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Research paper

Effect of aspirin on cholesterol crystallization: A potential mechanism for plaque stabilization

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ABSTRACT

Background and aims: Cholesterol crystals (CCs) have been found to be critical in the evolution and progression of atherosclerotic plaque leading up to rupture. This includes triggering inflammation and mechanically traumatizing the plaque and surrounding tissues. Thus, inhibition of crystal formation and degrading the crystals could be an important therapeutic approach in the prevention of cardiovascular events. Because of its physico-chemical properties we examined the effect of aspirin (ASA) on cholesterol crystallization.

Methods: A first experiment tested three amounts of cholesterol (1, 2, 3 g) with a wide range of ASA (0–60 mg) on cholesterol crystallization and volume expansion. A second experiment tested the effect of CCs with and without ASA in perforation of fibrous membrane during crystallization. A third experiment evaluated the effect of ASA on melting CCs in human atherosclerotic plaques. Scanning electron microscopy (SEM) was used to evaluate crystal morphology.

Results: Aspirin significantly inhibited cholesterol crystallization and volume expansion in a dose related fashion and even at physiologic levels (0.3 mg/ml). Moreover, ASA prevented perforation of fibrous membranes. By SEM, crystals in human atherosclerotic plaques were found melted with ASA.

Conclusions: Cholesterol volume expansion during crystallization was significantly inhibited and CCs were dissolved in the presence of ASA. Fibrous membranes were not perforated with ASA because of both these effects.

1. Introduction

Studies have demonstrated that cholesterol crystals (CCs) play a critical role in atherosclerotic plaque formation, inflammation and rupture [1–4]. We had previously demonstrated that cholesterol expands rapidly during crystallization forming sharp tipped crystals that can damage fibrous membranes [3,4]. When this process occurs in a confined space of an atherosclerotic plaque, it has the potential to cause plaque rupture. We and recently others have also demonstrated that statins have a direct effect on dissolving CCs and inhibiting their formation [5,6]. This property of statins may stabilize vulnerable plaques beyond their effects on lowering serum lipids. Because aspirin (ASA, acetyl salicylic acid) is an established agent that has been used to decrease the risk of cardiovascular events [7], we chose to evaluate its direct effects on cholesterol crystallization.

Aspirin is an organic chemical with alcohol and lipid properties. Such a chemical structure lends to potential interaction with other lipids such as cholesterol [8,9]. Thus, its chemical properties could then be tested to evaluate if ASA may also have a direct effect on inhibiting cholesterol crystal formation as we have seen with statins and ethanol

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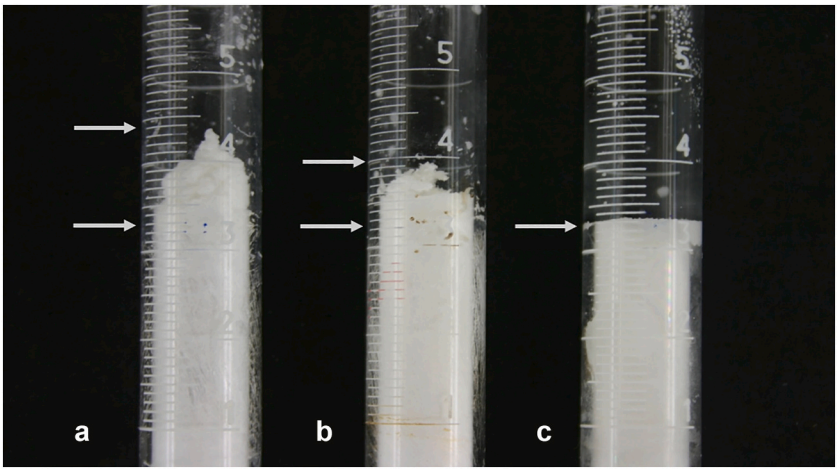

Fig. 1. Change in volume expansion (ΔVE) during crystallization. Graduated cylinders demonstrating crystal expansion with (a) pure cholesterol (3 g); (b) partial attenuation in ΔVE with cholesterol and aspirin at 10 mg; (c) total attenuation of ΔVE with cholesterol and aspirin at 50 mg.

[5,6,10]. Therefore, in this study we evaluated the direct effects of ASA on cholesterol crystal volume expansion and crystal formation in vitro and ex-vivo in human atherosclerotic plaques with formed CCs.

