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Impact of epicatechin on fibrin clot structure

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Abstract: Fibrin clot structure and function are major determinants of thromboembolic diseases. The study aim was to determine the impact of epicatechin (a flavonoid with cardiovascular protective effects) on fibrin clot structure and permeability.

Plasma samples from 12 healthy subjects were incubated with increasing concentrations of epicatechin. Turbidity of fibrin clot was analyzed by absorbance measurement at 405nm. The fibrin clot nanostructure was determined by scanning spectrometry (wavelength from 500 to 800nm) and fibrin fiber size by electron microscopy. Permeability was analyzed to assess the fibrin clot functional properties.

Epicatechin addition increased the maximum absorbance from 0.34 ± 0.066 (vehicle) to 0.35 ± 0.077 ($P=0.1$), 0.35 ± 0.072 ($P<0.05$) and 0.34 ± 0.065 ($P=0.5$) for 1, 10 and 100 μ M epicatechin, respectively. Epicatechin increased the fibrin clot fiber radius (nm) from 109.2 ± 3.2 (vehicle) to 108.9 ± 4.3 ($P=0.9$), 110.0 ± 3.6 ($P<0.05$) and 109.5 ± 3.3 ($P=0.4$), and the distance between protofibrils (nm) from 22.2 ± 1.5 (vehicle) to 22.1 ± 2.3 ($P=0.9$), 22.6 ± 1.8 ($P<0.05$) and 22.3 ± 1.8 ($P=0.9$) for 1, 10 and 100 μ M epicatechin respectively. Electron microscopy confirmed these changes. Fibrin clot permeability, expressed as Darcy's

constant (K_s , cm^2), increased from 2.97 ± 1.17 (vehicle) to 3.36 ± 1.21 ($P < 0.05$), 3.81 ± 1.41 ($P < 0.01$) and 3.38 ± 1.33 ($P = 0.9$).

Upon epicatechin addition, the fibrin clot structure became less dense and more permeable.

Keywords: Cardiovascular disease; coagulation; epicatechin; fibrin clot; permeability; turbidity;

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1. Introduction

The hemostatic fibrin clot is formed after generation of the thrombin peak on the platelet surface following coagulation activation (Monroe and Hoffman, 2006). Upon clot formation initiation, fibrinogen is cleaved by thrombin into fibrinopeptide A and B to form fibrin monomers. These monomers assemble into half-staggered double-stranded oligomers to form protofibrils. This is followed by lateral aggregation within and between protofibrils, leading to fibrin fiber formation (Mihalko and Brown, 2020). Clot function and structure (e.g. thickness, porosity, density and resistance to lysis) are essential for its physiological role in bleeding control, fibrinolysis and wound healing. Fibrin clot properties are altered in various diseases, including venous and arterial thromboembolic disease (Undas and Ariëns, 2011). Recent data indicate that the precise characterization of the fibrin clot is of particular interest for patient management, prognosis, and to predict the occurrence of complications (Baker et al., 2019; Undas, 2020). Specifically, clots display a less permeable structure (Cieslik et al., 2018) and higher density, and they seem to be resistant to lysis (Siudut et al., 2016; Undas et al., 2009). The fibrin clot quality can also be a risk factor for cardiovascular disease (Mills et al., 2002) with reduced clot permeability and fibrinolysis (Bridge et al., 2014), and can be a predictor of anticoagulation efficacy and adverse clinical outcome in patients with atrial fibrillation and acute coronary syndrome (Sumaya et al., 2018).

Several reviews and meta-analyses highlighted the positive effect of a diet rich in flavonoids on cardiovascular risk and mortality (Del Bo' et al., 2019; Mozaffarian and Wu, 2018; Ottaviani et al., 2018). There is a growing body of evidence on the beneficial properties for cardiovascular health of flavan-3-ols, one of the major classes of dietary flavonoids.

