical conditions including saturation, temperature, hydration, pH on cholesterol crystallization.

Methods: Graduated cylinders were filled with varying amount of cholesterol powder (1, 2 and 3 g) and dissolved in corn oil at 37 °C. Change in volume expansion (ΔVE) and time to crystallization were measured for each saturation. The same was repeated while varying temperature (22–44 °C); hydration (1–3 ml H2O); pH (5–8.4) and combination of saturation and temperature. Scanning electron microscopy was performed to evaluate crystal morphology and X-ray diffractometry to assess molecular structure of cholesterol.

Results: Increasing saturation raised both ΔVE (3 g: 0.53 ± 0.1 ml vs. 1 g: 0.14 ± 0.02 ml and 2 g: 0.3 ± 0.1 ml; p < 0.0001; p < 0.01) and rate of change over 3 min (3 g: 60% vs. 1 g: 14%). Crystal morphology was the same seen with crystals perforating human plaques. Temperature drop increased ΔVE (44 °C: 0.05 ± 0.01 ml vs. 22 °C: 0.5 ± 0.07 ml; p < 0.0001) and initiated earlier crystallization. Hydration resulted in greater ΔVE (3 ml: 0.7 ± 0.07 vs. 0 ml: 0.1 ± 0.05; p < 0.001) with corresponding changes in cholesterol molecular structure. Rising pH was associated with increased ΔVE (1.3 ± 0.03 ml vs. 0.1 ± 0.02 ml; p < 0.001). Combined increase in saturation and temperature had greater ΔVE than expected from the sum of each alone.

Conclusions: Physical factors influenced both volume and rate of cholesterol crystallization. This suggests that local factors may play an important role in triggering plaque rupture. Combination of several factors may even be a more powerful trigger for acute cardiovascular events.

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Keywords: Cholesterol crystals; Plaque rupture; Myocardial infarction; Stroke

1. Introduction

Plate rupture in the arterial wall is the primary event leading to thrombosis and acute cardiovascular syndromes [1–3]. A potential mechanism of triggering this process was recently defined by demonstrating that as cholesterol crystallizes from a liquid to a solid it forms sharp tipped crystals...
that expand in volume by up to 45% [4]. These crystals were shown to be capable of tearing up fibrous tissues similar in composition to the fibrous caps overlying the atheromatous plaque in the arterial wall [5]. The study was accomplished by melting cholesterol powder and then allowing crystals to form by cooling to room temperature. However, the events in humans would be expected to occur at 37 °C. Examination of human arterial plaques from patients who either died of heart attacks or who had strokes demonstrated similar findings of cholesterol crystals perforating the arterial intima causing damage and disruption of plaques [6] (Fig. 1). Therefore, it would be important to evaluate the physical conditions that can influence cholesterol crystallization under the same environmental conditions that are present in the human body.

In order to better define the in vivo conditions that could lead to cholesterol crystallization and plaque rupture we evaluated the physical stressors that could trigger this event. Therefore, this study was designed to evaluate the behavior of cholesterol crystallization while varying several physical parameters including cholesterol saturation, temperature, hydration and pH.

2. Methods

2.1. Effect of saturation on crystallization

Varying quantities of cholesterol powder (5-cholesten-3β-O; 3β-hydroxy-5-cholesten [C27H46O]: molecular weight = 386.7; 95–98% pure, Sigma–Aldrich) were used to evaluate effects of saturation on crystallization in an oil/water mixture as previously described by Jandacek et al. [7]. The oil/water phase boundary enhances crystallization as known to standard physical chemistry. Briefly, the process involves adding cholesterol powder to 5 ml of corn oil (Spartan Stores, Grand Rapids, MI) in graduated cylinders (Pyrex VISTA, Corning, Inc., Corning, NY) and dissolving by heating with a heat gun (HAG 1400-U, GAR-TEC, Germany). Cholesterol (1, 2, or 3 g) in 18 cylinders (six cylinders for each) was dissolved and 1 ml of H2O added. The peak volume expansion (ΔVE) of crystallization was then recorded while the cylinders were in a 37 °C water bath (VWR 1230 Universal Sheldon Manufacturing, Cornelius, OR). The temperature of the mixture was verified at 37 °C using a K-type thermocouple. The meniscus level of the liq-