2. Materials and methods

2.1. Effect of aspirin on cholesterol crystallization in-vitro

Purified cholesterol powder (5-cholesten-3β-ol; 3β-hydroxy-5-cholestene [$C_{27}H_{46}O$]: molecular weight = 386.7; 95–98% pure, Sigma, St. Louis, MO) was melted in 10 ml graduated cylinders (Pyrex VISTA, Corning Inc., Corning, NY) using a heating gun (HAG 1400-U, GAR-TEC, Baden, Germany) and volume expansion measured as previously described [5]. Briefly, the meniscus level of liquid cholesterol (V1) obtained upon melting was noted. The cylinder was then allowed to cool for 10 min at room temperature and maximal peak of CCs formed (V2) was noted. The peak volume expansion (ΔVE) was calculated by subtracting V1 from V2. Varying doses (0-60 mg) of aspirin (acetyl salicylic acid [$C_9H_8O_4$] Sigma-Aldrich Co. St. Louis, Mo) were then mixed with cholesterol, the mixture was then melted and ΔVE measured as mentioned above.

The experiment was repeated three times at varying quantities of cholesterol powder (1, 2, 3 g) using a range of ASA doses (0–60 mg) and results averaged for each dose. The crystals were then examined by scanning electron microscopy (SEM). The morphology of CCs formed with ASA was compared to cholesterol controls.

To match the therapeutic plasma concentrations of ASA, the experiment was repeated by dissolving ASA in distilled water at a concentration of 0.3 mg/ml and 3 mg/ml with cholesterol powder (3 g) and melted. The experiment was repeated eight times each with ASA solution or distilled water as a control. The ΔVE was measured and averaged for each and recorded.

A control experiment was performed using a combination of cholesterol powder (2 g) with a range of ASA doses (0–60 mg) and compared to sodium chloride (NaCl: 50 mg) with 2 g cholesterol in 3 cylinders.

2.2. Evaluate the effect of aspirin on membrane injury by cholesterol crystals

Rabbit pericardium was harvested from four normal New Zealand White rabbits after administering euthanasia using 1 ml of Euthasol (DelMarva Labs, Midlothia, Va., USA) intravenously via marginal auricular vein. These tissues were chosen because their composition and thickness were similar to the fibrous cap of an atherosclerotic plaque. Animal procedures were performed according to Michigan State University's Animal Care and Use Committee approved protocol (Institutional Animal Care and Use Committee # 03/18-034-01).

Cholesterol crystals were grown in 4 ml glass test tubes with a membrane spread taut over the tube orifice. Cholesterol powder (4 g) was placed in a graduated cylinder, melted using the heat gun and then poured into the test tube filling it to the rim. The liquefied cholesterol was then allowed to cool down at room temperature. Once the crystallization started, the membrane was quickly spread taut on the tube orifice and secured by a 4-0 silk tie around the tube creating a confined space. After crystallization, the membrane surfaces were then inspected, photographed by a digital camera and the membrane processed by SEM to examine for CCs.

2.3. Effect of ASA on cholesterol crystals in human atherosclerotic plaques

Fresh human atherosclerotic plaques from endarterectomy procedures were cut into equal halves and one half incubated in a water bath for 48 h at 37 °C using physiologic amount of ASA as present in humans (25 mg/ml physiologic buffered saline, PBS) and the other half incubated in PBS alone. Atherosclerotic plaques including carotid and peripheral artery were obtained from endarterectomy procedures. Specimens were de-identified, consistent with Sparrow Hospital and Michigan State University IRB approval (# 0518-exempt and no consent was required). Treated plaques were then prepared for light microscopy and SEM.

2.4. Microscopy

2.4.1. Light microscopy

Atherosclerotic plaques were fixed in 4% glutaraldehyde, then processed and embedded in paraffin blocks, serially sectioned and mounted on glass slides. These were then stained with hematoxylin and eosin and examined using a light microscope.

2.4.2. Scanning electron microscopy

Pericardial membrane and atherosclerotic plaques were fixed overnight in buffered 4% glutaraldehyde, then cut into 5 mm segments and air dried as previously described [5]. Samples were then mounted on stubs and gold coated in an EMSCOPE SC500 sputter coater (UK) and the surface examined using a JEOL scanning electron microscope (Model JSM-6300F, JEOL Ltd., Tokyo, Japan). Also, samples from both control and ASA in vitro experiments were collected and scanned for their morphology.