Epicatechin is one of the main representatives of this class, due to its wide distribution in a large variety of fruits and vegetables, such as tea and cocoa. Besides its anti-oxidant and anti-inflammatory activities (Natsume, 2018; Qu et al., 2020) that may affect fibrin clot (Undas et al., 2008), new evidence shows that epicatechin can also positively affect all hemostasis stages. Indeed, it has been reported that epicatechin decreases platelet

aggregation, has an anticoagulant effect, and displays pro-fibrinolytic activity. This suggests a promising role in the prevention of the thrombogenicity of atherosclerotic plaques (Abou-Agag et al., 2001; Sinegre et al., 2019). Epicatechin might also inhibit the action of thrombin (Bijak et al., 2014; Mozzicafreddo et al., 2006) that with fibrinogen, is a key molecule in fibrin clot formation and structure. To the best of our knowledge, no study has assessed whether epicatechin can modulate fibrin clot structure and function. Thus, the aim of this study was to determine epicatechin effect on the fibrin clot structure evaluated by turbidity, electron microscopy and permeability assays.

2. Materials and Methods

2.1. Subjects

Twelve healthy volunteers, 7 women and 5 men (mean age: 33 years [20-46]), were enrolled in 2018 to provide blood samples. Exclusion criteria were history of bleeding and thromboembolism, ongoing antiplatelet drug or anticoagulant therapy, and abnormal blood counts including thrombocytopenia <150 G/L and coagulation disturbances (fibrinogen <2.0 g/L, prothrombin time >15.5 s, activated partial thromboplastin time >40 s). All experiments were performed in accordance with the French laws and approved by the ethics committee of the university hospital of Clermont-Ferrand (Comité de Protection des Personnes Sud-Est VI, ref. AU765). Informed consents were obtained from all human participants of this study

2.2. Blood sampling and plasma preparation

Blood was collected by venipuncture in 0.109 M citrate tubes (Beckton Dickinson, le Pont de Claix, France) after discarding the first few milliliters. Platelet-poor plasma (PPP) was prepared by double centrifugation (2500 g, 21 °C for 15 min) with an intermediate step of plasma decantation, according to the International Society on Thrombosis and Haemostasis (ISTH) guidelines (Subcommittee on Control of Anticoagulation of the SSC of the ISTH,

2011). PPP samples were stored, at -80 °C until testing (less than 3 month). Before the experiments, frozen plasma samples were thawed in a water bath at 37 °C for 5 min.

A stock solution of epicatechin (12.5 mM in DMSO) was diluted with phosphate buffered saline to 0.1, 1 and 10 mM working solutions that were used to supplement the plasma samples to reach the target concentrations of 1, 10 and 100 µM with a constant 1/100 dilution. An equivalent volume of vehicle was added to the baseline samples without epicatechin. Before each experiment, plasma samples were incubated with epicatechin at 37 °C for 10 min.

2.3. Turbidity assay

Clot formation kinetics was monitored in 96-well plates with a flat bottom (Greiner Bio-One). As described by Pieters et al. (Pieters et al., 2018), plasma was diluted in buffer (final dilution 3 : 10) and coagulation was initiated by adding thrombin and calcium at the final concentration of 0.5 NIH U/ml and 15 mM, respectively. After stirring, absorbance was measured at 37°C on a spectrophotometer (Spark, Tecan, Switzerland) at 405 nm every 10 s for 120 min. The main parameters were the lag time (from the start to when absorbance increased 0.015 from baseline), the slope calculated at the midpoint, and the maximum absorbance. All tests were performed in duplicate with a coefficient of variation <5%.

2.4. Fibrin fiber nanostructure analysis

The fibrin clot nanostructure was investigated by scanning spectrometry as described by Yeromonahos et al. (Yeromonahos et al., 2010). Briefly, fibrin clots were formed in 96-well plates with a flat bottom (Greiner Bio-One) from diluted plasma (1 : 6) to which thrombin and calcium were added at the final concentration of 0.5 NIH U/ml and 10 mM, respectively. After incubation at 37°C for 90 min, clots were scanned over wavelengths ranging from 500 to 800 nm, every 1 nm (Spark). In parallel, in order to study the specific role of thrombin, the clot was also formed in the same way by replacing thrombin with reptilase (0.5 Batroxobine U/ml;

Stago, Aisnières, France). The primary endpoints included the average fiber radius, number of protofibrils in fibers, and distance between fibers. All tests were performed in triplicate with a coefficient of variation <5%.