Fig. 1. (Top left) SEM micrograph of diagonal coronary artery just below plaque rupture site from a 57-year-old male patient who died with acute coronary syndrome. (Top right; arrow) At higher magnification extensive crystals perforating the intimal surface are noted. (Bottom left) SEM of carotid artery plaque from patient after endarterectomy demonstrating plaque with thin thrombus layer. (Bottom right; arrow) At higher magnification extensive pile up of cholesterol crystals is noted over the carotid intima.
uid cholesterol was registered initially and then the ΔVE of crystal growth were measured at 0, 2, 5, 10 and 30 min. Also, time from start to end of crystallization was monitored and recorded. All of these experiments were conducted simultaneously.

2.2. Imaging of cholesterol crystals

2.2.1. Scanning electron microscopy

Crystals removed from the cylinders after crystallization were placed on filter paper and then dried for 12 h in a vacuum chamber (Speed Vac SC110, Savant Instruments Inc., Famingdale, NY) evacuated by a pump (VP110, Franklin Electric, Bluffton, IN). A sample of crystals was then mounted on stubs and gold coated in a sputter coater (EMSCOPE SC500, United Kingdom). The surfaces were then examined using a JEOL SEM (Model JSM-6300F, JEOL Ltd., Tokyo, Japan).

2.2.2. Confocal microscopy

Another sample of crystals was stained with fluorescent dye (cholesterol Bodipy-C12, Invitrogen, Eugene, OR) at a 1/100 dilution (75% ethanol) in a test tube for 3 min [8]. Samples were then transferred to a slide incubator chamber filled with buffered saline and cover slipped for visualization. Fluorescence images of the crystals were acquired using a Zeiss Pascal LSM microscope (Carl Zeiss, Inc., Jena, Germany). Fluorescence was excited using the 488 nm line of an argon ion laser. Green fluorescence emission was collected using a 505 long pass emission filter.

2.3. Effect of temperature on cholesterol crystallization

A fixed amount of cholesterol powder (2 g) was placed in 18 cylinders and dissolved as above in a corn oil/water mixture. The cylinders (3 for each temperature) were then placed in a temperature regulated water bath at 22, 34, 36, 37, 40 or 44 °C and ΔVE of cholesterol measured. Also, time for crystallization was monitored and recorded.

2.4. Effect of hydration on cholesterol crystallization

As the aqueous and lipid interface is prevalent in tissues and the arterial system, a set of 12 experiments was performed to assess the effects of increasing water (0, 1, 2 and 3 ml, three cylinders each) on the crystallization process. The baseline, no-water experiments used cholesterol powder as received and in the remaining 9 cylinders, increasing amount of water (1–3 ml) was added to 2 g of cholesterol powder while kept at 37 °C in a water bath. The meniscus level of the liquid cholesterol was registered and the ΔVE of crystals measured.

2.5. X-ray diffractometry

In order to assess the molecular structure of cholesterol following hydration, samples of cholesterol crystals formed with and without prior hydration were imaged using Cu Kα radiation to detect changes in the cholesterol crystal structure giving an indication of the level of hydration. This was done using a Sintag XDS 2000 θ2θ diffractometer (Sintag Inc., Cupertino, CA). Patterns were compared with simulated diffraction scans generated using the JEMS diffraction simulation package (P. Stadelmann, CIME-EPFS, Lausanne, Switzerland).