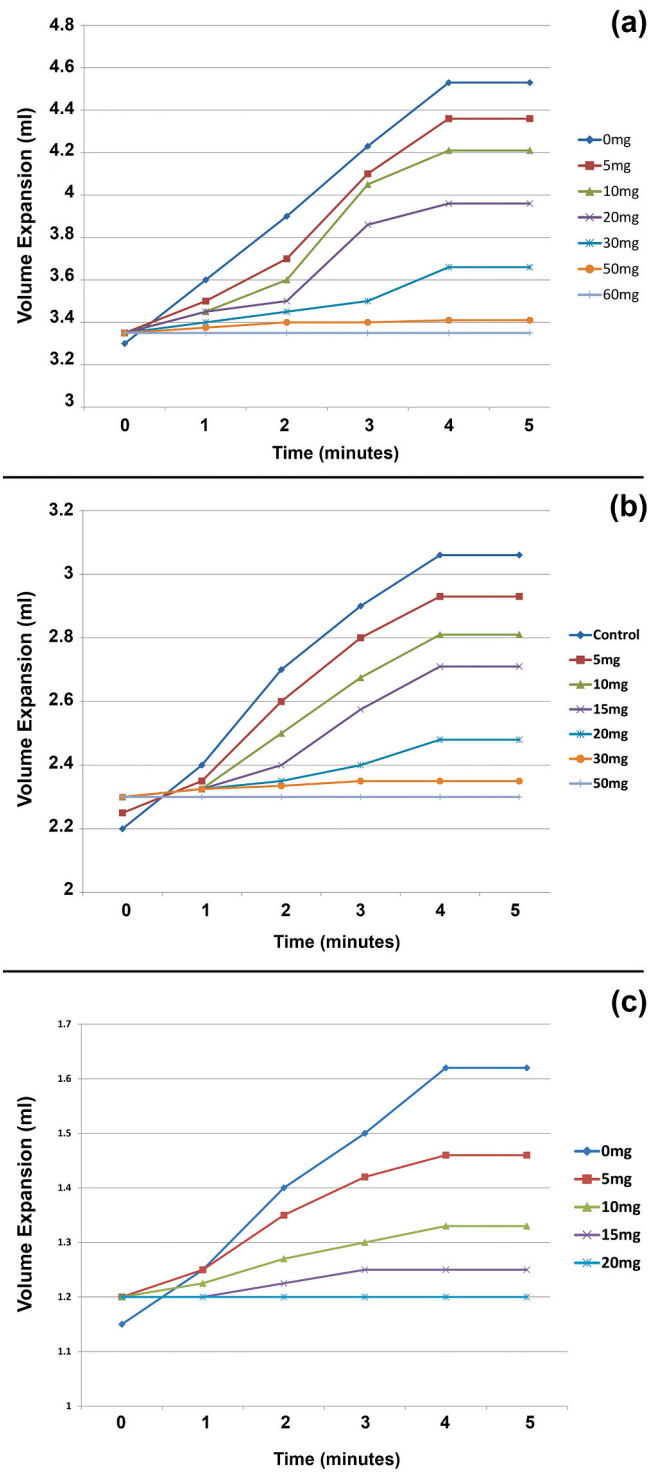

Fig. 2. Absolute volume expansion with and without aspirin. Effect of aspirin on volume expansion during cholesterol crystallization (a = 3 g, b = 2 g, c = 1 g cholesterol).

2.4.3. *Transmission electron microscopy*

Tissue fixed in buffered 4% glutaraldehyde was embedded in epoxy resin and ultrathin sections of 80 nm thickness were placed on copper grids, stained with uranyl acetate and lead citrate and examined by a transmission electron microscope (JEM 100-Cx II, JOEL Ltd).

2.5. *Statistical analysis*

Instat 3 (Graph Pad, San Diego) was used for statistical analysis. Data was represented as mean ± standard deviation. One way ANOVA with Tukey-Kramer multiple comparison post tests were performed to

Table 1
Effect of increasing dose of aspirin on cholesterol volume change.