2.5. Electron microscopy

The plasma clot structure was analyzed by electron microscopy. Clots were formed by adding thrombin and calcium at the final concentration of 0.7 NIH U/ml and 15 mM, respectively, in the presence of increasing final concentrations of epicatechin. Clots were washed with TBS for 2 h and fixed in 2% glutaraldehyde at 20°C for 2 h and gradually desiccated. Then, clots were sputter coated with 10 nm gold-palladium (JFC-1300, JEOL, Tokyo, Japan) and examined with an electron microscope (JSM-6060LV, JEOL). Experiments were performed in duplicate.

2.6. Fibrin clot permeability

Fibrin clot permeability was tested using a pressure-driven system, as described by Pieters et al. (Pieters et al., 2012). Briefly, fibrin clots were formed in a tube from 100 µl of plasma by addition of 10 µl of activation mixture containing thrombin and calcium at the final concentration of 1.0 NIH U/ml and 20 mM, respectively. After incubation at 37°C in a wet chamber for 2 h, tubes containing clots were connected to a reservoir containing Tris-HCL buffer (0.05 M Tris-HCL, 0.10 NaCl, pH 7.5). After an initial wash, the volume of buffer that passed through the clots, under constant pressure (4 cm H₂O), was measured for 2 h. The permeability coefficient (K_s ; cm²), which reflects the fibrin network in the clot and particularly the pore size, was determined using Darcy's law: $K_s = Q \times L \times \eta / (t \times A \times \Delta p)$, where Q is the volume of buffer collected in time t , L is the fibrin clot length, η is the liquid viscosity, A is the cross-sectional area of the clot container, and Δp the pressure drop. All tests were performed in quadruplicate with a coefficient of variation <10%.

2.7. Statistical analysis

Statistical analyses were performed with the Prism software, version 6 (GraphPad software, Inc., La Jolla, USA). Tests were two-sided, with a type I error set at $\alpha=0.05$. Data were presented as mean \pm standard deviation (S.D.). The statistical significance of differences between classes was determined with ANOVA or the Friedman test, followed by the appropriate multiple-comparison post-hoc, Tukey-Kramer or Dunn test.

3. Results

3.1. Epicatechin impact on turbidity assays

The turbidity kinetic analysis by measuring absorbance over time after incubation or not with epicatechin (Fig. 1) indicated that the mean maximum absorbance at 405 nm increased from 0.34 ± 0.066 for vehicle to 0.35 ± 0.077 ($P = 0.10$), 0.35 ± 0.072 ($P < 0.05$), and 0.34 ± 0.065 ($P = 0.5$) for 1 μM , 10 μM and 100 μM epicatechin, respectively. Epicatechin did not have any effect on lag time (in s) [83.0 ± 36.7 for vehicle versus 85.1 ± 30.3 ($P = 0.99$), 75.0 ± 36.7 ($P = 0.77$), 66.9 ± 34.8 ($P = 0.33$)] and on absorbance rate (s^{-1}) [2.0 ± 1.1 for vehicle versus 2.0 ± 1.1 ($P = 0.99$), 2.0 ± 1.4 ($P = 0.99$) and 2.2 ± 1.2 ($P = 0.62$), for 1 μM , 10 μM and 100 μM epicatechin, respectively].