2.6. Effect of pH on cholesterol crystallization

A sodium phosphate buffer (NaHPO4/NaH2PO4) was prepared and added to fifteen cylinders containing 2 g of cholesterol powder in a corn oil/water mixture. Various concentrations of 2 ml of buffer were added to alter pH levels to 5, 6.4, 7.4, 8.0 and 8.4. This was confirmed using a pH meter (Markson, Model 4603, Amber Science, Eugene, OR). All experiments were conducted at 37 °C in a water bath and ΔVE of liquid cholesterol crystal was recorded.

2.7. Combined effects of saturation and temperature on cholesterol crystallization

ΔVE was measured for 1 and 3 g at 22 and 44 °C (12 cylinders with 3 for each combination). These experiments were conducted as above using the corn oil/water mixture.

2.8. Statistical analysis

Instat 3 (Graph Pad, San Diego, CA) was used for statistical analysis. All continuous variables were represented as mean ± standard deviation. One-way ANOVA with Tukey–Kramer multiple comparison post-tests were performed to compare ΔVE among various groups of saturation, temperature, hydration and pH. SAS 9.1 statistical package was used to conduct a two-way ANOVA with interaction to compare ΔVE when the joint effect of temperature and saturation on crystallization was investigated. *p < 0.05 was used to report statistical significance in all tests.

Fig. 2. Effect of saturation on delta change in peak volume expansion of cholesterol *p < 0.001, 3 g vs. 1 g; *p < 0.01, 3 g vs. 2 g; **p < 0.05, 2 g vs. 1 g.
Table 1
Effect of cholesterol saturation and time required to achieve ΔVE

<table>
<thead>
<tr>
<th>Saturation (g)</th>
<th>ΔVE 10 min % (ml)</th>
<th>ΔVE 0–3 min % (ml)</th>
<th>ΔVE 3–5 min % (ml)</th>
<th>ΔVE 5–10 min % (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 (0.14)*</td>
<td>14 (0.02)</td>
<td>50 (0.07)</td>
<td>36 (0.05)</td>
</tr>
<tr>
<td>2</td>
<td>100 (0.32)†</td>
<td>47 (0.15)</td>
<td>35 (0.11)</td>
<td>18 (0.06)</td>
</tr>
<tr>
<td>3</td>
<td>100 (0.53)*†</td>
<td>60 (0.32)</td>
<td>32 (0.17)</td>
<td>8 (0.04)</td>
</tr>
</tbody>
</table>

ΔVE = Change in peak volume expansion, †p < 0.05; 2 g vs. 1 g, *p < 0.001; 3 g vs. 1 g, *p < 0.001; 2 g vs. 1 g.

3. Results

3.1. Effect of saturation on crystallization at 37 °C

By increasing saturation both the ΔVE and rate of expansion were greater. The ΔVE of 3 g saturated cholesterol solution was 3.8× and 1.76× larger when compared to 1 and 2 g ΔVE, respectively (0.53 ± 0.1 ml vs. 0.14 ± 0.02 ml and 0.3 ± 0.1 ml; p < 0.0001; p < 0.01 (Fig. 2). A 2 g saturated cholesterol solution had 2.15× greater ΔVE than 1 g (0.3 ± 0.1 ml vs. 0.14 ± 0.02 ml; p < 0.05). At 3 g saturation, 60% of ΔVE was achieved within 3 min when compared to

![Fig. 3. (Top) Cylinders with increasing cholesterol saturation (left to right) at 37 °C demonstrating greater peak cholesterol crystals expansion (arrows) in an oil/water mixture (middle). Increased cholesterol crystal crystallization with increasing saturation in an oil/water mixture at 37 °C (bottom left). Scanning electron microscopic image of cholesterol crystals formed in the tubes (bar = 20 μm). Crystal morphology is identical to that seen in human arteries shown in Fig. 1 (bottom middle and right). Fluorescence confocal microscopic images of crystals stained with Bodipyn (middle bar = 10 μm; right bar = 50 μm).](image-url)
only 14% for 1 g (Table 1). An example of crystallization with increasing saturation and crystal formation is demonstrated by SEM and confocal microscopy in Fig. 3.