Aspirin (mg)	0	5	10	15	20	30	50	60	p value
ΔVE (ml) 1 g choles	0.46 ± 0.02	0.26 ± 0.02	0.13 ± 0.02	0.05 ± 0.0	0	–	–	–	<0.001
ΔVE (ml) 2 g choles	0.86 ± 0.02	0.68 ± 0.02	0.51 ± 0.02	0.39 ± 0.01	0.16 ± 0.02	0.05 ± 0.0	0	–	<0.001
ΔVE (ml) 3 g choles	1.23 ± 0.05	1.01 ± 0.02	0.86 ± 0.02	–	0.61 ± 0.02	0.31 ± 0.02	0.06 ± 0.02	0	<0.001

ΔVE – volume change; choles - cholesterol.

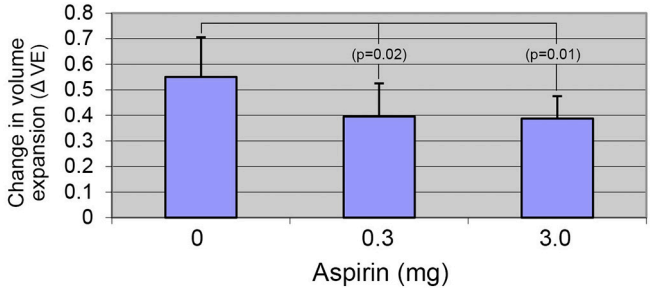

Fig. 3. Change in volume expansion (ΔVE) at physiologic level of aspirin. Reduction of ΔVE at physiologic levels of aspirin at 0.3 mg compared to higher dose at 3.0 mg.

compare peak volume expansions at various doses of ASA. Two tailed unpaired Student *t*-test was used to compare peak volume expansions after addition of NaCl and ASA. $p < 0.05$ was used to report statistical significance in all tests.

3. Results

3.1. Effect of ASA on cholesterol crystallization in vitro

Increasing dose strength of ASA progressively decreased ΔVE for all amounts of cholesterol used (1, 2, 3 g) (Figs. 1, 2), Table 1.

Significant reduction in ΔVE was noted with increasing doses of ASA when compared to 0 mg ASA control. The greater amount of cholesterol had a significantly greater volume expansion ($p<0.001$) and required more ASA to completely inhibit volume expansion.

ASA at therapeutic concentrations (0.3 mg/ml and 3 mg/ml) also caused significant reduction in ΔVE ($p = 0.003$; $p < 0.02$ respectively) (Fig. 3).

When 50 mg of NaCl was added to 2 g of cholesterol there was no significant change in ΔVE from distilled water alone as control (0.86 ± 0.02 vs. 0.81 ± 0.02 ml; $p > 0.05$).

By SEM, the crystal morphology with ASA was significantly altered with loss of the sharp tips and edges of CCs compared to controls (Fig. 4). Staining the pericardial membrane with toluidine blue and section by TEM revealed the collagen rich tissue (Fig. 5).

3.2. Effect of aspirin on membrane injury by cholesterol crystals

The addition of ASA attenuated cholesterol expansion and damage to the pericardial membrane in its path at the mouth of the tube by direct visualization and SEM (Fig. 6). In the control tubes, SEM revealed CCs perforating the membrane during crystallization while ASA prevented membrane damage by reducing cholesterol crystal expansion and altering crystal morphology. Crystal structure was markedly altered where normal sharp tipped CCs appeared to be blunted and smaller in the presence of ASA.

3.3. Effect of aspirin on human arterial plaques

SEM of the carotid artery plaques incubated in PBS (control) revealed dense, well-formed sharp-edged clusters of both rhomboidal and needle shaped CCs (Fig. 7). In contrast, ASA treated carotid samples revealed