3.2. Epicatechin impact on fibrin fiber nanostructure

Analysis of the fibrin fiber nanostructure thrombin-induced by absorbance measurement after incubation or not with epicatechin (Fig. 2) showed that the fibrin fiber radius (nm) [109.1 ± 3.2 for vehicle versus 108.9 ± 4.3 ($P = 0.99$), 110.0 ± 3.6 ($P < 0.05$) and 109.5 ± 3.3 ($P = 0.41$)] and the distance between protofibrils (nm) [22.2 ± 1.5 for vehicle versus 22.1 ± 2.3 ($P = 0.99$), 22.6 ± 1.8 ($P < 0.05$) and 22.3 ± 1.8 ($P = 0.86$) for 1 μM , 10 μM and 100 μM epicatechin, respectively] were significantly increased only in samples incubated with 10 μM epicatechin. The protein mass concentration ($\text{Da}\cdot\text{cm}^{-3}$) was significantly decreased only after incubation with 10 μM epicatechin [3.0 ± 0.4 for vehicle versus 3.0 ± 0.7 ($P = 0.89$), 2.8 ± 0.4

($P < 0.05$) and 2.9 ± 0.4 ($P = 0.94$) for 1 μM , 10 μM and 100 μM epicatechin, respectively], whereas the number of protofibrils was similar in all conditions (between 75 and 77).

No impact of epicatechin on the structural parameters of the clot reptilase-induced was found whatever the concentration (Fig. 3). These results support an impact of epicatechin on the structure of the fibrin clot through its action on thrombin.

The plasma clot structural analysis by electron microscopy after incubation or not with epicatechin (Fig. 4) showed that the fiber diameter ($n=100$ fibers/condition) was increased after incubation with 1 μM and 10 μM epicatechin: 81.7 ± 13.0 for vehicle versus 88.9 ± 10.9 ($P < 0.01$), 95.5 ± 12.0 ($P < 0.001$) and 78.5 ± 12.9 ($P = 0.37$) for 1 μM , 10 μM and 100 μM epicatechin, respectively.

3.3. Impact of epicatechin on fibrin clot permeability

The pore size within fibrin clots was estimated by measuring permeability (K_s ; cm^2) after incubation or not with epicatechin (Fig. 5). Permeability was significantly increased after incubation with 1 μM and 10 μM epicatechin: 2.97 ± 1.17 for vehicle versus 3.36 ± 1.21 ($P < 0.05$), 3.81 ± 1.41 ($P < 0.01$) and 3.38 ± 1.33 ($P = 0.99$) for 1 μM , 10 μM and 100 μM epicatechin, respectively.

4. Discussion

Cardiovascular diseases and venous thromboembolism are leading causes of death worldwide. There is growing evidence that the fibrin clot architecture is a major determinant of arterial and venous thromboembolic diseases and could represent an essential parameter in patient monitoring (Undas and Ariëns, 2011). The present study investigated epicatechin effect on fibrin clot structure and function. Epicatechin is a major flavonoid compound with extensively documented cardiovascular protective effects (Qu et al., 2020). Few previous studies examined the impact of other flavonoids (e.g. flavonols and flavones) on fibrin clot and reported an inhibitory effect on the polymer formation by turbidimetry and microscopy

analyses. Conversely, no information was available on epicatechin (Choi et al., 2015c, 2015a, 2015b).

Our turbidity data indicate that epicatechin increases the maximum absorbance, but does not affect the absorbance rate. The maximum absorbance value should reflect clot growth and partly the fibrin fiber diameter (Ząbczyk and Undas, 2017). However, its meaning is debated because of the many involved determinants and the different analytical conditions (Mihalko and Brown, 2020; Pieters et al., 2020). Nevertheless, our turbidity results are in agreement with our structural analysis of plasma clots that highlighted an increase in the fiber radius associated with higher distance between protofibrils upon incubation with epicatechin. Of note, the impact of epicatechin on the fibrin clot structure is comparable to the effect obtained by an anticoagulant therapy, although the effect size of epicatechin remains smaller (supplementary Table 1). Similarly, the electron microscopy analysis showed a progressive increase in fiber size after incubation with up to 10 μM epicatechin, and then a reduction with 100 μM epicatechin. These results are in agreement with the previously reported profibrinolytic effect of epicatechin (Sinegre et al., 2019) that seems to hinder the thrombogenic clot formation with thin fibers and ultimately prevents the normal course of the fibrinolytic process (Collet et al., 2000). Finally, these structural data on the fibrin clot are consistent with the observed permeability characteristics where porosity increased after incubation with up to 10 μM epicatechin, whereas higher concentrations did not have any effect. These results corroborate the potential beneficial effects of epicatechin on clot sensitivity to lysis, and were obtained using different experimental methods without and with clot desiccation (electron microscopy) (Undas, 2020, 2016).