3.2. Crystal morphology

Cholesterol crystals by SEM were found to be in clusters with pointed tipped ends measuring 2.5–5 μm and 10–20 μm at the base. By fluorescence staining, crystals were noted to be clumped and were stained green with the Bodipy dye. The morphology was similar to that noted by SEM (Fig. 3; bottom) and the crystals seen in human arterial plaques (Fig. 1).

3.3. Effect of temperature on crystallization at 2 g cholesterol saturation

A drop in temperature increased ΔVE. The ΔVE at 22°C was 10× higher when compared to 44°C (0.5 ± 0.07 ml vs. 0.05 ± 0.01 ml; *p < 0.0001). Significant differences in ΔVE were noted between 37°C vs. 22°C and 40°C (0.27 ± 0.04 ml vs. 0.5 ± 0.07 ml and 0.1 ± 0.02 ml; *p < 0.01). Also, 40°C vs. 44°C was 0.1 ± 0.02 ml vs. 0.05 ± 0.01 ml (p < 0.05) (Table 2, Fig. 4A). Also, there was a significant difference around physiologic temperatures noted between 34°C vs. 36°C and 37°C (0.4 ± 0.02 ml vs. 0.3 ± 0.02 ml; *p < 0.01; 37°C vs. 40°C (0.27 ± 0.04 ml vs. 0.1 ± 0.02 ml; *p < 0.01). Also, 40°C vs. 44°C was 0.1 ± 0.02 ml vs. 0.05 ± 0.01 ml (p < 0.05) (Table 2, Fig. 4A). Also, there was a significant difference around physiologic temperatures noted between 34°C vs. 36°C and 37°C (0.4 ± 0.02 ml vs. 0.3 ± 0.02 ml; *p < 0.01; 37°C vs. 40°C (0.27 ± 0.04 ml vs. 0.1 ± 0.02 ml; *p < 0.01).

![Fig. 4. (A) Effect of temperature on delta change in peak volume expansion of cholesterol.](image)

![Fig. 4. (B) Effect of temperature on peak volume expansion of cholesterol.](image)

Table 2

<table>
<thead>
<tr>
<th>Physical trigger</th>
<th>Temperature (°C)</th>
<th>Peak volume expansion (ΔVE) ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>22°C</td>
<td>0.5 ± 0.02</td>
<td>*p &lt; 0.0001, 22°C vs. 44°C; *p &lt; 0.05, 34°C vs. 36°C; *p &lt; 0.05, 44°C vs. 40°C</td>
</tr>
<tr>
<td>30°C</td>
<td>1 ml</td>
<td>*p &lt; 0.0001, 24°C vs. 44°C; *p &lt; 0.05, 34°C vs. 36°C; *p &lt; 0.05, 44°C vs. 40°C</td>
</tr>
<tr>
<td>36°C</td>
<td>2.5 ml</td>
<td>*p &lt; 0.0001, 24°C vs. 44°C; *p &lt; 0.05, 34°C vs. 36°C; *p &lt; 0.05, 44°C vs. 40°C</td>
</tr>
<tr>
<td>40°C</td>
<td>3 ml.</td>
<td>*p &lt; 0.0001, 24°C vs. 44°C; *p &lt; 0.05, 34°C vs. 36°C; *p &lt; 0.05, 44°C vs. 40°C</td>
</tr>
</tbody>
</table>

ΔVE = Change in peak volume expansion; temperature: *p < 0.0001, 22°C vs. 44°C; *p < 0.05, 34°C vs. 36°C; *p < 0.05, 44°C vs. 40°C; *p < 0.01, 37°C vs. 40°C; *p < 0.01, 40°C vs. 44°C; hydration: *p < 0.0001, 3 ml vs. 0 ml and *p < 0.001, 2 ml vs. 0 ml and 1 ml; *p < 0.01; 8.4 vs. 8.0.
Fig. 5. Effect of hydration on delta change in peak volume expansion of cholesterol † * p < 0.001, 3 ml vs. 0 and 1 ml; † † p < 0.001, 2 ml vs. 0 and 1 ml.