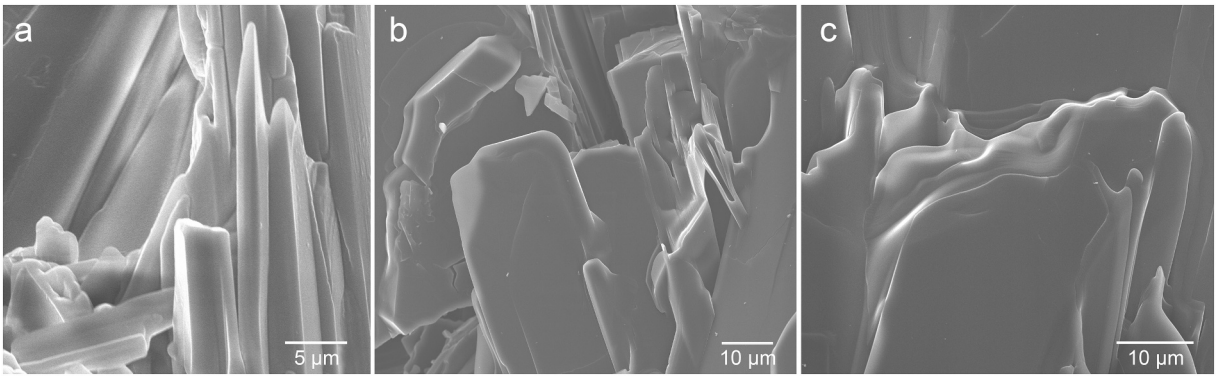

Fig. 4. Effect of aspirin on cholesterol crystal morphology (a) SEM demonstrating cholesterol crystals with sharp tipped edges; (b, c) SEM demonstrating blunted tipped cholesterol crystals when formed in the presence of aspirin.

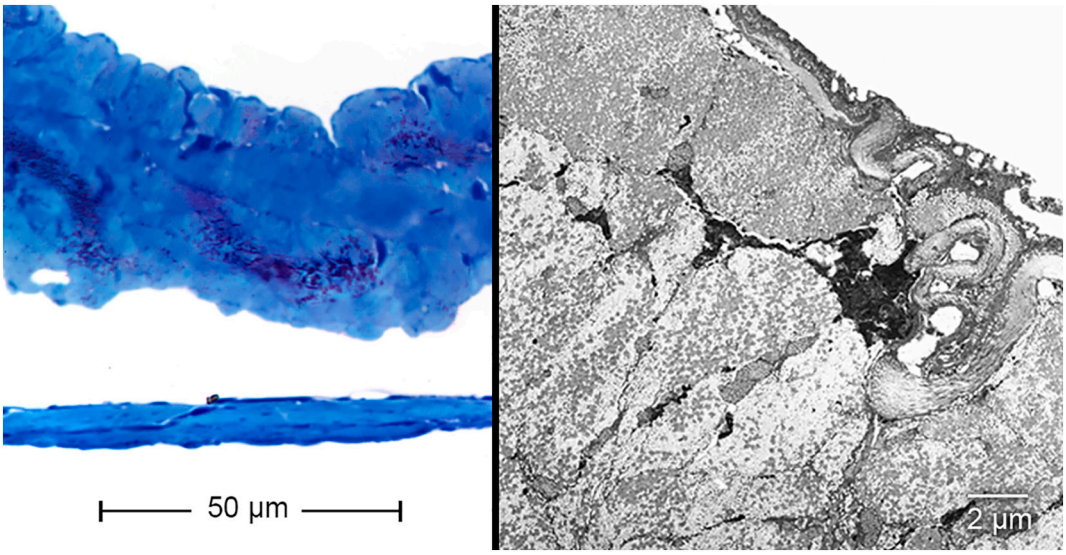

Fig. 5. Composition of pericardial membrane. Histology of pericardial membrane stained with toluene blue and transmission electron microscopy of the same tissue demonstrating extensive collagen composition. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

significant dissolving, less dense crystals with blunted tips and altered geometry. The otherwise sharp edged, dense rhomboid and needle shaped crystals appeared to be melting, fragmented, less dense crystals with rounded edges with ASA. The samples treated with ASA showed significantly less dense cholesterol crystal load when compared to their matched halves incubated in PBS.

4. Discussion

The cardiovascular protective effect of ASA has been attributed primarily to its antiplatelet properties [11,12]. However, in vivo ASA may have a more complex action on the vasculature that remains to be elucidated. Cyrus et al. demonstrated that ASA has additional in vivo properties which include decrease in the size of atherosclerotic lesions with less macrophages and cholesterol, consistent with a more stable plaque phenotype [13]. Also, Ranke et al. demonstrated that the natural course of early carotid atherosclerosis can be slowed with ASA treatment in a dose-dependent fashion [14]. In another study it was proposed that ASA has more profound effects on plaque stability by inhibiting endothelial dysfunction [15]. All these studies suggest that ASA has more profound effects on atherosclerotic plaques independent of its antiplatelet properties.