Altogether, our results suggest that epicatechin at low (1 μM) to moderate (10 μM) concentrations positively modulates fibrin clot formation, while epicatechin appears to be ineffective at the highest concentration (100 μM). This bell-shaped response curve has already been described for flavonoids, arguing a possible desensitization at high concentrations (Claude et al., 2014). Our previous work showed that epicatechin decreases

thrombin generation (Sinegre et al., 2019), and this could constitute a preferential mechanism for the epicatechin-mediated formation of thicker fibrin fibers (Domingues et al., 2016). This hypothesis is consistent with the absence of impact on nanostructure of reptilase-induced fibrin clot and the reported effects of drugs, particularly anticoagulants, that decrease thrombin generation (Undas, 2014; Undas and Zabczyk, 2018). Moreover, fibrinogen is also a major determinant of the clot and thrombotic disease (Ariëns, 2013), and its concentration, polymorphisms or mutations can alter the fibrin clot quality (Allan et al., 2012; Lim et al., 2003). Fibrinogen post-translational modifications, notably oxidation to which fibrinogen is particularly susceptible, also may impact fibrin clot structure, permeability, and sensitivity to lysis (de Vries et al., 2020). Therefore, due its anti-inflammatory and antioxidant properties, epicatechin might also interfere with the fibrin clot characteristics (Undas et al., 2008).

The choice to work on plasma of healthy subjects could induce a potential bias, therefore our results must be confirmed in patients with thromboembolic diseases. Moreover, Ottaviani et al. (Ottaviani et al., 2016) established epicatechin pharmacokinetic profile and described more than 20 different metabolites in plasma. These epicatechin-derived metabolites originate from the action of phase II and gut microbial enzymes. Some of these derivatives might be involved in mediating epicatechin beneficial properties on fibrin clot *in vivo*; however, as these molecules are not commercially available, they could not be tested in this study. Therefore, rather than a short *in vitro* exposure at high epicatechin concentrations, the effect of long-term *in vivo* exposure to physiological epicatechin concentrations including its metabolites deserves to be explored.

In conclusion, our study demonstrated that epicatechin affects the fibrin clot structure making it more permeable, less dense, and consequently more sensitive to lysis. Due to the fibrin clot importance in cardiovascular diseases, as the thrombogenesis end point, an *in vivo* study needs to be carried out to confirm epicatechin beneficial role and to overcome the incapacity to test *in vitro* the plasma metabolites of epicatechin.

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Figures legends

Figure 1: Impact of epicatechin on turbidity parameters of fibrin clots.

A. Lag time (s). B. Maximum absorbance at 405 nm. C. Rate of absorbance (s^{-1}). NS, not significant, *P < 0.05 compared with vehicle.

Figure 2: Impact of epicatechin on the nanostructure of a thrombin-induced fibrin clot.

A. Fiber radius (nm). B. Distance between protofibrils (nm). C. Protofibril number. D. Protein mass concentration ($Da \cdot cm^{-3}$). NS, not significant, *P < 0.05 compared with vehicle.

Figure 3: Impact of epicatechin on the nanostructure of a reptilase-induced fibrin clot.

A. Fiber radius (nm). B. Distance between protofibrils (nm). C. Protofibril number. D. Protein mass concentration ($Da \cdot cm^{-3}$). NS, not significant.

Figure 4: Impact of epicatechin on fiber size within the fibrin clot by electron microscopy.

The clot structure is analyzed with an electron microscope x10 000. A. Vehicle. B. 1 μ M Epicatechin. C. 10 μ M Epicatechin. D. 100 μ M Epicatechin.

Figure 5: Impact of epicatechin on fibrin clot permeability.

Pore size is expressed as Darcy's constant (Ks). *P < 0.05, **P < 0.01 compared with vehicle. #P < 0.05 compared with 1 μ M Epicatechin.