0.3 ± 0.05 ml and 0.27 ± 0.04 ml; p < 0.05). A drop in temperature (22 and 34 °C) initiated crystallization earlier, within the first minute when compared to 37 °C or higher temperatures (40 and 44 °C) (Fig. 4B).

3.4. Effect of hydration at 37 °C and 2 g cholesterol saturation

Increasing hydration significantly increased ΔVE of crystallized cholesterol. ΔVE with both 3 and 2 ml of H2O was 7 × and 2.1 × higher than with 0 and 1 ml of H2O (0.77 ± 0.07 and 0.7 ± 0.02 ml vs. 0.1 ± 0.05 and 0.33 ± 0.07 ml respectively; p < 0.001). However, no significant difference was noted between 2 and 3 ml H2O (Table 2; Fig. 5).

3.5. X-ray diffractometry

X-ray diffractometry (XRD) indicated major changes in the cholesterol signature between the samples of cholesterol crystals with and without hydration. Fig. 6A presents an XRD scan of the cholesterol without hydration and Fig. 6B with hydration. While the XRD peaks appear in the same positions, indicating the same cholesterol unit cell, the differences in the peak height suggest significant differences in the atom/molecule locations within the unit cell. This was related to the addition of OH groups to the basic cholesterol molecule.

3.6. Effect of pH

Alkaline pH was associated with higher peak volume cholesterol crystallization. At pH 8.4 the ΔVE was 13 ×, 3.7 ×, 2.4 × and 1.2 × higher than that of pH of 5.0, 6.4, 7.4, 8.0, respectively (1.3 ± 0.03 ml vs. 0.1 ± 0.02 ml, 0.35 ± 0.05 ml and 0.54 ± 0.05 ml; p < 0.001 and 1.1 ± 0.05 ml; p < 0.01) (Table 2; Fig. 7).

3.7. Combined effects of saturation and temperature

When the two triggers were combined, ΔVE greatly exceeds the sum of expansion due to the individual contribution of each trigger alone. Higher ΔVE were noted
when comparing the extreme of cholesterol saturation and temperature. Expansion with 3 g of cholesterol saturation at 22 °C was greater than with 3 g at 44 °C (1.3 ± 0.05 ml vs. 0.88 ± 0.03 ml; \( p < 0.0006 \)). However, 1 g saturation at 22 °C was similar to 1 g at 44 °C (0.2 ± 0.1 ml vs. 0.2 ± 0.1 ml; \( p = \text{ns} \)).

4. Discussion

The underlying cause of heart attacks and strokes is related to plaque rupture and thrombosis. The mechanism of this process was recently proposed by us as the tearing of fibrous tissue in the artery due to expansion of cholesterol when crystallizing from a liquid to a solid [4–6]. However, the triggers for cholesterol crystallization are unknown. In this study we experimentally evaluated physical conditions that are known to influence crystallization, including: saturation, temperature, hydration state and pH.

In our earlier work we also demonstrated that by avoiding ethanol in tissue processing it was possible to demonstrate that the process of crystallization was widely disseminated within the arterial wall near and at the site of plaque rupture [5,6]. This was confirmed in arteries of patients who had died of acute cardiovascular syndrome. Cholesterol crystals were seen perforating plaques at those sites. Prior investigation has demonstrated the presence of cholesterol in the atheromatous plaques in various physical states including liquid, liquid-crystal and crystal state [9]. Also, Jandacek et al. reported on the formation of cholesterol crystals at 37 °C by dissolving cholesterol in corn oil and exposing this solution to a water interface causing crystallization [7]. We used a similar approach in order to evaluate the effect of saturation on crystallization at 37 °C. Saturation is a basic pre-requisite for crystallization and may be a major driver to plaque rupture. One of the primary features of a vulnerable plaque is a large lipid pool [10–12]. Also, in the early studies on ruptured plaques, Davies et al. reported that the extra-cellular lipid pool size was a critical feature of the ruptured plaque [10]. These observations fit into our model where even in a test tube, the amount of cholesterol in solution becomes a critical factor in the extent of ΔVE as well as the rate of cholesterol crystallization. It further confirms that a large cholesterol load is a critical feature in the plaque vulnerability to rupture [13–15]. Moreover, clinical data have demonstrated that softer lesions are most prone to acute events [16]. In fact this would also fit the composition of the plaques with the most liquid cholesterol content.