Our study demonstrated that ASA can directly inhibit cholesterol crystallization and dissolve CCs in a dose-dependent fashion that can help prevent plaque rupture. First, the volume expansion of cholesterol was significantly reduced and completely attenuated at high doses of ASA (>20 mg/1 g cholesterol). Second, the sharp tipped crystal morphology was altered to blunted and rounded morphology with melting noted in the CCs. Third, these effects on CCs were noted at physiologic doses of ASA. Fourth, the melting effect of ASA was noted in CCs in human atherosclerotic plaques. Thus, these effects can be viewed as potential mechanisms that could explain the more complex mechanism of action of ASA independent of the thromboxane A_2 pathway [16]. The increasing doses of ASA progressively decreased the volume expansion of cholesterol which is consistent with the study by Ranke that indicated the course of carotid atherosclerosis can be slowed by ASA in a dose-dependent fashion [14]. However, in that study the beneficial effects of ASA were attributed to its anti-inflammatory effects but given that CCs trigger inflammation, inhibiting the formation of CCs would therefore reduce the inflammation.

Inflammation is a response to injury and in this case, CCs are the injurious agent. Treatment that is focused primarily on the inhibition of the inflammation response is fraught with major complications, typically systemic infections and potential for activation of cancer [17,18]. However, blocking the underlying cause for the inflammation by inhibition and/or dissolving of CCs may prove to be more effective and a safer approach. In our study, we demonstrated that larger quantities of cholesterol required higher doses of aspirin to suppress volume expansion during crystallization. This implies the need to investigate the effectiveness of higher doses of aspirin and enhanced modes of delivery of aspirin that can penetrate large lipid cores.

The volume expansion of cholesterol upon crystallization from liquid to solid state has significant implications in plaque rupture and thrombosis [3–5]. A large lipid core with a thin fibrous cap is known to be the hallmark of a vulnerable plaque [19]. Crystallization and expansion of the supersaturated core lipid pool in the compact environment of the vulnerable atherosclerotic plaque can lead to disruption of the plaque cap which in turn leads to arterial thrombosis. Thus, we propose that approaches that inhibit cholesterol crystallization can provide direct therapeutic benefit. Currently, we are conducting experiments to quantify the effect of ASA on the extent of crystallization of cholesterol, the crystallization behavior and the molecular structure of cholesterol at different temperatures.

We have previously demonstrated that the local physico-chemical conditions can influence cholesterol crystallization. These include a small drop in local temperature (1–2 °C), increasing cholesterol saturation, a basic pH and hydration of the cholesterol molecule to the monohydrate form [20]. Basic physical chemistry principles teach that certain molecules can interfere with the formation of the crystal lattice and disrupts the crystal. This could explain the alteration in the cholesterol crystal formation in the presence of ASA. The alcohol and lipid domains of the ASA molecule have the features needed to interact with cholesterol as already demonstrated in the cell membrane [8]. This feature may not be unique to aspirin and may be shared with a variety of other compounds which can inhibit cholesterol crystallization and may have major therapeutic implications. Both statins and α-cyclodextrin