The effect of temperature is also another well-known key factor to crystal formation. Cholesterol crystallization occurs with greater ΔVE and at a faster rate with decreasing temperature. This points to a potential link for increased acute cardiovascular events noted with exposure to cold temperature, especially during snow shoveling [17,18]. Also, cardiovascular events have been known to cluster in the early morning hours [19]. Although humans are warm blooded mammals, there is diurnal temperature variation of a few degrees centigrade with lower temperatures occurring in the early morning hours [20]. Given our data with an overall trend of greater crystallization with lower temperatures around physiological ranges 34–37 °C this raises the possibility that even a small drop in temperature (i.e. 1 °C) may be sufficient to trigger cholesterol crystallization especially in combination with other physical factors.

The predominant form of cholesterol detected in atherosclerotic plaques taken from humans is cholesterol monohydrate as was demonstrated by X-ray diffraction studies by Bogren and Larson [21]. Because the hydration of the cholesterol molecule could impart some water property behavior, we evaluated the amount of hydration on crystal growth and expansion as would be expected for water transforming into ice [22]. Our study clearly supported this hypothesis and indicates that more hydration was associated with greater volume expansion. We demonstrated X-ray diffraction changes in the Bragg peak intensities between no water and hydrated cholesterol molecules. This is critical given the extensive water environment available in tissues and circulating blood.

Finally, pH is another local physical factor that we tested since that can vary with inflammation as well as shifting electrolytes within the plaque. It may be difficult to reconcile this feature because we demonstrated an increasing pH enhances crystallization but inflamed plaques seem to be more acidic. In fact, some investigators have already attempted the use plaque pH as a potential measure for vulnerability to rupture [23].

Many of our observations fit into the clinical paradigm of cardiovascular events. One specifically relates to an increased occurrence of heart attacks after a febrile illness [24]. The rise of body temperature to 40 °C could help dissolve cholesterol into a liquid state forming a supersaturated solution within the plaque which after body temperature returns to normal (37 °C) would trigger crystallization. Another relates to the potential of bleeding into an atheromatous plaque [25]. In addition to increasing the cholesterol load from the RBC membranes, the increase in the amount of water in the plaque can make the crystals grow to a larger volume as we have shown. Although further studies would be needed to confirm these findings in vivo, the physical conditions we investigated are known to occur and can greatly influence crystallization as we have demonstrated.

Overall, we have provided a strong paradigm for the role of local physical factors that may trigger cholesterol crystallization alone or in combination. We have already demonstrated that expanding cholesterol crystals can tear fibrous membranes and coronary and carotid plaques in the arterial wall of humans (4–6). By evaluating various physical features that can trigger cholesterol crystallization we have provided a potential mechanism for plaque rupture. Thus, the conclusion of these findings is that shifts in environmental factors including local saturation of cholesterol, temperature, pH and hydration status could alone or in various combinations lead...
to crystallization with sudden and forceful volume expansion triggering plaque rupture and thrombosis causing heart attacks and strokes. However, it should be recognized that the in vivo conditions can be much more complex as was demonstrated by the combination of two physical factors (saturation and temperature). Therefore a better understanding of these physical parameters may be critical in explaining many of the clinical observations regarding acute cardiovascular events.

Conflicts of interest

None.

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References