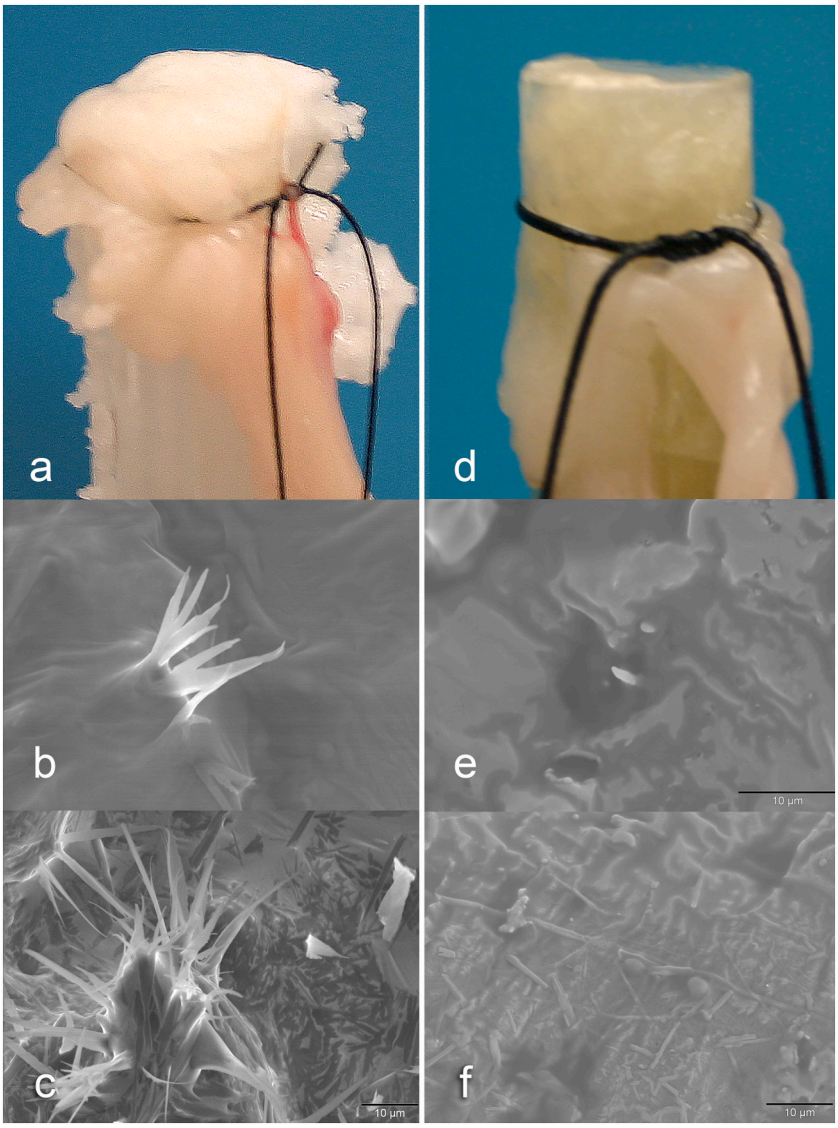

Fig. 6. Effect of aspirin on fibrous tissue injury. (a) Tube with cholesterol crystals expanding and disrupting pericardium during crystallization. (b, c) SEM of cholesterol crystals perforating the pericardial membrane during crystallization. (d) Tube with cholesterol crystals and aspirin (50 mg) with minimal encroachment on the pericardial membrane. (e, f) SEM of cholesterol crystals formed in the presence of aspirin demonstrating very few crystals perforating the membrane surface.

have been shown to have similar effects on volume expansion and CCs formation [5,6].

Limitations of our study include the in vitro setting of the experiments. However, studies of human plaque and aspirates from culprit coronary arteries during acute myocardial infarction reveal similar crystal morphologies and composition as our in vitro model [3,21]. Never-the-less, the observations in our study are compelling and suggest the role of cholesterol as a direct mechanical injurious agent that can be altered by ASA. In this study we used a simple yet effective model to evaluate aspirin on cholesterol crystals formation. This model was verified by the similar findings in ruptured human plaque [4].

In conclusion, we have demonstrated that ASA can alter the physical state of cholesterol crystallization. This is based on the reduction in the forceful rapid volume expansion as well as altered crystal geometry by the blunting of sharp-edged crystals that can damage the plaque cap. This can be an additional mechanism that explains the role of ASA in prevention of plaque rupture. Our model demonstrates a potential tool that can be used to evaluate other agents to prevent acute cardiovascular events by reducing mechanical damage induced by the physical state changes of CCs within atherosclerotic plaques while attenuating their inflammatory response.

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CRediT authorship contribution statement

LF, SK, KA, AV, and GSA study concept and design; SK, KA, and AV performed experiments; LF, AL, AV, and GSA performed analysis and interpretation of data; LF, AL, and GSA drafted the manuscript; LF, AL, SK, KA, AV, and GSA performed critical revision of the manuscript for important intellectual content.

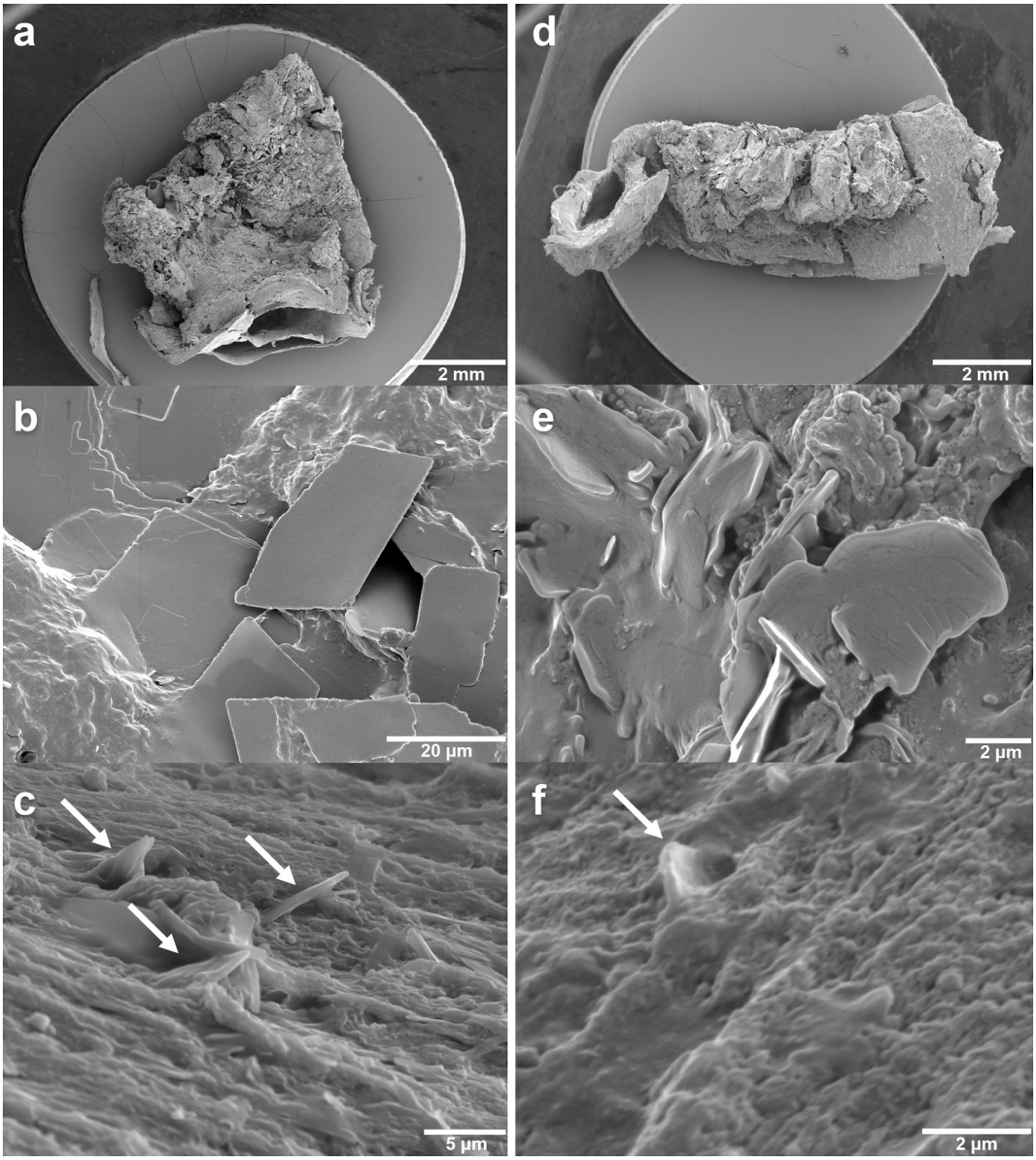

Fig. 7. Effect of aspirin on cholesterol crystal morphology. Scanning electron micrographs of matching segments of human carotid plaques (left: a, b, c) low power and higher power demonstrating presence of sharp edged crystals in plaque incubated in physiologic buffered saline. (right: d, e, f) Segment of plaque incubated physiologic buffered saline with aspirin demonstrates plaque with loss of sharp edges and fewer crystals perforating the intimal surface.

Declaration of competing interest

Dr. George Abela is a speaker for Espiron and consultant to Amarin. No other author has any disclosures.

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